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DNA
Damages and Repair Mechanisms

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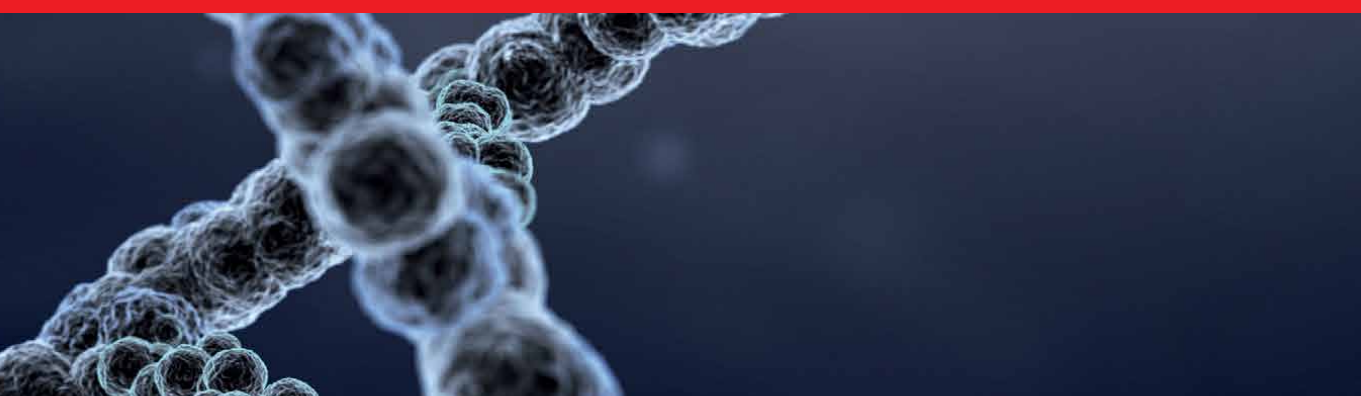

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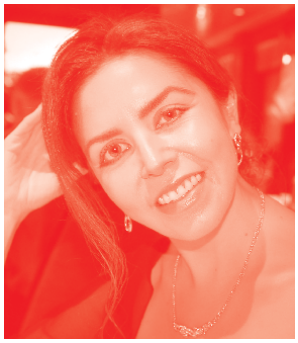

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Meet the editor

Dr. Payam Behzadi was born in 1973 in Tehran-Iran. He is currently an assistant professor in the Department of Microbiology, College of Basic Sciences, Shahr-e-Qods Branch, Islamic Azad University, Iran. He obtained an MSc in Microbiology in 2004 and a Ph.D. in Molecular Biology in 2016. He has authored and edited more than twenty chapters and academic books and more than seventy original and review articles. His scientific research interests include urinary tract infections, antibiotics, bioinformatics, genetics, gene profiling, molecular biology, and cellular and molecular immunology. Dr. Behzadi trains as an ice skater in his free time.

Contents

Preface

Deoxyribonucleic acid (DNA) is the cornerstone of life. It consists of the sugar ribose, phosphate, and the bases of thymine, cytosine, adenine, and guanine. Among these constructive materials, bases have the most important role in transferring genetic information from one generation to another. Hence, the language of information located on DNA molecules is genetic, which, by the contribution of RNA polymerase, changes into mRNA molecules. By the participation of ribosomes, the mRNAs are then translated into proteins. Thus, the genetic language of DNA is translated into proteins.

There are several cellular, genetic, and environmental factors that can damage DNA molecules. This damage may lead to destructive mutations and lesions predisposing individuals to different infectious diseases, autoimmune diseases, and cancers. As such, there are several DNA repair mechanisms that protect DNA molecules from a variety of damages, lesions, and destructive agents.

DNA modifications can occur spontaneously or via cellular activities such as errors in DNA replication or RNA transcription processes. Moreover, reactive oxygen species (ROS), reactive nitrogen species (RNS), alkylation agents, X-rays, ionizing radiation, and ultraviolet (UV) beams are the most recognized DNA-modifying agents that result in lesions and damages to one or both DNA strands.

In contrast to DNA-modifying agents, there is a wide range of DNA repair mechanisms, such as base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ) that have evolved in different organisms.

This book is a collection of chapters covering DNA structural bioinformatics, DNA damages and lesions, and related DNA repair mechanisms. The volume is divided into three main sections: "Biochemistry and Bioinformatics," "DNA Damage-Repair Mechanisms," and "DNA Repair: Cancers and Diseases."

The first section consists of Chapter 1: "Where Quantum Biochemistry Meets Structural Bioinformatics: Excited Conformationally-Tautomeric States of the Classical A·T DNA Base Pair." This chapter uses quantum biochemistry and structural bioinformatics to reveal the conformation and configuration of DNA and its base pairs at the quantum level.

The second section includes five chapters: Chapter 2 "Origin of DNA Repair in the RNA World"; Chapter 3 "Super-Resolution Radiation Biology: From Bio-Dosimetry towards Nano-Studies of DNA Repair Mechanisms"; Chapter 4 "DNA Damage and Repair Mechanisms Triggered by Exposure to Bioflavonoids and Natural Compounds"; Chapter 5 "Recent Perspectives in Radiation-Mediated DNA Damage and Repair: Role of NHEJ and Alternative Pathways"; and Chapter 6 "Interstrand Crosslink Repair: New Horizons of DNA Damage Repair."

The third section includes four chapters: Chapter 7 "DNA Repair Defects in Sarcomas"; Chapter 8 "Epigenetics and DNA Repair in Cancer"; Chapter 9 "Genomic Instability and DNA Repair in Cancer"; and Chapter 10 "The Striatal DNA Damage and Neurodegenerations."

This book is the result of collaboration and cooperation among numerous experts in the field. I am sincerely grateful for their contributions. I would also like to thank Ms. Mia Vulovic, Lucija Tomicic-Dromgool, and Martina Usljebrka Kauric at IntechOpen for their support and cooperation during the preparation of this valuable book.

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Section 1

Biochemistry and Bioinformatics

Chapter 1

Where Quantum Biochemistry Meets Structural Bioinformatics: Excited Conformationally-Tautomeric States of the Classical A·T DNA Base Pair

Ol'ha O. Brovarets', Kostiantyn S. Tsiupa and Dmytro M. Hovorun

Abstract

This Chapter summarizes recent quantum-chemical (QM) investigations of the novel conformational and tautomeric states on the potential energy hypersurface of the classical A·T/A·U nucleobase pairs. For the first time, it was observed 28 local minima for each base pair excluding enantiomers - planar, non-planar base pairs and structures with wobble geometry. Considered excited conformationally-tautomeric states of the classical A·T DNA base pair have been revealed in the Nucleic Acid Database by structural bioinformatics. These data shed light on the biological significance of the unusual A·T/A·U nucleobase pairs for the functioning of the nucleic acids at the quantum level.

Keywords: quantum biology, A·T and A·U nucleobase pairs, tautomeric state, conformational state, wobble geometry, quantum-chemical calculation, structural bioinformatics

1. Introduction

Since the discovery of the spatial organization of the DNA molecule by James Watson & Francis Crick [1, 2], it is traditionally believed that canonical Watson-Crick A·T and G·C DNA base pairs are quite conservative structures. These classical DNA base pairs almost do not have tautomeric variability and essential conformational mobility at the dynamical behavior of DNA molecule [2]. Generally, it is suggested that bases in the *anti*-conformation able to form a pair according to the so-called Watson-Crick (WC) scheme joined through the three intermolecular hydrogen (H) bonds [3]. At the same time, many biologists-contemporaries questioned the proposed Watson-Crick conformation, since X-ray resolution does not allow to establish for sure the precise conformation of the base pairs constituting to the DNA double helix. Exactly by this reason Maurice Wilkins – the third author of the discovery of the DNA structure – explained the reason why Rosalind Franklin doubted in modeling the structure of DNA [4]. And even forefathers of the

discovery noted that suggested by them structure "must be regarded as unproved until it has been checked against more exact results" [1]. Also, Linus Pauling opposed Watson-Crick model of the paring of the bases "because of existing uncertainty about the detailed structure of nucleic acid" (personal correspondence to the Nobel Committee for Chemistry and Physics).

After some time, in 1959 Karst Hoogsteen fixed in crystal state a novel structure for the 1-methylthymine. 9-methyladenine base pair [5], which was named afterwards with the same name – Hoogsteen base pair, in which A purine base adopts the *syn*-conformation formed by flipping of its orientation on 180 degree according the T DNA base. Moreover, the distance between the glycosidic atoms C1′–C1′ is shorter for Hoogsteen base pair in comparison with the classical WC base pair.

Altogether, the A·T DNA base pair can acquire four biologically significant classical configurations – Watson-Crick A·T(WC), reverse Watson-Crick A·T(rWC), Hoogsteen A·T(H) and reverse Hoogsteen A·T(rH) [5–26], due to the rotation of one of the bases in the Watson-Crick A·T(WC) base pair according to the other on 180° around:

- the (A)N1–N3(T) axis, leading to the formation of the reverse Watson-Crick A·T(rWC) or so-called Donohue DNA base pair [6], registered in the bioactive parallel-stranded DNA [7–12];

- the (A)C9-N9 axis from the *anti-* to *syn*-conformation, representing Hoogsteen A·T(H) base pair [5] involved into a number of biologically important processes such as recognition, damage induction and replication [11–22];

- the (A)N7–N3(T) axis in the Hoogsteen base pair forming the reverse Hoogsteen A·T(rH) or so-called Haschemeyer–Sobell base pair [23–26].

Discussed DNA base pairs are not static structures in the composition of DNA [27, 28]. Thus, the spontaneous A·T(WC)↔A·T(H) conformational transition has been experimentally registered by the NMR spectroscopy on the DNA regions enriched by the classical A·T nucleobase pairs [22]. Despite numerous theoretical investigations, microstructural nature of these transitions still remains incomprehensible [20, 29].

Recently, in the literature especial attention has been paid to the searching and careful investigation of the novel conformational and tautomeric states of the classical A·T base pair [30–36], since it can expand their functionality. Generally saying, the topic of the prototropic tautomerism has attracted especial attention, in particular in the area of drug design [37], in physics of crystals [38], in the various created databases [39–41], multinuclear magnetic resonance [42], in NMR spectroscopy [43] as well as biologically important molecules [44–46].

This Chapter summarizes previous investigations, in particular performed by quantum-mechanical (QM) modeling [47–53]. Thus, it was established that the planar classical Watson-Crick A·T DNA base pairs – Watson-Crick A·T(WC), reverse Watson-Crick A·T(rWC), Hoogsteen A·T(H) and reverse Hoogsteen A·T(rH) structures possess unique ability to perform conformationally-tautomeric transitions [47–53]. It occurs *via* the non-planar transition states, *through* the structural or conformational rearrangements and intramolecular proton transfer along the intermolecular H-bonds.

These novel excited conformational and tautomeric states occur due to the quantum effects, e.g. amino group pyramidalization because of electron conjugation

of the lone electron pair of nitrogen amino atom with π-electron system of the ring [54–56]. This data enables us to suggest the potential energy surface of the classical A·T base pairs and also to predict pathways of their interconversions. Moreover, this modeling could be used for the understanding and description in details of the physico-chemical mechanisms of the DNA functioning, in particular DNA "breathing", which has significant biological role [27, 28].

Also, obtained data would enable to make new insights into the understanding the DNA and RNA structural biology, which are based on their conformational and tautomeric variety. By the methods of structural bioinformatics it was revealed unusual conformationally-tautomeric states of the A·T DNA base pair in the Nucleic Acid Database, confirming their existence in biological systems. Altogether, further this could be extended to the area of epigenetics and experimental verification.

2. Methods

2.1 Computational methods

Equilibrium geometries of the investigated DNA base pairs, as well as their harmonic vibrational frequencies have been calculated at the B3LYP/6–311++G(d, p) level of theory [54–58], using Gaussian'09 package [59]. Applied level of theory has proved itself successful for the calculations of the similar systems [60–62]. A scaling factor that is equal to 0.9668 has been applied in the present work for the correction of the harmonic frequencies of all complexes [63, 64].

All calculations have been carried out in the continuum with ε = 1, that adequately reflects the processes occurring in real biological systems without deprivation of the structurally functional properties of the bases in the composition of DNA, and in the continuum with ε = 4, which satisfactorily models the substantially hydrophobic recognition pocket of the DNA-polymerase machinery as a part of the replisome [65–70].

Single point energy calculations have been performed at the MP2/6–311++(2df, pd) level of theory [71, 72].

The Gibbs free energy G for all structures was obtained in the following way:

$$G = E_{el} + E_{corr}, \quad (1)$$

where E_{el} – electronic energy, while E_{corr} – thermal correction.

Electronic interaction energies ΔE_{int} have been calculated at the MP2/6–311++G (2df,pd) level of theory as the difference between the total energy of the base pair and energies of the monomers and corrected for the basis set superposition error (BSSE) [73, 74] through the counterpoise procedure [75, 76].

Bader's quantum theory of Atoms in Molecules (QTAIM) [77–82], using program package AIMAll [77], was applied to analyze the electron density distribution. The presence of the bond critical point (BCP), namely the so-called (3,-1) BCP, and a bond path between hydrogen donor and acceptor, as well as the positive value of the Laplacian at this BCP ($\Delta\rho > 0$), were considered as criteria for the H-bond formation [77–82]. Wave functions were obtained at the level of QM theory used for geometry optimization.

The atomic numbering scheme for the DNA bases is conventional [3]. In this study mutagenic or rare tautomeric forms are denoted by the asterisk [83–92].

2.2 Bioinformatical analysis

It was created original author's algorithm in order to reveal the unusual A·T base pairs in the Nucleic Acid Database by Rutgers University [93, 94]. This algorithm is based on the comparison of the calculated structure of the A·T base pairs at the $\varepsilon = 4$ with structure of the analogical base pairs in the Nucleic Acid Database.

3. Obtained results

It was found out novel *tautomerization pathways* for the formation of the *rare tautomers of the A or T DNA bases*:

- A·T(WC)↔A*·T(w)/A·T*$_{O2}$(w)/A·T*(w) *via* the sequential proton transfer and shifting of the bases relatively each other [47];

- A·T(rWC)/A·T(H)/A·T(rH)↔A·T*(rw$_{WC}$)/A·T*(w$_H$)/A·T*(rw$_H$) mutagenic tautomerization *via* the sequential proton transfer [48];

- A·T(w$_{WC}$)↔A·T*($w^{\perp}_{WC}$), A·T(w$_{rWC}$)↔A·T*$_{O2}$($w^{\perp}_{rWC}$), A·T(w$_H$)↔A·T*($w^{\perp}_{H}$), A·T(w$_{rH}$)↔A·T*$_{O2}$($w^{\perp}_{rH}$) reactions of tautomerization [49];

- A·T(WC) / A·T(rWC)↔A*·T(rw$_{WC}$) / A*·T(w$_{WC}$), A·T(H) / A·T(rH)↔A*$_{N7}$·T(rw$_H$) / A*$_{N7}$·T(w$_H$) reactions *via* sequential proton transfer through the quasi-orthogonal transition states, as well as between the formed base pairs by the participation of the rare tautomers: A*·T(rw$_{WC}$)↔A·T*(rw$_{WC}$) and A*·T(w$_{WC}$)↔A·T*$_{O2}$(w$_{WC}$), A*$_{N7}$·T(rw$_H$)↔A·T*(rw$_H$) and A*$_{N7}$·T(w$_H$)↔A·T*$_{O2}$(w$_H$) through the double proton transfer (DPT) [50].

Also, we found out new pathways of the *conformational transformations* of the *Watson-Crick A·T(WC), reverse Watson-Crick A·T(rWC), Hoogsteen A·T(H) and reverse Hoogsteen A·T(rH) base pairs*:

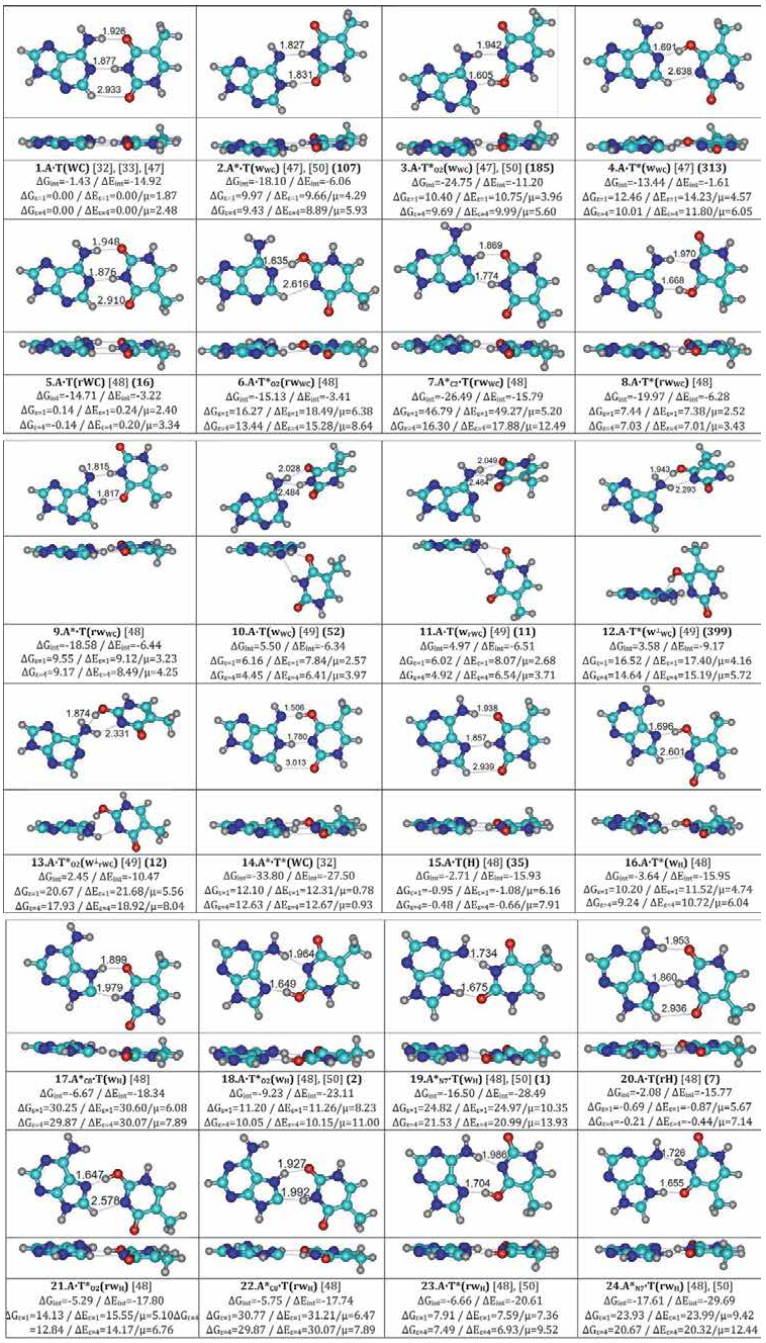
- A·T(WC)↔A·T(w$_{WC}$), A·T(rWC)↔A·T(w$_{rWC}$), A·T(H)↔A·T(w$_H$) and A·T(rH)↔A·T(w$_{rH}$) conformational transformations (Gibbs free energies of activation 7.13, 7.26, 7.67 and 7.44 in the continuum with $\varepsilon = 4$) [51], leading to the novel non-planar conformational states – ***A·T(w$_{WC}$), A·T(w$_{rWC}$), A·T(w$_H$)*** and ***A·T(w$_{rH}$)*** (**Figure 1**). This opens up new perspectives for the understanding of the physico-chemical mechanisms of the opening of the base pairs, which precede the melting of DNA molecule and also describe in details the "breathing" of DNA molecule [27];

- A·T(w$_{WC}$)↔A·T(w$_H$) and A·T(w$_{rWC}$)↔A·T(w$_{rH}$), which define the conformational transitions - ***A·T(WC)***↔A·T(w$_{WC}$)$_{R,L}$↔A·T(w$_H$)$_{L,R}$↔***A·T(H)*** and ***A·T(rWC)***↔A·T(w$_{rWC}$)$_{R,L}$↔A·T(w$_{rH}$)$_{L,R}$↔***A·T(rH)***, occurring through the wobble conformers as intermediates [52];

- A·T(w$_H$)↔A·T(w$_{rWC}$), A·T(w$_{WC}$)↔A·T(w$_{rH}$), A·T(w$_{WC}$)↔A·T(w$_{rWC}$), A·T(w$_H$)↔A·T(w$_{rH}$) conformational transitions (Gibbs free energies of activation 3.20, 3.70, 12.04 and 10.69 kcal·mol^{-1} in the continuum with $\varepsilon = 1$ at T = 298.15 K), which define the conformational interconversions: A·T(WC)↔A·T(rWC) / A·T(rH) and A·T(H)↔A·T(rH) / A·T(rWC) [53].

So, on the potential energy surface of the classical A·T/A·U base pair it was received ***28 various conformationally-tautomeric states*** (**Figure 1**, **Table 1**):

- *Planar structures (C_s point symmetry) with wobble geometry:* WC & rWC – 2.A*·T(w_{WC}), 3.A·T*$_{O2}$(w_{WC}), 4.A·T*(w_{WC}), 6.A·T*$_{O2}$(rw_{WC}), 7.A*$_{C2}$·T(rw_{WC}), 8. A·T*(rw_{WC}), 9.A*·T(rw_{WC}) and H & rH – 16.A·T*(w_H), 17.A*$_{C8}$·T(w_H), 18. A·T*$_{O2}$(w_H), 19.A*$_{N7}$·T(w_H), 21.A·T*$_{O2}$(rw_H), 22.A*$_{C8}$·T(rw_H), 23.A·T*(rw_H), 24.A*$_{N7}$·T(rw_H);



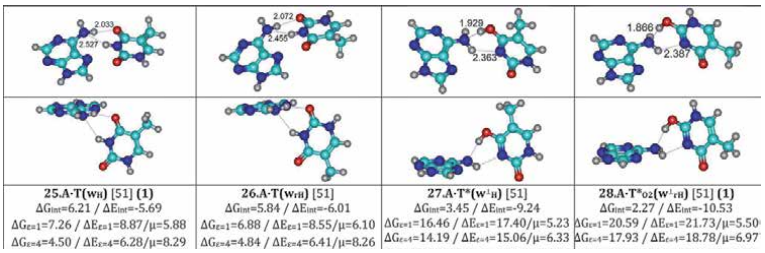


Figure 1.
Unusual A·T base pairs formed through the newly discovered conformationally-tautomeric transformations at the MP2/6–311++G(2df,pd)//B3LYP/6–311++G(d,p) level of QM theory. Graphs of the A·T base pairs are presented for the data in the continuum with ε = 4. ***Definitions:*** *ΔG relative Gibbs free and ΔE electronic energies (in kcal·mol^{-1}) in vacuum, ε = 1 (upper row) and also in the continuum with ε = 4 (lower row); ΔE_{int} electronic and ΔG_{int} Gibbs free energies of the interaction in free state (MP2/6–311++G(2df,pd)//B3LYP/6–311++G(d,p) level of QM theory, in kcal·mol^{-1}). Intermolecular AH…B H-bonds are designated by dotted lines, their lengths H…B are presented in angstroms. Number of the unusual A·T base pairs, which have been identified in the Nucleic Acid Database [62, 63] by structural bioinformatics, is presented in brackets in bold.*

- *Non-planar structures (C_1 point symmetry):* WC & rWC – 10.A·T(w_{WC}), 11.A·T(w_{rWC}), 12.A·T*($w^{\perp}_{WC}$), 13.A·T*$_{O2}$($w^{\perp}_{rWC}$); and H & rH – 25.A·T(w_H), 26.A·T(w_{rH}), 27.A·T*($w^{\perp}_{H}$), 28.A·T*$_{O2}$($w^{\perp}_{rH}$).

Notably, that Gibbs free and electronic energies of the A·T/A·U base pairs are in the wide range of values, which insignificantly decrease at the transition from the continuum with ε = 1 to the continuum with ε = 4, while dipole moment increases at this (**Table 1**).

We have carefully scanned all 28 unusual conformationally-tautomeric states of the A·T DNA base pairs in the Nucleic Acid Database by Rutgers University using original author's methodology for structural bioinformatics analysis. It was identified most part of the theoretically investigated by us excited conformationally-tautomeric states of the classical A·T DNA base pair (**Figure 1**, **Table 1**).

4. Discussion of the obtained results

Let us start the discussion and more detailed analysis of the obtained results from the consideration of the traditional area of the biological applications of the prototropic tautomerism of the DNA bases [54], as well as their role in the origin of the spontaneous point mutations – transitions and transversions at the DNA biosynthesis – so-called replication errors [58–63]. This physico-chemical model should satisfy strict conditions. Saying shortly, in order to point on the most important things, from one side – barriers of the mutagenic tautomerization of the base pairs should not be quite high in view of the quite rigid kinetic requirements for the incorporation into the double strand of DNA by the DNA-polymerase during the one act of replication ($\sim 10^{-4}$ s) [54]. At this, the lifetime of the tautomerized states of the pairs should exceed characteristic time of the inertial DNA-polymerase machinery ($\sim 10^{-9}$ s). Only at this condition the inertial replicational DNA-polymerase machinery would successfully dissociate tautomerized base pairs into the monomers, in particular into the rare tautomeric forms.

From the other side, these barriers should be quite high in order to overcome resistance of the stacking interactions and sugar-phosphate backbone of DNA on the way of the incorporation of the tautomerizing base pair into the double structure of DNA [54].

A·T pair	$\Delta G_{\varepsilon=1}$ [a]	$\Delta E_{\varepsilon=1}$ [b]	$\mu_{\varepsilon=1}$ [c]	$\Delta G_{\varepsilon=4}$ [a]	$\Delta E_{\varepsilon=4}$ [b]	$\mu_{\varepsilon=4}$ [c]	A·U pair	$\Delta G_{\varepsilon=1}$ [a]	$\Delta E_{\varepsilon=1}$ [b]	$\mu_{\varepsilon=1}$ [c]	$\Delta G_{\varepsilon=4}$ [a]	$\Delta E_{\varepsilon=4}$ [b]	$\mu_{\varepsilon=4}$ [c]
1. A·T(WC) [47]	0.00	0.00	1.87	0.00	0.00	2.48	**1. A·U(WC)**	0.00	0.00	1.69	0.00	0.00	2.25
2. A*·T(w_{WC}) [47]	9.97	9.66	4.29	9.43	8.89	5.93	**2. A*·U(w_{WC})**	10.35	9.92	4.83	9.67	9.04	6.52
3. A·T*$_{O2}$(w_{WC}) [47]	10.40	10.75	3.96	9.69	9.99	5.60	**3. A·U*$_{O2}$(w_{WC})**	10.96	11.33	4.59	10.15	10.41	6.32
4. A·T*(w_{WC}) [47]	12.46	14.23	4.57	10.01	11.80	6.05	**4. A·U*(w_{WC})**	11.82	13.53	4.09	9.57	11.38	5.42
5. A·T(rWC) [48]	0.14	0.24	2.40	−0.14	0.20	3.34	**5. A·U(rWC)**	0.34	0.42	2.78	0.26	0.28	3.77
6. A·T*$_{O2}$(rw_{WC}) [48]	16.27	18.49	6.38	13.44	15.28	8.64	**6. A·U*$_{O2}$(rw_{WC})**	16.95	19.02	6.98	10.15	10.41	6.32
7. A*$_{C2}$·T(rw_{WC}) [48]	46.79	49.27	5.20	16.30	17.88	12.49	**7. A*$_{C2}$·U(rw_{WC})**	46.71	49.42	5.85	16.66	17.76	13.23
8. A·T*(rw_{WC}) [48]	7.44	7.38	2.52	7.03	7.01	3.43	**8. A·U*(rw_{WC})**	6.99	6.94	1.97	17.73	17.25	1.97
9. A*·T(rw_{WC}) [48]	9.55	9.12	3.23	9.17	8.49	4.25	**9. A*·U(rw_{WC})**	9.55	9.05	2.69	20.29	19.36	2.69
10. A·T(w_{WC}) [49]	6.16	7.84	2.57	4.45	6.41	3.97	**10. A·U(w_{WC})**	6.12	8.15	2.50	5.06	6.39	4.10
11. A·T(w_{rWC}) [49]	6.02	8.07	2.68	4.92	6.54	3.71	**11. A·U(w_{rWC})**	6.25	8.18	2.63	5.31	6.52	3.50
12. A·T*($w^{\perp}_{WC}$) [49]	16.52	17.40	4.16	14.64	15.19	5.71	**12. A·U*($w^{\perp}_{WC}$)**	16.02	16.86	3.67	14.24	14.87	5.20
13. A·T*$_{O2}$($w^{\perp}_{rWC}$) [49]	20.67	21.68	5.56	17.93	18.92	8.04	**13. A·U*$_{O2}$($w^{\perp}_{rWC}$)**	21.38	22.32	6.21	18.58	19.40	8.85
14. A*·T*(WC) [50]	12.10	12.31	0.78	12.63	12.67	0.93	**14. A*·U*(WC)**	11.96	12.04	0.73	12.42	12.45	0.83
15. A·T(H) [48]	−0.95	−1.08	6.16	−0.48	−0.66	7.91	**15. A·U(H)**	−0.59	−0.96	6.34	−0.18	−0.57	8.12
16. A·T*(w_H) [48]	10.20	11.52	4.74	9.24	10.72	6.04	**16. A·U*(w_H)**	9.32	10.97	4.45	9.19	10.34	5.67
17. A*$_{C8}$·T(w_H) [48]	30.25	30.60	6.08	29.87	30.07	7.89	**17. A*$_{C8}$·U(w_H)**	30.17	30.50	5.80	29.91	30.06	7.52
18. A·T*$_{O2}$(w_H) [48]	11.20	11.26	8.23	10.05	10.15	11.00	**18. A·U*$_{O2}$(w_H)**	11.91	11.91	8.65	10.23	10.62	11.46
19. A*$_{N7}$·T(w_H) [48]	24.82	24.97	10.35	21.53	20.99	13.93	**19. A*$_{N7}$·U(w_H)**	25.32	25.39	10.79	21.66	21.25	14.40
20. A·T(rH) [48]	−0.69	−0.87	5.67	−0.21	−0.44	7.14	**20. A·U(rH)**	−0.40	−0.74	5.38	−0.19	−0.38	6.79
21. A·T*$_{O2}$(rw_H) [48]	14.13	15.55	5.10	12.84	14.17	6.76	**21. A·U*$_{O2}$(rw_H)**	14.82	16.10	5.40	13.25	14.52	7.11
22. A*$_{C8}$·T(rw_H) [48]	30.77	31.21	6.47	29.87	30.07	7.89	**22. A*$_{C8}$·U(rw_H)**	31.18	31.49	6.68	30.62	30.66	8.88

A·T pair	$\Delta G_{\varepsilon=1}$[a]	$\Delta E_{\varepsilon=1}$[b]	$\mu_{\varepsilon=1}$[c]	$\Delta G_{\varepsilon=4}$[a]	$\Delta E_{\varepsilon=4}$[b]	$\mu_{\varepsilon=4}$[c]	A·U pair	$\Delta G_{\varepsilon=1}$[a]	$\Delta E_{\varepsilon=1}$[b]	$\mu_{\varepsilon=1}$[c]	$\Delta G_{\varepsilon=4}$[a]	$\Delta E_{\varepsilon=4}$[b]	$\mu_{\varepsilon=4}$[c]
23. A·T*(rw_H) [48]	7.91	7.59	7.36	7.49	6.93	9.52	**23. A·U*(rw_H)**	7.48	7.11	6.88	6.81	6.62	8.91
24. A^*_{N7}·T(rw_H) [48]	23.93	23.99	9.42	20.67	20.32	12.44	**24. A^*_{N7}·U(rw_H)**	23.69	23.83	8.91	20.77	20.26	11.75
25. A·T(w_H) [49]	7.26	8.87	5.88	4.50	6.28	8.29	**25. A·U(w_H)**	7.35	8.12	5.12	4.75	6.47	7.92
26. A·T(w_{rH}) [49]	6.88	8.55	6.10	4.84	6.41	8.26	**26. A·U(w_{rH})**	6.88	8.53	6.02	4.84	6.54	8.21
27. A·T*($w^{\perp}_H$) [49]	16.46	17.40	5.23	14.19	15.06	6.33	**27. A·U*($w^{\perp}_H$)**	16.00	16.90	4.83	14.01	14.68	5.97
28. A·T^*_{O2}($w^{\perp}_{rH}$) [49]	20.59	21.73	5.50	17.93	18.78	6.97	**28. A·U^*_{O2}($w^{\perp}_{rH}$)**	21.28	22.36	5.88	18.19	19.25	7.25

[a] *Relative Gibbs free energy of the base pair (T = 298.15 K), kcal·mol^{-1}.*
[b] *Relative electronic energy of the base pair, kcal·mol^{-1}.*
[c] *Dipole moment of the base pair, Debay.*

Table 1.
Energetic and polar characteristics of the conformers and tautomers of the A·T/A·U nucleobase pairs obtained at the MP2/6–311++G(2df,pd) // B3LYP/6–311++G(d,p) level of QM/PCM theory in the isolated state (ε = 1) and in the continuum with ε = 4 under normal conditions (see ***Figure 1****).*

Nowadays, just one single model satisfies these strict conditions [47]. According to this model (**Figure 1**), mutagenic tautomerization of the bases in the A·T(WC) base pair is controlled by the transition states, which represent itself tight ion pairs $A^{+}\cdot T^{-}$, and is realized through the step-by-step proton transfer along the intermolecular H-bonds and is assisted by the lateral changing of the configuration of the pair – its transition from the Watson-Crick configuration to the wobble or shifted [47]. In fact, complementary A base plays a role of catalysator of the intramolecular mutagenic tautomerization of the T base within the A·T(WC) base pair. Below it would be outlined experimental confirmations that wobble structures of the A·T base pair, containing mutagenic tautomeric forms of the T base, are real objects of the structural biology. This fact, in our opinion, experimentally confirms reality of the tautomeric mechanisms of the origin of the replication errors [47].

We have demonstrated for the first time, that others three biologically important configurations of the A·T base pair – A·T(rWC), A·T(H) and A·T(rH) [47] – tautomerises by the abovementioned and described mechanism of the tautomerization, forming wobble pairs by the participation of the mutagenic tautomers (**Figure 1**). Moreover, we have arrived to the conclusion by the comparison of their energetical characteristics, that *Nature* quite consciously choose evolutionary the most remote A·T(WC) base pair for the building of the carrier of the genetic information in the form of the right-handed DNA [47].

In this regard, it arises quite logical question – "Whether *Nature* uses prototropic tautomerization of the DNA bases beyond the borders of classical tautomeric hypothesis?" Let us say – for the supporting of the unusual DNA structures. Principle of economy of thinking (*Entia non sunt multiplicanda praeter necessitate*), which is quite often applied by the living nature, enables in principle, affirmatively answer on the quite interesting question. Below we would provide number of examples of the application in the structural biology of all without exception wobble configurations of the A·T pair by the participation of the mutagenic tautomers.

Biological role of the prototropic tautomerism of the DNA bases is not limited by the presented here examples. It is quite more complex and wider. Let us attract readers' attention to the one more so-called unusual role of the tautomeric-conformational transformations in the DNA structural transitions. However, their mechanism of action could be explained only at the macroscopical level.

In the work [61] at the example of the hypoxanthine dimer it was revealed novel way of the conformationally-tautomeric transformations of the structures, which are joined by the neighboring antiparallel H-bonds, through the quasi-orthogonal transition state with the changing of the mutual orientation of the dimmers on 180 degree. Conformationally-tautomeric transitions of such a nature have been fixed in all without exception four configurations of the classical A·T DNA base pair [53]. Combining these data with previous, concerning the WC/H↔w_{WC}/w_{H} conformationally-tautomeric transitions [50], we have obtained joined picture of the WC/H↔rWC/rH at the quantum level:

- A·T(WC)↔A·T*(rw_{WC})↔A·T(rWC)↔A·T*$_{O2}$(w_{WC})↔A·T(WC);
- A·T(H)↔A·T*(rw_{H})↔A·T(rH)↔A·T*$_{O2}$(w_{H})↔A·T(H),

as well as experimental confirmation (see below) of the existence of these structures in real macromolecular biosystems.

Bioinformatical analysis. This data convincingly evidence on the real occurrence of these base pairs in the real biological systems [93, 94] and thus – on their biological importance. This situation remains for a long time the hidden side of the classical A·T DNA base pair. However, it became successfully resolved in the current work.

5. Conclusions

Concluding, we can state that it was received the most complete up to now quantum map of the biologically-important conformationally-tautomeric transitions of the classical A·T/A·U nucleobase pair, which enable to classify it as a ***quantum choreography with all further going consequences***. But it is not a pursuit of a new term, but rather an attempt to realize the molecular logic of the quantum evolution at its initial stages, when it was formed its behavior, which is evolutionary programmed in its electronic structure.

For the first time, it was shown for the classical A·T DNA base pair that prototropic tautomerism of the DNA bases is responsible both for the origin, as well as for the supporting of the unusual local structures in the constitution of DNA and in complexes with proteins and small biomolecules. Moreover, prototropic tautomerism of the classical A·T DNA base pair significantly expands its conformational possibilities and its impact on the biological importance.

It is connected with the fact that presented mechanisms of the tautomerization are assisted by the significant changing of the geometry of the tautomerizing base pair. This means that they are conformationally-tautomeric transitions by their essence.

This conclusion is confirmed by the structural bioinformatics. Thus, it was identified hundredth of the structures containing tautomers of the DNA bases. This fact points that all described exited conformationally-tautomeric states of the A·T and A·U nucleobase pairs, corresponding to local minima, are real structures.

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Section 2

DNA Damage-Repair Mechanisms

Chapter 2

Origin of DNA Repair in the RNA World

Harris Bernstein and Carol Bernstein

Abstract

The early history of life on Earth likely included a stage in which life existed as self-replicating protocells with single-stranded RNA (ssRNA) genomes. In this RNA world, genome damage from a variety of sources (spontaneous hydrolysis, UV, etc.) would have been a problem for survival. Selection pressure for dealing with genome damage would have led to adaptive strategies for mitigating the damage. In today's world, RNA viruses with ssRNA genomes are common, and these viruses similarly need to cope with genome damage. Thus ssRNA viruses can serve as models for understanding the early evolution of genome repair. As the ssRNA protocells in the early RNA world evolved, the RNA genome likely gave rise, through a series of evolutionary stages, to the double-stranded DNA (dsDNA) genome. In ssRNA to dsDNA evolution, genome repair processes also likely evolved to accommodate this transition. Some of the basic features of ssRNA genome repair appear to have been retained in descendants with dsDNA genomes. In particular, a type of strand-switching recombination occurs when ssRNA replication is blocked by a damage in the template strand. Elements of this process appear to have a central role in recombinational repair processes during meiosis and mitosis of descendant dsDNA organisms.

Keywords: RNA world, RNA virus, recombination repair, copy-choice, synthesis-dependent strand annealing (SDSA), DNA repair, archaea, genome damage, strand-switching, self-replication, single-stranded RNA

1. Introduction

Protocellular organisms may have come into existence 2.5 to 3.5 billion years ago [1, 2]. Woese [3] proposed that the genomes of the early protocellular forms of life were individual strands of RNA rather than DNA, and that these RNA strands were present as separate genome segments, rather than being linked together end-to-end as is generally the case for genes in DNA. The idea that, during an early period in the evolution of life, genetic information was stored and transmitted solely by RNA molecules has come to be known as the "RNA world hypothesis." This hypothesis is currently being tested by many investigators. Of particular significance, Horning and Joyce [4] have demonstrated that the replication of genetic information and its conversion into functional molecules can be accomplished with RNA in the complete absence of protein. RNA molecules with catalytic activity are called ribozymes. An RNA ribozyme developed by Horning and Joyce can act as an RNA polymerase to replicate RNA [4].

Persistence and replication of even the simplest forms of RNA life must have depended on preserving the information content of the RNA genome from damage (a form of informational noise). Damage to the RNA genome likely occurred in a variety of ways including spontaneous hydrolysis, exposure to UV light and exposure to reactive chemicals. Natural selection would have acted to promote the evolution of RNA sequences that allowed solutions to this problem of informational noise. While free living organisms with ssRNA genomes are unknown in today's world, viruses with ssRNA genomes are currently common. The present day ssRNA viruses also need to cope with informational noise in the form of damage to their RNA genome. Therefore, such ssRNA viruses can serve as models for understanding the adaptive solutions that early ssRNA protocells may have developed for coping with genome damage. Numerous ssRNA viruses have been shown to be capable of exchanging sequence information between individual genomes within an infected cell [5]. This information exchange, or genetic recombination, can occur by reassortment of genome segments or during genome replication by a process of strand-switching to form a progeny genome with information from two parental genomes. The process of strand-switching is often referred to as "copy-choice" recombination. The term "copy-choice" embodies the idea of template-switching during genome replication, although the term was introduced before the DNA/RNA nature of genetic information was understood. Lederberg [6] and Bernstein [7] were among the first to explicitly propose copy-choice mechanisms of recombination. The two recombination processes, segment reassortment and copy-choice, allow the formation of an undamaged progeny genome even when one or both parental genomes contain damage. In the sense that both segment reassortment and copy-choice restore genetic sequence information that is damaged in the parental genomes, these are informational repair processes. Although information is restored in progeny, the parental genomes may retain their physical damage. Thus when "repair" is discussed at the level of ssRNA organisms it is the genetic information content of damaged parental genomes that is restored or "repaired" during formation of the progeny genome.

The role of RNA segment reassortment in genome repair is discussed by Bernstein et al. [8] and the role of copy choice recombination in an RNA genome repair is discussed by Hu and Temin [9].

As the early protocells with RNA genomes evolved they likely went through a series of adaptive transitions that eventually led to the double-stranded DNA (dsDNA) genome. The archaea are a group of prokaryotes with a dsDNA genome that likely evolved prior to the emergence of eukaryotes. These organisms are capable of a process, genetic transformation, during which cells exchange DNA to repair DNA double-strand breaks via homologous recombination [10]. In eukaryotes, during meiosis and mitosis, most recombination events occur by a repair process termed "synthesis-dependent strand annealing" (SDSA) [11] that is basically a form of copy-choice recombination (see Section 6.1.). In addition, single-strand damages that block the movement of the DNA polymerase during replication can be repaired by a mechanism that includes copy-choice recombination [12, 13]. Thus strand-switching copy-choice mechanisms that likely emerged in early ssRNA protocells appear to have evolved into fundamental processes for maintaining the information content of dsDNA genomes.

While the capability for recombinational repair is retained as a major mechanism for dealing with DNA damages, organisms with a dsDNA genome, including humans, have also evolved other repair processes that take advantage of the duplex nature of the DNA genome [14]. For such organisms, damages in one strand can be repaired by removal of the damaged section and its replacement by copying information from the other strand, as occurs in the well-studied processes of mismatch repair, nucleotide

excision repair and base excision repair [14]. Other processes for dealing with DNA damages in organisms with DNA genomes include direct reversal of UV photolesions and alkylated bases, repair of DNA crosslinks by Fanconi anemia proteins, and a mechanism for tolerating damages termed translesion synthesis [15].

The aim of this review is to outline how genome repair processes emerged in the earliest evolved protocells that likely had RNA genomes, and how these processes further evolved in the transition from the RNA world to the DNA world.

2. Genome repair in the RNA world

Since the actual sequence of evolutionary adaptive events in the RNA world that gave rise to genome repair occurred in organisms that are probably long extinct, and it is unlikely that events at the nucleic acid level are preserved in the fossil record, the sequence of evolutionary events proposed here is necessarily speculative. However, the proposed evolutionary sequence is based on the established activities of extant RNA viruses. These activities are reviewed in sections 2.1, 2.2, 3 and 4. Thus it is assumed, as discussed by Bernstein et al. (pgs. 342-345) [8], that the adaptations that extant RNA viruses use to repair genome damage can illuminate how early life in the RNA world also coped with genome damage.

In early protocellular organisms the genome is thought to have consisted of ssRNAs (genes) that formed folded structures with catalyic activity (ribozymes) [16]. If two or more such ssRNAs were present in a protocell they presumably functioned interdependently to promote the viability and reproduction of the protocell. A key ribozyme in early protocellular organisms would likely have been a polymerase that could catalyze RNA replication [4]. A persistent problem for early protocellular organisms would probably have been damage to their ssRNA genomes. The damaging stresses on protocellular organisms likely would have included hydrolytic reactions, exposure to UV light and interaction with reactive chemicals in the environment. For example, Sagan [17] analyzed the flux of solar UV light that penetrated the earth's primitive reducing atmosphere. His analysis indicated that unprotected microorganisms of the type existing today would receive a mean lethal dose at 2600 angstroms within 0.3 seconds and that this vulnerability could have posed a major problem during the early evolution of life. A protocell that has only one copy of each ssRNA (a haploid protocell) would be very vulnerable to damage, since damage to even one base in a ssRNA sequence might be lethal to the protocell by either blocking replication of the ssRNA or interfering with an essential ssRNA ribozyme function [8].

One possible adaptation for dealing with genome damage would be to maintain two or more copies of each ssRNA gene in each protocell, yielding a diploid or polyploid state. Genome redundancy would allow replacement of a damaged gene by an additional replication of an undamaged homologous gene. However, for a simple protocellular organism, the proportion of available resource budgeted to the maintenance of two or more genomes would have been a large portion of its total resource budget. When resources are limited, the protocell's reproductive rate would likely be inversely related to ploidy number. The fitness of the protocell would be diminished by the costs of genome redundancy. Coping with damage to the ssRNA genome while minimizing the costs of genome redundancy would likely have been a fundamental problem in the early evolution of cellular life [8].

When the costs of maintaining genome redundancy verses the costs of genome damage were balanced against each other in a cost–benefit analysis, it was found that under a wide range of conditions the selected strategy would be for each

protocell to be haploid, but to periodically fuse with another haploid protocell to form a transient diploid [18]. This strategy allows the haploid state to be retained to maximize reproductive rate, while the periodic fusions would allow otherwise lethally damaged protocells to be mutually reactivated. Reactivation can occur if at least one undamaged copy of each ssRNA gene is present in the transient diploid and this leads to production of a viable progeny protocell. In order for two (rather than just one) viable progeny protocells to be produced, an extra replication of the gene(s) homologous to damaged gene(s) would have to occur before division of the fused diploid protocell. The process of recovering from potentially lethal damage in one ssRNA genome by reassorting information with another homologous ssRNA genome can be regarded as a primitive form of genome repair [8, 18]. This proposed cycle for coping with genome damage, although hypothetical, is based on the way that ssRNA viruses with segmented RNA genomes deal with genome damage as discussed below in Section 2.1.

The events that contributed to the evolution of genomic repair in ssRNA protocells can also be viewed as an early stage in the evolution of sexual reproduction since these events include the coming together of two genomes from separate parents to generate progeny genomes containing shared genetic information [18].

2.1 Recombination in influenza virus and hantavirus

Influenza virus (Family *Bunyavirales*) is an example of a virus with a segmented ssRNA genome (**Figure 1**). Influenza virus has a genome comprised of eight physically separated ssRNA segments [19]. These eight segments of single-stranded RNA code for seven virion structural proteins and three non-structural proteins. During infection of a host cell by two viruses, recombinant progeny can be formed as the result of exchange of segments of the virus ssRNA, a process termed reassortment [19].

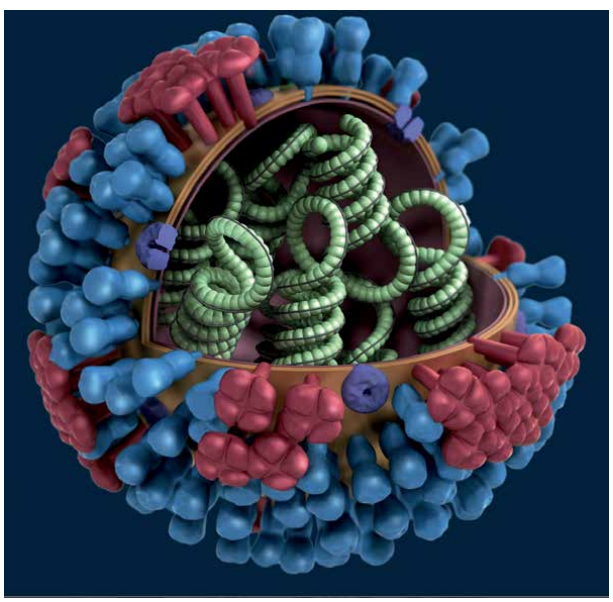

Figure 1.
Influenza virus. An enveloped virus with an outer lipid membrane and glycoprotein "spikes." Influenza A or B viruses have eight genome segments inside the virion. https://pixnio.com/science/microscopy-images/influenza/3-dimensional-model-of-influenza-virus In the public domain.

Upon infection, influenza virus induces a host response involving increased production of reactive oxygen species, and this can damage the virus genome [20]. Consider two individual viruses each with a lethal damage in its genome. If either of these viruses infects a host cell the infection aborts and no progeny viruses are produced. However, if these two damaged viruses infect the same host cell, the multiple infection may lead to reactivation (production of viable progeny). This phenomenon is known as "multiplicity reactivation" and is thought to reflect acts of recombination that allow an undamaged genome to be reconstituted from damaged ones [21]. Multiplicity reactivation has been demonstrated in influenza virus infections after induction of RNA damage by UV-irradiation [22] and ionizing radiation [23]. In these studies, recombination by reassortment of genome segments likely played a role in the observed multiplicity reactivation.

Hantaviruses (Order *Bunyavirales*; Family *Hantaviridae*), another group of segmented ssRNA viruses, are also able to undergo reassortment [24, 25]. Reovirus (Family *Reoviridae*), a segmented double-stranded RNA virus, can also undergo multiplicity reactivation after its genome is damaged by exposure to UV light [26]. Substantial evidence in model virus systems indicates that multiplicity reactivation is a recombinational repair process for overcoming a variety of types of genome damage (reviewed in [27, 28]). If, under natural conditions, virus survival is ordinarily vulnerable to oxidative or other damage, then multiplicity reactivation likely acts as an adaptive genomic repair process.

Recombination by reassortment is a simple way of restoring an undamaged genome from multiple lethally damaged genomes and thus is a primitive form of genomic repair. Lehman [29] has reviewed evidence supporting the view that recombination is an evolutionary development as ancient as the origins of life.

In addition to the role of recombination in genome repair, recombination also has a role in viral evolution by generating new genetic combinations that can be tested by natural selection. An infrequent new genetic combination may be selectively advantageous. However, RNA is very vulnerable to damage. Because of the reactivity of the oxygen and nitrogen atoms of the nucleobases [30], RNA molecules are especially susceptible to certain types of chemical damage from sources such as reactive oxygen species, UV light, and alkylating agents; and the oxygen atoms of the ribose and the phosphodiester backbone are also vulnerable to chemical damage [30]. In early protocells, repair of RNA genome damage likely provided a considerable and immediate selective advantage while new recombinant genetic combinations may have been adaptively beneficial only infrequently.

2.2 Intragenic recombination in segmented ssRNA influenza virus and hantavirus

In influenza virus infections, genome segment reassortment is not the only mechanism of recombination. Intragenic homologous recombination can also occur between a pair of homologous viral genes [31]. Homologous recombination occurs by template-switching (copy-choice) during viral genome replication [32].

In addition to influenza viruses, ssRNA hantaviruses are also capable of recombination by both segment reassortment and by homologous recombination [33].

In the evolution of repair processes in the RNA world, template-switching (copy-choice) recombination was likely an important advance since it allows two damaged homologous genes to generate an undamaged homolog. However, at present there is insufficient evidence available to determine whether copy-choice recombination emerged before or after the emergence of genome segment reassortment as a mechanism of genome repair [31].

3. Repair of RNA genomes by copy-choice recombination

3.1 Copy-choice recombination

Figure 2 indicates how an accurate undamaged progeny single-stranded genome can be generated from a damaged parental genome by strand-switching (copy-choice) recombination. As shown in this **Figure 2**, (1) during synthesis of a progeny strand by a replicative polymerase, a damage in the (green) template strand (strand being copied) blocks polymerase progression. (2) If another (orange) homologous template is available, the polymerase may switch templates, thereby bypassing the damage. (3) The newly synthesized strand may then release from the second template strand. (4) The newly synthesized strand can return and pair with the original template. (5) The polymerase may then complete the replication using the original template. (6) These steps can generate a new recombinant genome without damage [9, 34].

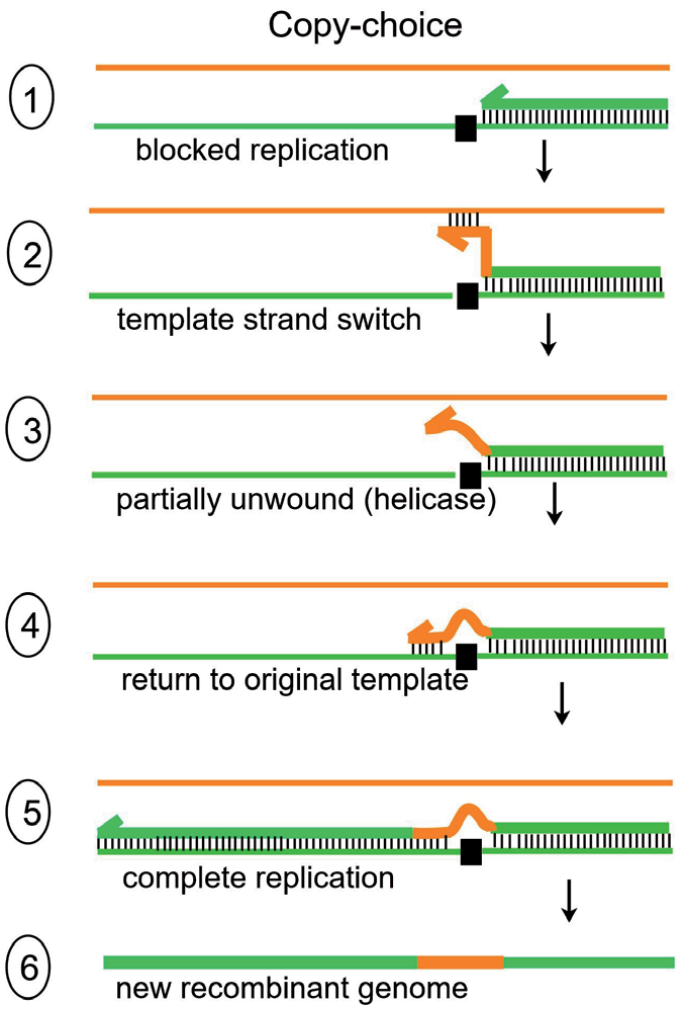

Figure 2.
Copy-choice recombination.

3.2 Poliovirus and coronavirus

Poliovirus (Family *Picornaviridae*; Genus *Enterovirus*) is a positive ssRNA ((+) ssRNA) virus that can undergo genetic recombination when there are at least two ssRNA viral genomes in the same host cell. RNA recombination is considered to be a major driving force in determining the course of poliovirus evolution [35]. RNA-dependent RNA polymerase (RdRp), an enzyme encoded in the viral genome, catalyzes genome replication. Kirkegaard and Baltimore [34] presented results strongly supporting a copy-choice mechanism for RNA recombination for poliovirus. By this mechanism the RdRp switches between (+)ssRNA templates during synthesis of the progeny negative strand (−)ssRNA (**Figure 2**). Recombination in RNA viruses is considered to be an adaptive mechanism for maintaining genome integrity [36].

To regenerate the next generation of (+)ssRNA strands, the (−)ssRNA strands are also copied and this may also be accompanied *infrequently* by strand switching [34].

When cells are infected by two or more viruses containing genome damage the viruses may undergo multiplicity reactivation. Polioviruses are able to undergo

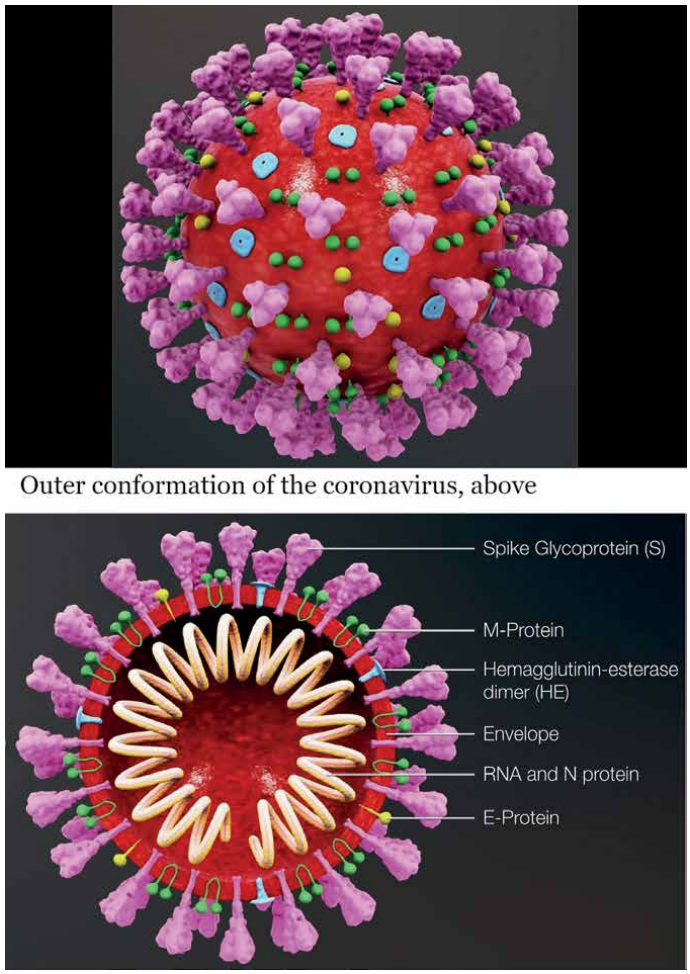

Figure 3.
Coronavirus. Modified from https://commons.wikimedia.org/wiki/File:3D_medical_animation_coronavirus_structure_vie.png with license https://www.scientificanimations.com/CC BY-SA (https://creativecommons.org/licenses/by-sa/4.0)

multiplicity reactivation [37]. That is, when polioviruses were irradiated with UV light and then allowed to infect host cells at a multiplicity of two or greater, viable progeny are produced at UV doses that inactivate the virus in single infections. As noted above, multiplicity reactivation occurs in various different virus systems, and has been shown to be a form of recombinational repair [27, 28].

Coronaviruses (Family *Coronaviridae*) (see **Figure 3**) are (+)ssRNA enveloped viruses. The genome size of coronaviruses ranges from about 26 to 32 kilobases, one of the largest among RNA viruses. They have characteristic club-shaped spikes that project from their surface, which in electron micrographs create an image reminiscent of the solar corona, from which their name derives.

RNA recombination appears to be a major driving force in the evolution of (+) ssRNA coronaviruses. Recombination contributes to genetic variability within a coronavirus species, the capability of a coronavirus species to jump from one host to another and, infrequently, the emergence of a novel coronavirus [38]. The mechanism of recombination in coronaviruses likely involves template-switching during genome replication [38]. Also, the (+)ssRNA plant carmoviruses and tombusviruses frequently undergo recombination by RdRp template-switching (copy-choice) [39]. A key step in the evolution of repair in the RNA world appears to have been the emergence of template-switching (copy-choice) recombination as a major mechanism for dealing with genome damage.

4. Reverse transcription of the RNA genome to DNA in HIV

Human immunodeficiency virus (HIV (Family *Retroviridae*) (**Figure 4**) is a positive single-stranded RNA ((+)ssRNA) virus. Each HIV virus particle encapsidates two (+)ssRNA genomes.

During infection of a host cell, genome replication is catalyzed by reverse transcriptase, an RNA-dependent DNA polymerase [40]. During reverse transcription, recombination between the two genomes can occur [9]. The reverse transcriptase can switch between the two parental RNA genomes by copy-choice recombination [40], and such events may occur throughout the genome. Thus the two infecting genomes from each virus can cooperate to form a complementary negative single-strand DNA copy that has recombined information from the two parental RNA genomes. Recombination is necessary for efficient HIV replication and the maintenance of genome integrity [40]. During each replication cycle, from 5 to 14 recombination events may occur per genome [41]. The recombination events are "clustered" so that one recombination event is correlated with another that is close by. This clustering is apparently caused by correlated template-switches, known as high negative interference, during minus-strand DNA synthesis [42]. That is, once a switch is made from template *a* to template *b*, then another switch is made very soon (not at some random time) back to template *a*. Template-switching in HIV is considered to be a repair mechanism for salvaging damaged genomes that is essential for maintaining genome integrity [9, 40].

After the first single strand DNA copy is synthesized, another round of replication generates a duplex DNA molecule which can integrate into the host DNA genome to form a provirus [9].

4.1 HIV recombination can sometimes produce genetic variation

Recombination of the viral genomes can introduce genetic variation among progeny HIV that contributes to the evolution of resistance when humans are treated with anti-retroviral therapy [43]. Viral genome recombination may also

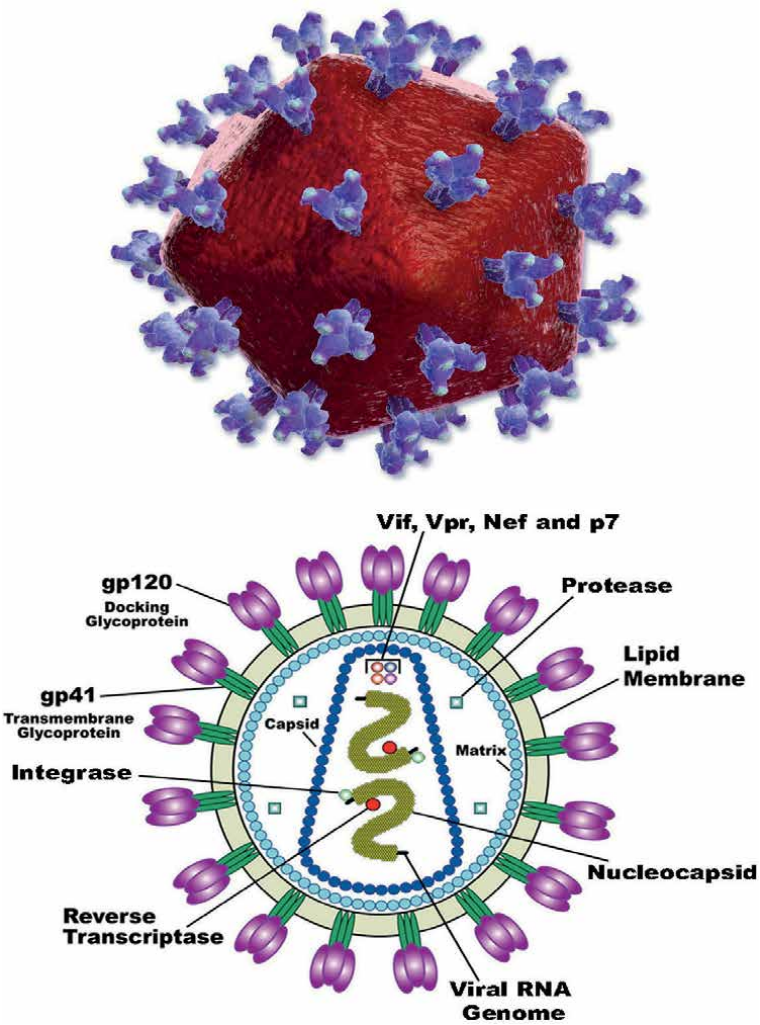

Figure 4.
Human Immunodeficiency virus (HIV). Top image indicates outer conformation of the virion. Lower image shows the two RNA genomes present within the virion, the reverse transcriptase and other components of the virion. Top image: https://commons.wikimedia.org/wiki/File:HIV.png BruceBlaus/CC BY-SA (https://creativecommons.org/licenses/by-sa/4.0) Bottom image: https://commons.wikimedia.org/wiki/File:HI-Virion-en.png US National Institute of Health (redrawn by en:User:Carl Henderson) / Public domain.

play a role in overcoming the immune defenses of the human host. The sequence of events necessary to produce genetic variation by recombination that is adaptively beneficial to HIV are considered next.

For an adaptive benefit of genetic variation to be realized, the two RNA genomes contained in an individual infecting virus particle would have to be derived from separate progenitor viruses of differing genetic constitution. In general, only viruses that have packaged two genetically different RNA genomes can produce a recombinant genome with a genotype distinctly different from that of its parents [44] . For this to occur multiple events are required [44]. These events are: (1) A human host cell would need to be infected by two viruses of genetically different lineages, and the genomes of these two different viruses would have to produce progeny genomes. (2) Two different progeny RNA genomes produced from such an infection would have to be co-packaged into the same progeny virus particle. (3) When this progeny virus infects a new host cell, template-switching would have to occur during reverse

transcription to generate a recombinant DNA copy. (4) The recombinant DNA would then need to integrate into the DNA genome of the infected cell. (5) The recombinant provirus would next have to be able to produce replication-competent virus progeny for the impact of the recombination to be observed.

How often cells in HIV patients are infected by more than one HIV (double-infection) is not known, and it is unknown how often mixed packaging occurs under natural conditions [44, 45]. As discussed above, from 5 to 14 strand-switching recombination events occur in each infection cycle. These events, in most cases, occur between genomes with the same genetic constitution. Thus it is apparent that although recombination can, under some circumstances, produce variation that is adaptive, the great majority of recombination events do not produce significant adaptive variation.

4.2 Recombination as a repair process

Infection by HIV results in chronic ongoing inflammation associated with reactive oxygen species production [46]. Thus a strategy for dealing with oxidative damages to the HIV genome would be adaptively beneficial. Each HIV particle contains two homologous templates, rather than one. Temin [9] considered it likely that recombination is an adaptation for repair of damaged RNA genomes. Also, template-switching by the reverse transcriptase was suggested by Bonhoeffer et al. [47] to be a repair process for dealing with breaks in the ssRNA genome. Copy-choice recombination by the reverse transcriptase could produce a DNA copy of the genome that is free of damage even if both parental ssRNA copies in each virus are damaged. This benefit of recombination can be realized at each infection cycle even if, as is usually the case, the two genomes do not differ, or are closely similar genetically, and little if any new genetic variation will be produced [9, 45]. If recombination in HIV infections is primarily an adaptation for genome repair, the generation of recombinational variation would be an occasional natural consequence, but not the principle driving force, for the evolution of template-switching [47].

4.3 HIV as a model for the transition from ssRNA to dsDNA genomes

Early organisms may have evolved through a stage, like HIV, where their genome in the form of ssRNA was replicated to form a hybrid RNA: DNA duplex which upon further replication formed dsDNA. A laboratory evolved RNA polymerase ribozyme that synthesizes RNA has also been shown to act as a reverse transcriptase to synthesize DNA [48]. A ribozyme like this may have evolved in nature and been instrumental in the transition from the RNA to the DNA world. It could have arisen as a secondary function of an RdRp.

While oxidative stress appears to be a principle damaging stress for the HIV genome, the damaging stresses on organisms that were undergoing the early evolutionary transition from RNA to DNA genomes would likely have been different. The genome damages in the transition from RNA to DNA genomes could have arisen, as described above, from hydrolytic reactions, UV light or environmental reactive chemicals, but undoubtedly there would have been some kinds of significant damages. Thus during the transition from the RNA world to the DNA world there was very probably a continuous need to cope with genome damage. The copy-choice mechanisms that had a repair function in the RNA world may have continued to operate as repair functions during the transition to the dsDNA world. The selective pressure of genome damage on genome repair as the genetic material transitioned from RNA to DNA is discussed further in Bernstein et al. (pgs. 342-345) [8].

5. Recombination in archaea acts in DNA repair

In the previous sections it was proposed that genome repair processes emerged in the RNA world and that, after going through several evolutionary stages, such repair processes were present in organisms with DNA genomes. The archaea are single-celled microorganisms whose genome is DNA. These organisms are regarded as descendants of a form of life that arose subsequent to organisms with RNA genomes but prior to eukaryotes [49].

The evolution of the eukaryotic cell appears to trace back to the establishment of a symbiotic relationship between a host anaerobic archaeal cell and an internalized bacterium capable of aerobic metabolism [50]. The eukaryotic cell emerged at least 1.5 billion years ago [51]. Eukaryotic genes of archaeal origin appear to have a more central role in basic cellular functions than genes of eubacterial origin [49]. Thus the manner in which present day archaea deal with genome damage may throw light on how genome repair processes that arose in the RNA world became adapted for repair in both the archaeal and the eukaryote DNA world.

Recent findings show that cells of archaeal species, particularly *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius,* under stressful environmental conditions that cause DNA damage, aggregate and transfer DNA from one cell to another through direct contact [52, 53]. Exposure of *S. solfataricus* to UV irradiation strongly induces type IV pili formation which facilitates cellular aggregation [54, 55]. This induced cellular aggregation mediates intercellular chromosome marker exchange with high frequency. UV irradiated cultures were found to have recombination rates exceeding those of uninduced cultures by up to three orders of magnitude. The UV-inducible DNA transfer process and subsequent homologus recombination are considered to represent a repair mechanism for maintaining chromosome integrity [54, 56, 57]. Also in *S. solfataricus*, exposure to bleomycin or mitomycin C, agents that cause double-strand breaks and other damages, induces cellular aggregation [54]. In *S. acidoclaldarius,* genes that facilitate DNA transfer are upregulated by DNA damaging UV irradiation [52]. DNA damage can be lethal to a cell unless repaired. DNA transfer between neighboring archaeal cells appears to be an adaptation for aiding survival of nearby (and likely genetically related) damaged cells by facilitating recombinational repair.

The repair capabilities of archaea suggest that ancestral organisms arising early in the DNA world underwent processes that allowed DNA damage in one cell to be repaired by transfer of DNA sequence information from a neighboring cell in order to facilitate recombinational repair.

6. Eukaryotes

Eukaryotes are capable of several different types of DNA repair process:

a. The DNA damage may be enzymatically directly reversed. There are three known direct reversal mechananisms (Yi C) [58]: (1) Photolyase catalyzed direct reversal of UV light-induced photolesions; (2) O^6 alkylguanine-DNA alkytransferase catalyzed direct reversal of a set of of O^6 alkylated DNA damages; and (3) direct reversal of N-alkylated base adducts by AlkB family dioxygenases. Direct reversal mechanisms are specific for a small subset of DNA damages and thus have limited applicability.

b. Single-strand damages may be excised and the proper information restored by copying the other undamaged strand. This can occur by any one of several

well-studied processes. These include mismatch repair (MMR), nucleotide excision repair (NER) and base excision repair (BER) [14]. These processes appear to have arisen in the archaea [59], but are most well understood in the eukaryotes. This option was not available to organisms with ssRNA genomes because the double-stranded state exists only transiently during replication. In any case the enzymes that carry out such repair processes in organisms with DNA genomes are not known to be encoded in the ssRNA virus genomes. Thus this type of mechanism was not likely present during the early evolutionary stages in ssRNA genome containing organisms.

c. Double-strand damages in double-stranded DNA, such as double-strand breaks, can be repaired without the presence of an homologous template by such processes as non-homologous end joining (NHEJ) and microhomology mediated end joining (MMEJ). These processes depend on the duplex nature of DNA but not on strict homology. NHEJ can be accurate if the ends of the DNA in double-strand breaks do not need processing. However, if the ends need processing before rejoining then mutations are very likely to be introduced [60]. MMEJ is inaccurate and is always associated with a DNA deletion [61]. Thus these processes are inaccurate and generate mutations and are not applicable to ssRNA genomes.

d. Homologous recombinational repair is possible when two templates are present and adjacent. Such repair may occur for various types of DNA damage. For double-strand breaks in mitosis, homologous recombinational repair, either by the less common breakage and exchange mechanism or by the more frequently used SDSA (copy-choice) mechanism [11], are the only accurate forms of repair available. Template switching can occur during mitosis when two sister chromatids are present and adjacent after DNA synthesis and before cell division.

During meiosis homologous chromosomes originating from different parents align intimately with each other. This is followed by transfer of sequence information between homologs, homologous recombination. The main mechanism is SDSA (copy-choice recombination), a central characteristic of meiosis (see Section 6.1). Less frequent homologous recombination by breakage and exchange of chromosomes also occurs during meiosis.

Copy-choice recombination is also an important general mechanism for dealing with DNA damages that block the movement of the DNA polymerase during DNA replication (see Section 6.2).

6.1 Meiotic and mitotic recombination

The results of numerous studies in a wide range of eukaryotes indicate that during meiosis a variety of DNA damages are repaired by recombinational repair (reviewed in [62]). In somatic cells, mitotic recombination also facilitates DNA repair. Molecular models of recombination have been revised over the years as relevant evidence accumulated. Our current understanding of recombination reflects the work of several groups of investigators that have provided evidence that SDSA is a major mechanism of recombination [11, 63–65]. Furthermore, SDSA is a type of copy-choice mechanism since it involves switching from one template to another during strand synthesis and the return to the original template after a short distance (compare **Figure 5** to **Figure 2**).

Figure 5 illustrates the series of steps that occur by the meiotic SDSA process in the repair of a double-strand break (DSB) in one chromosome using information

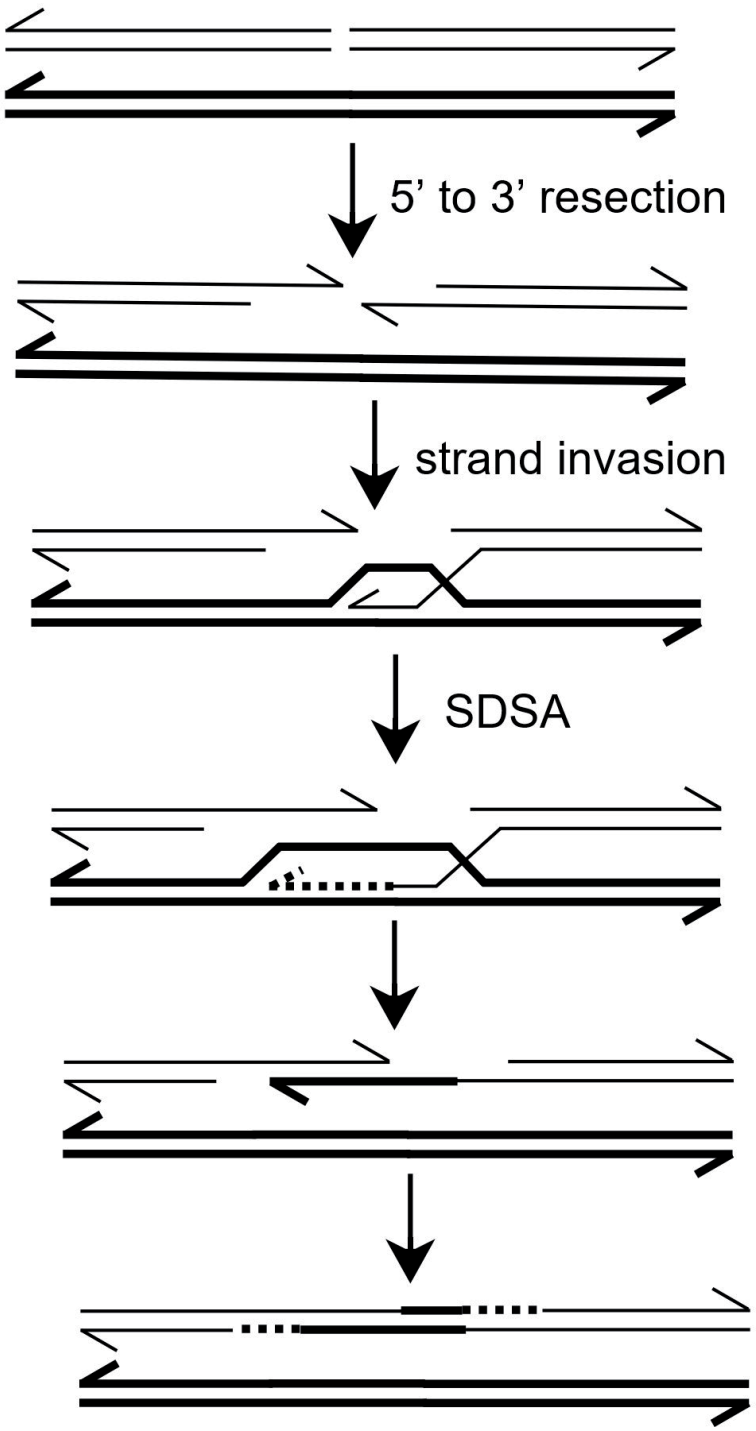

Figure 5.
Synthesis-dependent strand annealing (SDSA) in the repair of a double-strand break.

from an adjacent undamaged homologous chromosome. As shown in the figure, the steps include strand invasion by a broken strand to form a D-loop, the further extension of the strand by DNA synthesis, and then the reassociation of the transferred strand with its original pairing partner. These strand-switching and DNA synthesis events associated with repair of a damage are similar to the copy-choice

recombination described above for ssRNA viruses. Thus a central feature of eukaryotic recombination in meiosis and mitosis, strand-switching copy-choice recombinational repair, may have evolved from the simpler repair-related copy-choice events postulated above for ssRNA protocells based on the known processes in ssRNA viruses. Experimental evidence demonstrating that SDSA is a major recombination pathway in meiosis was presented by McMahill et al. [64].

The process of SDSA can accurately repair genome damage by copying the information lost in a damaged template strand from another intact homologous template strand without the need for physical breakage and exchange of DNA. Evidence bearing on the role of SDSA during meiotic recombination was reviewed by Bernstein et al. [66]. An alternative mechanism for recombinational repair termed the Double-Strand Break Repair (DSBR) model also explains some types of recombination events, but in contrast to SDSA recombination, the DSBR model does require physical breakage and exchange of DNA strands [67]. However,

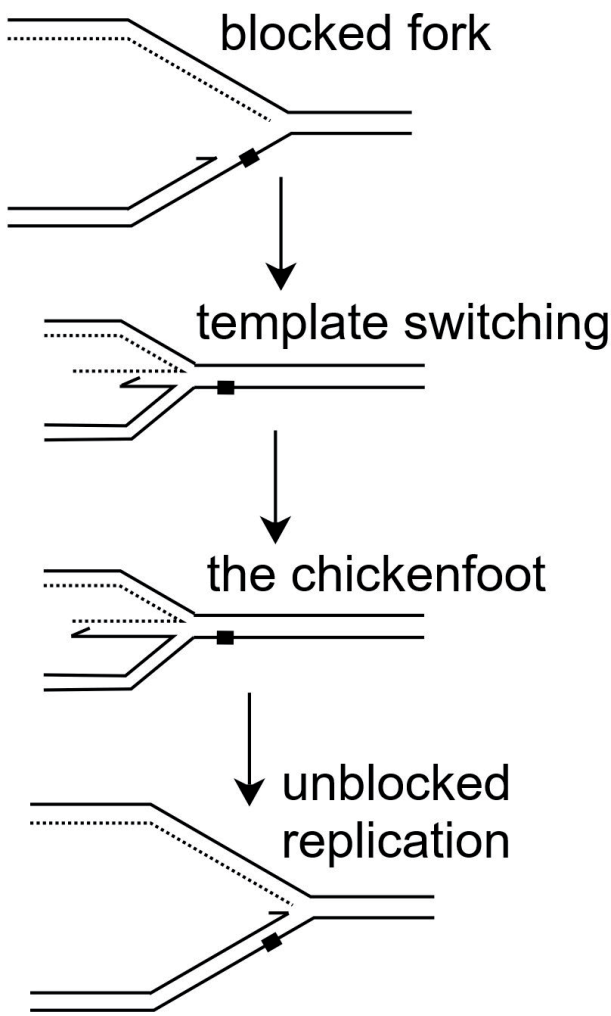

Figure 6.
Bypassing a DNA damage during replication. This mechanism involves reversal of the replication fork, where the newly replicated strands dissociate from their previous templates and anneal to form a cruciform intermediate, known as the "chicken foot" structure. Further replication of the previously blocked strand can then continue, leading to the bypass of the damaged site.

both the SDSA and DSBR models include a step in a which a DNA strand switches at a site of damage from one complementary partner strand to another and then continues synthesis with the new partner as template. Thus both models have elements of copy-choice recombination.

With respect to mitotic recombination in somatic cells, Andersen and Sekelsky [11] reviewed evidence that DSBR is a minor pathway for recombinational repair, and that the SDSA model appears to describe mitotic repair more accurately.

6.2 DNA replication

During DNA replication, a DNA damage in a template strand may be present and act as roadblock to the movement of the DNA polymerase as it extends synthesis of a new complementary strand. A blocked replication fork may be accurately bypassed by the mechanism illustrated in **Figure 6** [12, 13]. When movement of the replicative polymerase is blocked by a damage, the polymerase can switch template strands (mediated by a helicase) [12, 13] to form a structure referred to as a "chickenfoot" intermediate. As synthesis of the new strand proceeds along the alternate template it synthesizes the DNA region that is complementary to the damaged site in its original partner strand. The newly forming strand may then unwind and then re-associate with its original partner to continue synthesis along its original track. Polymerase-mediated strand-switching to deal with a damaged template during DNA synthesis appears to be an important general mechanism in eukaryotic cells [64]. This mechanism can be regarded as a type of copy-choice recombinational repair, and it too may have evolved from simpler copy-choice processes in ssRNA protocells.

7. Conclusions

Given the copy-choice genomic repair mechanism present in today's ssRNA viruses, it appears that copy choice as a repair process may have emerged as early as 3.5 to 2.5 billion years ago when RNA was apparently the only genetic material. It is possible that the capability for strand-switching was a property of the earliest ribozyme polymerases.

In early protocells, the ssRNA genomes may have been segmented, as some ssRNA viruses are in the present day. Two protocells with damaged segmented genomes could have been able to generate undamaged progeny after fusion and then reassortment of segments. Present day ssRNA segmented genome viruses can repair damage in their genomes through both copy choice and segment reassortment.

The early stages of the evolution of genome repair proposed here are based on known capabilities of extant RNA viruses. Currently it is not known if these RNA viruses are the actual evolutionary descendants of early RNA life forms, or if they arose later. It has only been assumed here that the problem of dealing with damage to an RNA genome arises in the two cases, and that the solutions to this problem would be similar.

The earliest ssRNAs that formed folded structures that acted as ribozymes can be designated plus (+) strands. Such a ribozyme strand could have had polymerase activity and acted as an RdRp. The progeny ssRNAs that it synthesizes would be complementary to the corresponding parental (+) strands, and can be designated minus (−) strands. During the synthesis of (−) strands template-switching may have occurred.

When the ssRNA genome evolved to a dsDNA form, elements of the earlier copy-choice recombinational repair processes appear to have been retained. In addition, the informational redundancy inherent in double-stranded DNA allowed the emergence of novel excision repair pathways (MMR, BER and NER) that could use the information in one strand to repair damage in the other strand. Other mechanisms (e.g. NHEJ and MMEJ) also emerged to deal with double-strand damages when an homolgous genome was not available. As eukaryotes evolved from unicellularity to multicellularity, and within an organism the germline became segregated from the somatic cell line, copy-choice recombinational repair was retained in the germline as a central feature of meiosis. Recombinational repair was also retained during mitosis, and as a general process for overcoming damage roadblocks to DNA replication.

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Chapter 3

Super-Resolution Radiation Biology: From Bio-Dosimetry towards Nano-Studies of DNA Repair Mechanisms

Jin-Ho Lee and Michael Hausmann

Abstract

Past efforts in radiobiology, radio-biophysics, epidemiology and clinical research strongly contributed to the current understanding of ionizing radiation effects on biological materials like cells and tissues. It is well accepted that the most dangerous, radiation induced damages of DNA in the cell nucleus are double strand breaks, as their false rearrangements cause dysfunction and tumor cell proliferation. Therefore, cells have developed highly efficient and adapted ways to repair lesions of the DNA double strand. To better understand the mechanisms behind DNA strand repair, a variety of fluorescence microscopy based approaches are routinely used to study radiation responses at the organ, tissue and cellular level. Meanwhile, novel super-resolution fluorescence microscopy techniques have rapidly evolved and become powerful tools to study biological structures and bio-molecular (re-) arrangements at the nano-scale. In fact, recent investigations have increasingly demonstrated how super-resolution microscopy can be applied to the analysis of radiation damage induced chromatin arrangements and DNA repair protein recruitment in order to elucidate how spatial organization of damage sites and repair proteins contribute to the control of repair processes. In this chapter, we would like to start with some fundamental aspects of ionizing radiation, their impact on biological materials, and some standard radiobiology assays. We conclude by introducing the concept behind super-resolution radiobiology using single molecule localization microscopy (SMLM) and present promising results from recent studies that show an organized architecture of damage sites and their environment. Persistent homologies of repair clusters indicate a correlation between repair cluster topology and repair pathway at a given damage locus. This overview over recent investigations may motivate radiobiologists to consider chromatin architecture and spatial repair protein organization for the understanding of DNA repair processes.

Keywords: ionizing radiation, DNA damage, DNA repair, super-resolution localization microscopy, chromatin nano-architecture, spatial repair protein organization, molecular cluster analysis, molecular topologies

1. Introduction

Past efforts in epidemiological (nuclear power industry, atomic bomb explosions, nuclear reactor accidents, etc.) and clinical (diagnostic imaging, radiation

oncology, radiation therapy planning etc.) research strongly contributed to the current understanding of ionizing radiation effects on human organs, tissues, and cells [1, 2]. In principle radiation biology is based on effects of instantaneous (10^{-18} s) [3], stochastic damaging interactions of ionizing radiation with cells, a main target being the genetic material, i.e. chromatin in the cell nucleus [4]. In this context, radio-sensitivity and radio-resistance as opposing terms describe the extent of individual cellular susceptibility or 'response' upon radiation exposure which are highly dependent on physical (e.g., radiation type, dose, dose rate, etc.), chemical (e.g., hydroxyl radicals, etc.) and biological (e.g., developmental and proliferative state of the affected cell type) factors. As the overall organismal radiation response results from the entirety of all individual radiation responses on the single cell level, deeper understanding of the underlying, complex molecular mechanisms and dynamics of radiation induced DNA damaging and repair on the cellular level is highly relevant for fundamental and applied radiation biology (for review see [1, 2, 5]).

Hence, cytometric analyses based on fluorescence microscopy have become the method of choice to study damaging effects of ionizing radiation and DNA repair. This has contributed a lot to today's knowledge. However, conventional fluorescence microscopy is limited to average lateral resolutions around 200 nm laterally and 600 nm axially [6] and thus is limited to the bulk analysis of molecular cellular processes and structures. In parallel, super-resolution fluorescence microscopy techniques have rapidly evolved during the last few decades and turned out to be powerful tools to study cellular structures and molecular architectures on the nanoscale (for review see [6–8]). Methods based on stochastic reversible photobleaching [9–15] of single molecules called Single Molecule Localization Microscopy (SMLM) [16] reach effective resolutions down to 10 nm and have become popular among modern super-resolution imaging techniques as their realization is highly practical and straightforward using established specimen preparation methods of standard fluorescence microcopy [17]. As such resolutions allow the detection of single molecules, such as nucleosomes [18], proteins [19, 20], receptors and junction proteins [21, 22], or even single chromatin loops [23] etc., super-resolution microscopy opens new avenues for the research of radiation induced damaging and repair processes [5, 24].

With this article, we attempt to introduce the novel SMLM approach to radiation biophysics and radiation biology. We start with a brief summary on the basics of ionizing radiation, induction of DNA damage and damage repair mechanisms, to follow up with some standard radiobiology analysis methods. We further provide an overview of the working principles of selected sub-diffraction microscopy techniques with a focus on SMLM. Finally, the successful application of localization microscopy in radiation biology research is demonstrated along examples of current works.

2. Effects of ionizing radiation on cells and cell nuclei

Ionizing radiation penetrates through material and deposits enough energy to ionize molecules or atoms by liberating electrons. The effects of ionizing radiation on biological materials are highly dependent on the dose, the dose rate and type of radiation. In living cells, ionizing radiation hits all kinds of biomolecules, such as desoxyribonuleic acids (DNAs), aminoacids (proteins), lipids (membranes), carbohydrates, etc. However, most harmful consequences to living organisms show damages inflicted to their genomic DNA, especially in the form of DNA double-strand breaks (DSBs) [25, 26]. Especially follow-up effects of false strand repair may lead to significant dysfunctional development as for instance tumor genesis.

2.1 Ionizing radiation

Ionizing radiation (IR) includes all high energy/speed (> 1% speed of light) ions (e.g. carbon ions), atom nuclei (e.g. alpha particles), subatomic particles (e.g. beta particles, protons or neutrons) and high-energy electromagnetic waves (e.g. high energy ultraviolet (UV) rays, X-rays and gamma rays), that carry enough energy to directly or indirectly ionize atoms or molecules by liberating electrons from them, and to break molecular bonds [27].

The most common types of ionizing radiation occurring under environmental circumstances are caused by radioactive decay and can be divided into three groups: alpha, beta and gamma radiation [27]. Alpha radiation is made up of particles comprising two protons and two neutrons (helium nucleus) that carry energies in the range of up to several MeV. Due to its large particle size, alpha radiation has the lowest penetration depth through biological materials and the highest energy deposition per distance traveled. Beta particles are made up of electrons or positrons, thus exerting higher penetration depths and lower energy depositions compared to alpha particles. Gamma rays are high energy electromagnetic waves that exhibit the highest penetration and compared to particles, lowest energy deposition per track unit in biomaterials among these three types of IR. Due to the dispersed and low energy deposition in tissue, gamma and beta radiation are often referred to as low linear energy transfer (LET) radiation, whereas alpha particles belong to high LET radiation [27].

For clinical diagnosis and therapy in radiology or radiation oncology [28, 29], typically artificial radiation sources are applied, as for instance to produce X-rays in the energy range of keV to MeV, electrons and positrons, protons, and heavy ions (carbon or nitrogen). Like alpha particle, protons and heavy ions belong to high LET radiation. The advantage of protons and especially heavy ions is based on the characteristic absorbance with a Bragg peak at the end of the particle track where most of the particle energy is deposited. This energy positioning peak can exactly be localized in the tumor volume so that intact cells and tissues in the tumor surroundings are excluded from radiation damaging [30].

2.2 Dose measures

The absorbed dose D of ionizing radiation is quantified by the amount of energy deposited per unit mass of the penetrated material and is measured in units of Joule per kilogram (J/kg) or Gray (Gy) [27]. It describes an universal energy absorption for all types of ionizing radiation and is most commonly used in radiophysical research, whereas a radiation type specific dose also called the equivalent dose H calculated by multiplication with a weighting factor W_R (e.g. $W_R = 1$ for gamma radiation and $W_R = 20$ for alpha radiation) is often used in radio-biology, radio-medicine, or radiation protection and safety. The equivalent dose can be further weighted by a tissue weighting factor W_T to result in the effective dose E, which describes radiobiological effects considering the used radiation type and the tissue/organ of interest. Both, the equivalent dose and effective dose are quantified in units of Sievert (Sv) and do not represent physically measurable quantities but rather a value based on clinical and epidemiological outcome that is typically used in radiation safety [31].

2.3 Damages of DNA induced by ionizing radiation

Among all kinds of ionizing radiation induced biological effects, damages to chromatin especially the DNA molecules in the nucleus of cells are thought to be the

most severe with respect to cellular survival and carcinogenesis [2, 5, 32, 33]. DNA base oxidation, single strand breaks (SSBs) and double strand breaks (DSBs) are the most common ionizing radiation induced damages to the DNA molecule, that affect genome integrity and DNA biochemistry [34].

DSBs of DNA belong to the most complex and severe types of DNA damages as they directly affect genome integrity and the way of cellular survival [35–37]. Single strand breaks (SSBs) induced by ionizing radiation and base damages occur more frequently than double strand breaks [34]. It can be estimated to about 40 DSBs/Gy and about 1,000 SSBs/Gy. SSBs are less severe to genome integrity as an intact template strand is still available for complementarity-aided, error-free repair of the lesion. But DSBs are also simply formed by two or more opposing SSBs in close proximity or combinations of different DNA damage types [26].

Induction of DSBs in native chromatin is rapidly followed up by phosphorylation of nearby histones of the H2A variant H2AX at serine residues at position 139 [38]. This results in the generation of plenty γH2AX molecules around a DSB damage site, where about 2 Mbp of DNA are usually phosphorylated [39]. This leads to the formation of focus structures of sizes in the range of micrometers, which can be visualized under a fluorescence microscope [40]. These phosphorylated histones serve as signal and anchor points for many downstream recruited proteins of certain DNA damage response and repair machineries [41]. As the number of γH2AX foci is quantitative for DNA damage, counting of specifically labeled foci has been established as a measure for dose-efficiency and correlated to cell survival [42].

Single ionizing radiation induced DNA lesions can be caused by direct or indirect hits [43]. Ionizing radiation penetrating through a cell nucleus can hit and ionize atoms in a DNA molecule itself with a certain probability. However, the most prominent primary reaction underlying all ionizing radiation induced DNA damages is the radiolysis mediated formation of reactive oxygen species (ROS), e.g. •OH radicals, O2•- radicals and H2O2, which can further inflict reducing damage and thus lesion to the DNA [44]. Ionizing radiation, especially high LET radiation, is known for its property to efficiently induce highly complex damages to DNA. Such complex DNA damage sites composed of multiple lesions in close proximity on both strands are also termed locally multiple damage sites (LMDS) [45].

3. DNA double strand break repair mechanisms

Living organisms developed highly efficient and customized ways to repair the severe damages inflicted to their genome. The DNA DSB sites are rapidly (within seconds to minutes) recognized and marked by proteins of an initial response, which serve as signals and docking sites for more specialized proteins of DNA repair pathways. The fate of repair type depends on the concerted presence of pathway specific damage response proteins [1, 2, 46–48]. The main two ways by which cells respond to DNA double-strand breaks are non-homologous end joining (NHEJ; also called canonical NHEJ = cNHEJ) and homologous recombination (HR). NHEJ mediated DSB repair is fast and can be error-prone, but it can be flexibly performed throughout all cell cycle phases. HR works error-free, but is mostly restricted to late S and G2 phases as a homologous sister chromatid is required as a repair template [49–52]. Recent data, however, have suggested that active genes may employ HR also in G1 phase, by utilizing the nascent RNA as a template for precise repair (reviewed in [53]). As the DNA-end resection is inhibited in G1 cells, an alternative model with cNHEJ taking the advantage of the same principle (RNA-templated repair) has also been proposed. Interestingly, DNA repair by HR is preferred in lower eukaryotic life forms, whereas NHEJ is predominantly observed

in mammalian organisms. Alternative low abundant DSB repair pathways are the alternative end joining pathways (a-NHEJ; also called back-up EJ), micro-homology mediated EJ (MMEJ) and single strand annealing (SSA). One main difference between all DSB repair mechanisms is the extent of initial DNA end-resection at the damage site [26, 54–59]. The DNA damage response (DDR) against DSBs is subject to intensive radiobiological investigation and fluorescence microscopy of in situ DSB repair proteins serves as state of the art biological dosimetry.

3.1 The initial response

After the induction of a DSB, damage response proteins are rapidly recruited and accurately determine the fate of the DSB towards a repair pathway that best deals with the damage site in a certain genomic and cellular context. The chromatin remodeling p53-binding protein (53BP1) protects the break site from extensive end resection [60], thereby promoting repair by non-homologous end joining [61], whereas BRCA1 facilitates extensive end resection for repair by homologous recombination [52, 62].

3.2 Non-homologous end joining

The NHEJ repair pathway is initiated with binding of the Ku70-Ku80 heterodimer complex to the DNA ends of the DSB site, which serves as a linkage between damage site and further damage response proteins [61, 63]. In a second step, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited to the Ku complex forming the DNA-PK complex. On-going recruitment of X-ray complex (XRCC4)/DNA Ligase IV (X4LIG4) complex and XLF to the DNA-PK complex forms the core NHEJ complex [64]. DNA-PKcs sterically protects the break site for repair and phosphorylates other repair proteins [65, 66] and H2AX [41]. Furthermore, DNA-PK auto-phosphorylation results in a conformational change of the core complex, thereby enabling DNA end processing by nucleases and dissociation of the DNA-PKcs subunit [67, 68]. Finally, ligation of the DNA ends is mediated by the X-ray complex (XRCC4)/DNA Ligase IV (X4LIG4) complex and XLF [69–71]. Artemis endonuclease [72, 73], polynucleotide kinase (PNK) [74], DNA polymerase (pol) μ and λ can be additionally involved in NHEJ repair depending on the chemical properties of the DNA damage site [75].

3.3 Homologous recombination

To initiate repair by HR, the free damaged DNA ends at the DSB site must be sensed and bound by a protein complex comprised of MRE11, RAD50 and NBS1 (MRN complex) [76]. Next, the protein kinase Ataxia Telangiectasia Mutated (ATM) [77] is recruited to the MRN complex at the damage site [78], which auto-phosphorylates and phosphorylates components of the neighboring chromatin. Most prominent phosphorylations are those of the histone variant H2AX (γH2AX), one of the earliest and a very sensitive marker of cellular response to DSBs [38]. End resection is initiated by the single-strand endonuclease and exonuclease activity of the Mre11 protein [52, 79] of the MRN complex. RAD50 further stimulates Mre11 nuclease activity and Nbs1 interacts with CtIP [80], another protein that is essential for the initiation of MRN complex mediated end resection [81]. Exonuclease 1 (Exo1) and Dna1/BLM are recruited by CtIP to continue end resection [82–84] until it gets attenuated by RPA coating of resected ssDNA ends [85]. BRCA2 in combination with BRCA1 and PALB2 dismantles the ssDNA ends from RPA coats enabling binding and forming of the RAD51 nucleoprotein filament, which stimulates

homology search and strand invasion [86]. Sister chromatid strand recombination via Holiday junctions is further facilitated by RAD54A and its paralog RAD54B [87, 88], finally resulting in conservative repair of the DNA lesion.

3.4 Alternative repair pathways

a-NHEJ or b-NHEJ has been described in slightly different ways which are not well distinguished [56–58]. Mostly, in the presence of short micro-homologies (>4 bp) after CtIP-MRN mediated end resection, repair via an alternative end joining (MMEJ) can take place [89]. This is initiated by Poly(ADP-ribose) polymerase 1 (PARP1) and followed up by DNA polymerase θ (pol θ) mediated strand extension starting at the paired micro-homology site. Ligase1 and Ligase2 are supposed to perform the final ligation of DNA ends [90, 91].

When the damage site is flanked by larger regions with non-allelic sequence homologies, repair by single-strand annealing is also possible. The absence of Ku proteins and even more extensive end resection to expose the homologous regions as single strands are necessary for SSA repair [92]. Again, RAP binding to the resected ends promotes RAD52 mediated annealing of homologous regions. Nuclease XPF-ERCC1 trims the remaining non-homologous overhangs and DNA Ligase 1 connects the DNA ends [93].

Several studies indicate, that damaged genomic Alu elements use micro-homologies for single-strand annealing, thereby often leading to translocations [94, 95]. Such nonconventional damage repair processes might explain a significant portion of the observed deletion events associated with malignancies [59]. In fact, in vitro model systems could already demonstrate Alu mediate non-allelic homology dependent DSB repair [96].

4. Radio-sensitivity and biological dosimetry

Radio-sensitivity can be assessed on different scales ranging from whole organs and tissues over single cells to molecular markers and mechanisms. Based on clinical and experimental findings on DNA damage induction and response mechanisms, the dose effect of ionizing radiation on biological material is commonly described with a linear-quadratic model [97, 98]. At low dose ranges (below 1 Gy), radiation damages are supposed to linearly increase with the applied dose, whereas at higher doses the probability for multiple hits increases and complex DNA damage spots dominate.

In this chapter, we summarize some established methods to study cellular and molecular effects of ionizing radiation. In the past, sophisticated assays were developed to detect and quantify radiation induced damages to the cell and nuclear DNA ranging from techniques to assess overall cell survivability, large-scale chromosomal damages and rearrangements over sensitive detection of DNA break sites to modern state of the art technologies that can visualize the formation of damage foci in situ with the help of suitable biomarkers.

a. Colony forming assays (CFAs) based on clonogenic survival (also called clonogenic assay) (see [99] and citations therin) as a method to quantify cell survival after radiation exposure was firstly described by Puck and Marcus in 1956 [100]. CFAs measure the ability of cells to divide after treatment with agents that impair cellular reproduction, e.g. radiation (**Figure 1**). Since then, colony forming assays were improved for many different cell types and are widely used as a "gold standard" in radiobiological studies. In practice, irradiated

cells are plated at higher dilutions so that single cells are well separated. Upon incubation colonies form each originating from a single cell. Thereby, colonies comprising 50 cells or more are considered for estimating the survival fraction. Treatments such as exposure to ionizing radiation damages the reproductive survivability of cells and thus results in a lower number of colony formation events at the same number of plated cells [103].

b. A fraction of ionizing radiation induced DNA double strand breaks results in heavy genomic rearrangements that can be detected on metaphase chromosomes. False rearrangements of multiple centromeric regions between chromosomes can lead to dicentric, acentric, centric ring conformations [104, 105] that can be visualized under a conventional fluorescence microscope (**Figure 2a**). The good reproducibility and comparability lets the so called dicentric assay stand among the gold standards of biological dosimetry [106]. Nowadays dicentric assays are further developed towards biological dosimetry in the low dose range (< 500 mGy).

c. The micronucleus test is a method to assess and detect chromosomal breakages in interphase nuclei developed by Schmid et al. in 1975 [107]. Radiation damage can result in major chromosomal aberrations and loss on the centromeric region by wrong rearrangement of DNA double strand breaks (**Figure 2a**). These heavily damaged acentric chromosomes can form separated globular structures outside the main nucleus in interphase. As micronucleus formation can be readily detected in interphase nuclei, analysis can be performed much faster and serves as an efficient alternative for the analysis of instable chromosome aberrations [108].

d. In 1984, Ostling and Johanson published a micro-electrophoresis technique that could visualize DNA damages in single cells [109]. First, cells are embedded in agarose and lysed with non-ionic detergents under high salt concentrations, so that only nucleoids (supercoiled DNA loops attached to the nuclear matrix) remain. Ionizing radiation-induced breaks relax and locally unwind the supercoiled DNA structure, thereby partly linearizing the strand at the break site. When voltage is applied linearized DNA segments (SSB) protrude from the nucleoid and migrate faster towards the anode while the nucleoid core remains assembled. The nucleoid and its tail resemble a comet, when stained with 4′,6-diamidino-2-phenylindole (DAPI) or other quantitative DNA fluorescence dyes and visualized under the fluorescence microscope, thus leading to the term 'comet assay' [110, 111]. Alkaline variations of the comet assay

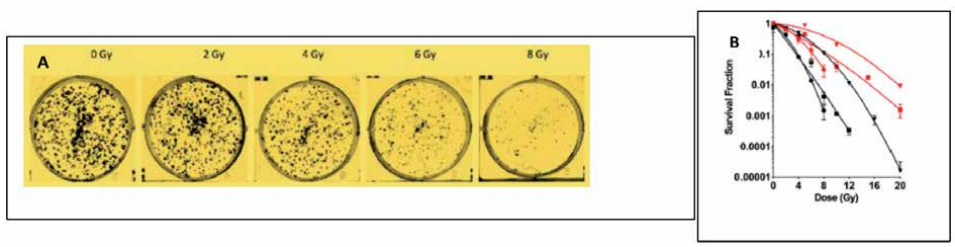

Figure 1.
(A) Example of colony formation after cell exposure to different doses of X-ray irradiation. (B) Typical survival curves for cell colonies after irradiation with different types of photon and particle radiation. Linear-quadratic cell survival curves are fitted and can be used to calculate the relative biological effectiveness. Note: These figures are modified and were originally published under CC BY license in [101, 102].

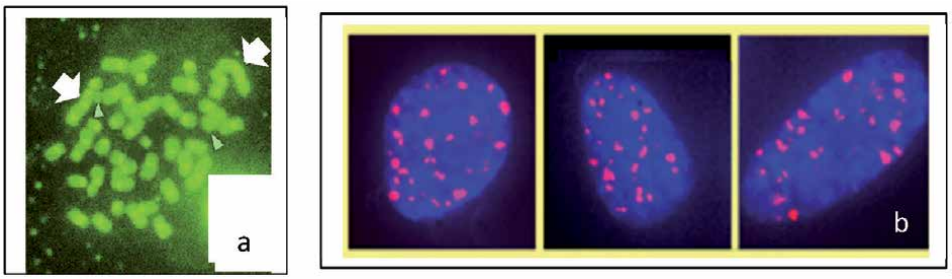

Figure 2.
(a) Example of a lymphocyte metaphase plate with centromeres highlighted by FISH. The cells were irradiated with 3 Gy X-rays. The big arrows show two dicentric chromosomes. The small arrow heads label the corresponding acentric fragments. (b) Typical examples of fibroblast nuclei (stained with a specific DNA dye) with γH2AX foci after exposure to high dose irradiation. The foci are labeled by specific antibodies.

[110] were introduced, that can detect DNA damages over an extended dose range (0.25 Gy to 2 Gy) than under neutral pH conditions (1 Gy - 3 Gy) [111]. Modern approaches extend the method by automatization of experimental procedures and image analysis [112–114], thereby enabling statistically robust high-throughput detection of DNA damages for potential clinical applications.

e. DNA damage response proteins like γH2AX, 53BP1, RAD51 etc. accumulate at initial damage sites and rapidly form foci-like structures in the nucleus (see for example **Figure 2b**). Antibody staining and fluorescence microscopy of such damage response proteins is an established tool to visualize and quantify DNA damage repair foci at single cell resolution. One advantage of this technique is the ability to assess molecular dynamics of DNA damage repair by visual observation of foci formation at different time points after irradiation.

5. Super-resolution radiation biology

Fluorescence microscopy of potent marker labels is a powerful analysis tool to assess cellular effects of ionizing radiation on the single cell level by optical examination. Due to past efforts, a myriad of fluorescent probes exists for the molecular labeling of almost any known biological target structure (e.g. specific antibodies against γH2AX, 53BP1, MRE11, RAD51 or other repair proteins as well as against heterochromatin or euchromatin etc.). This opens the door to analyze molecular mechanisms underlying fundamental biological functions by optical investigation, e.g. DNA damage response and repair dynamics upon ionizing radiation exposure [1, 2, 5].

5.1 Super-resolution microscopy

A variety of novel super-resolution microscopy techniques were invented in the last few decades [115]. With the help of novel super-resolution microscopy techniques, the molecular effects of ionizing radiation in single cells can be studied on the nanoscale. Nano-labeled molecular structures can be resolved in biological specimens down to a precision of 10 nm (1/50 of the wavelength of visible light), which is in the range of single nucleosomes, antibodies, receptors, etc. (see for example [20, 21]).

In order to improve the resolution in light microscopy, a prerequisite is to circumvent the diffraction limit of light, a physical phenomenon firstly described by

Ernst Karl Abbe and John William Strutt, 3rd Baron Rayleigh, during the late 19th and early 20th century [115]. In diffraction limited fluorescence/light microscopy, the Abbe or Rayleigh criterion (**Figure 3**) is commonly used to define a resolution measure describing the minimal distance D between two point-like light sources with wavelength λ that can be resolved:

$$D = 0.61\lambda / NA \quad (1)$$

Therein λ is the wavelength and NA the Numerical Aperture of the objective lens (NA = n sin(α/2); n = refraction index; α = lens aperture angle). Conventional fluorescence microscopy techniques that use objective lenses with high numerical aperture NA (≥ 1.4) are available today. In confocal laser scanning microscopes, they typically achieve resolutions down to 200 nm in lateral and 600 nm in axial direction. However, modern super-resolution microscopy using the same objective lenses circumvent this physical limit by sophisticated interaction with fluorescence signals so that they can visualize biological specimen down to resolutions in the order of 10 nm, which is in the range of single nucleosomes, antibodies, receptors, etc. [16].

A complete overview of super-resolution microscopy techniques is beyond the aim of this article. However, we want to mention some meanwhile very well established ones:

Sophisticated near-field super-resolution methods, e.g. total internal reflection fluorescence (TIRF) microscopy (TIRFM) [118, 119] or near-field scanning optical microscopy (SNOM, NSOM) [120, 121], belong to the first techniques breaking the diffraction limit by novel techniques working in the optical near field of fine crystal tips probing the specimen without an microscope objective lens. Unfortunately, near-field techniques are technically restricted to the visualization of surfaces of cells, membranes or isolated organelles [122–124].

More recently evolved far-field super-resolution fluorescence microscopy techniques use objective lenses available from establishes microscope manufactures and can be separated into two principle approaches. The first is based on the spatially modulated excitation of fluorophores, e.g. by point spread function engineering as in stimulated emission depletion (STED) [125] or by excitation through a series of illumination patterns as in structured illumination microscopy (SIM) [126]. A second group of super-resolution techniques is based on optical isolation of fluorescent molecules through switchable intensities [17] or intrinsic differences in spectral signatures [127]. The latter techniques often referred to as single molecule localization microscopy (SMLM) in general, can be practically implemented with customary microscope parts and standard objective lenses [16]. Spectral precision distance microscopy (SPDM) an early development of the 1990s [128] is the one and only localization microscopy method, that establishes optical isolation of molecular labels through constant differences in absorption and emission spectra of different fluorophores, that are applied in a combinatory labeling strategy [127, 129]. Most localization microscopy methods, however, rely on stochastic spectral modulations of single fluorophore molecules, such as photo-activated localization microscopy (PALM) [12], fluorescence PALM (FPALM) [13], stochastic optical reconstruction microscopy (STORM) [15, 130], direct STORM (dSTORM) [131], ground state depletion microscopy followed by individual molecule return (GSDIM) [132], SPDM with physically modifiable fluorophores ($SPDM_{phymod}$) [14, 17], etc. In the following chapters, we will describe single molecule localization microscopy in more details as being applied in radiation biophysics and we will provide examples indicating wide applications in nano-probing biomolecules and molecular mechanisms.

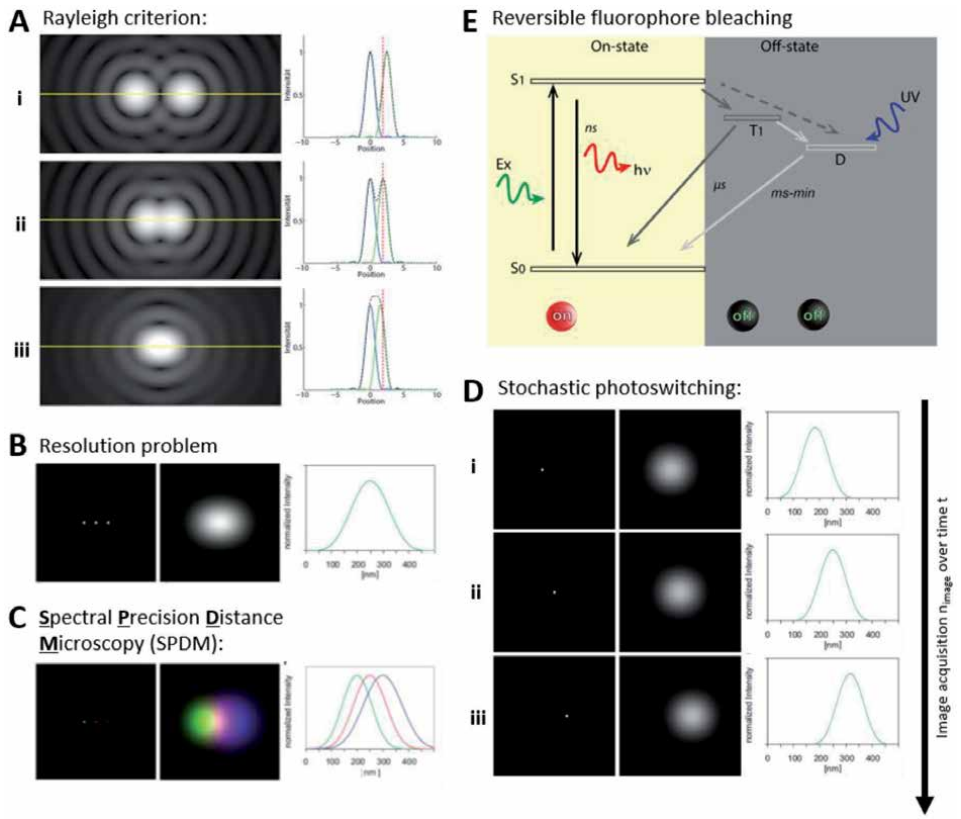

Figure 3.
Rayleigh criterion for the diffraction of two point-like light sources and single-molecule localization microscopy techniques to circumvent the diffraction limit. (A) The resolution limit of two adjacent point-like sources of light is defined by the distance between these two light points; the first intensity minimum of one light point overlaps with the main intensity maximum of the other light point. (B) The diffraction limited resolution of fluorescence microscopy illustrated by an example of three point-like signal sources within a distance below the resolvable range. (C) Working principle of SPDM by spectral isolation of labeling molecules. Here the spatial positions of three point-like fluorescent light sources can be separated by three different colors green, red and blue (from left to right). (D) Working principles of most single molecule localization microscopy methods rely on spectral modulation that switches most fluorophores into a dark state in a stochastic manner. Thereby, detection of only a sparse subpopulation of labels that are either totally isolated or lie apart at distances greater than the diffraction limit is possible. A series of acquisitions, each representing another stochastic sparse subpopulation of signals, can be summarized to result in a complete image below diffraction limit. (E) Minimal Jablonski diagram showing the electronic states and transitions involved in the intrinsic stochastic blinking of fluorophores. Note: These figures are modified and were originally published under CC BY license in [116, 117].

5.2 Single molecule localization microscopy for radiation biophysics

Single molecule localization microscopy is one the most popular super-resolution techniques, because it can be practically realized with standard optical setup and standard specimen preparation methods using commercially available fluorophore labels. Fundamental to all SMLM techniques is the stochastic sampling of signals. The intrinsic blinking nature of a variety of available fluorophores at excitation with high laser powers (in the range of several kW/cm^2), enables SMLM with conventional dyes like GFP / YFP, Alexa488, Alexa568, etc. [16, 17, 133].

Apart from conventional fluorescence, which is based on rapid, repetitive excitation (10–15 s) and red-shifted emission (10–9 s) between the ground state S_0 and excited singlet state S_1, fluorescent molecules additionally undergo inter-system crossing (ISC) [134] from S_1 to dark triplet states T_1 [135]. From there, fast

relaxation (10–3 s) to the original S_0 ground state enables re-entry to new cycles of normal fluorescence (**Figure 3E**, left). Further transition from the T_1 state into a second dark state D also occurs, which takes longer (ms to min) to recover to the ground state S_0 (**Figure 3E**, right) [116, 136]. This reversible photobleaching via the long lived dark state D results in a limited number of stochastically blinking fluorochromes at resolvable time scales that can be used in single molecule localization microscopy to determine sub-diffraction positions of single fluorescing molecules [133].

For image acquisition, a time series of raw diffraction limited images (several hundreds to a few thousand) from the same region of interest are registered and efficiently searched for blinking events under a user-defined intensity threshold to discriminate signals from background. Then, the intensity profile of each blinking event is fitted by a Gaussian curve and the barycenter point of the signal source is calculated. Notably, the localization precision of such a point merely depends on its intensity/background ratio [137].

A major advantage of SMLM approaches lies in the data format. The point matrix containing the lateral x and y coordinates of each localization signal allows all kinds of mathematical and statistical analyses (**Figure 4**). Most prominent are analyses based on Ripley's point-to-point distance information which can be used for the elucidation of signal densities, cluster formation, and spatial organization of labels [139]. Recently, novel mathematical approaches like persistent homology determinations were introduced to investigate topological similarities [138]. Computation of the coordinate matrix into an image with user-defined resolution and visual enhancements is then possible. If provided, multi-color analyses on the single molecule level can be performed to study more complex molecular mechanisms and dynamics.

5.3 Applications of single molecule localization microscopy in radiation biophysics and biological dosimetry

5.3.1 γH2AX clustering and chromatin arrangements at DNA damage sites

Phosphorylated histone variant γH2AX molecules at the site of DSBs and their accumulation into γH2AX-foci are well-established markers of DNA damage response and repair. Most recent studies performed SMLM of γH2AX specific antibodies in HeLa cells that were exposed to different doses of γ-radiation and fixed at different time points after radiation exposure [140]. Quantitative analysis resulted in a linear quadratic increase in measured γH2AX localization signal points and cluster numbers with increasing doses of radiation exposure (**Figure 5A**, **B**). With increasing repair time, the number of γH2AX clusters decreases; thereby successfully demonstrating repair dynamics and cell recovery by γH2AX-cluster relaxation on the molecular level. As dose responses and molecular dynamics for γH2AX clusters and raw γH2AX signal points well correlate with past observations, this study can serve as a benchmark standard for future super-resolution radiobiology experiments.

Similar studies indicated that the γH2AX cluster size remained constant during repair also at later times post irradiation, i.e., at later times only the number of clusters reduced. This typical size was about 400 nm in diameter after photon irradiation and nearly independent from dose or the cell types analyzed [140, 141]. For α-particle irradiation from radioactive decay [142], the γH2AX cluster size along the particle track was about 200–300 nm; this size could be also observed for γH2AX clusters induced by environmental stress as for instance the deficiency of folat during long time culturing [143].

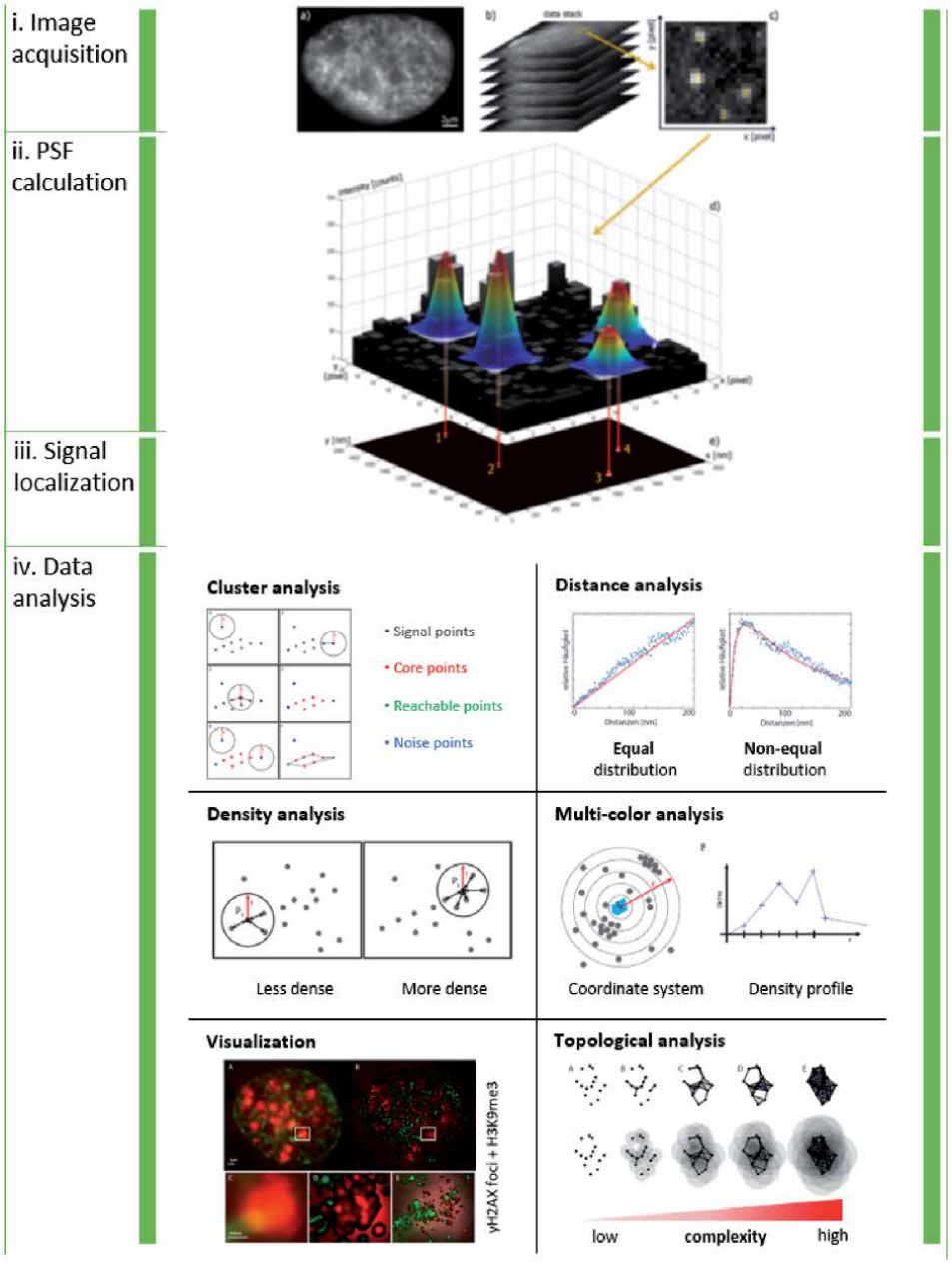

Figure 4.
General workflow of single molecule localization microscopy and data analysis. Serial images are acquired from the same region of interest (i). The point-spread function of each blinking event in each single image is gauss fitted to estimate the intensity maximum (ii), which represents the idealized lateral coordinates of the signal source (iii). The result is a data table containing the coordinates of all detected signal points. The matrix representation of data allows mathematical and stiatistical analysis of clustering, distance distributions, signal densities, multi-color signal distributions, enhanced visualization and topology (iv). Note: These figures are modified and were originally published under CC BY license in [16, 117, 138].

After exposure to photon radiation (different doses and energies) the SMLM analysis of heterochromatin around γH2AX clusters using specific antibodies against H3K9me3 methylation sites, revealed a fast relaxation of the chromatin and slower re-condensation after finishing the repair processes [144]. The degree of

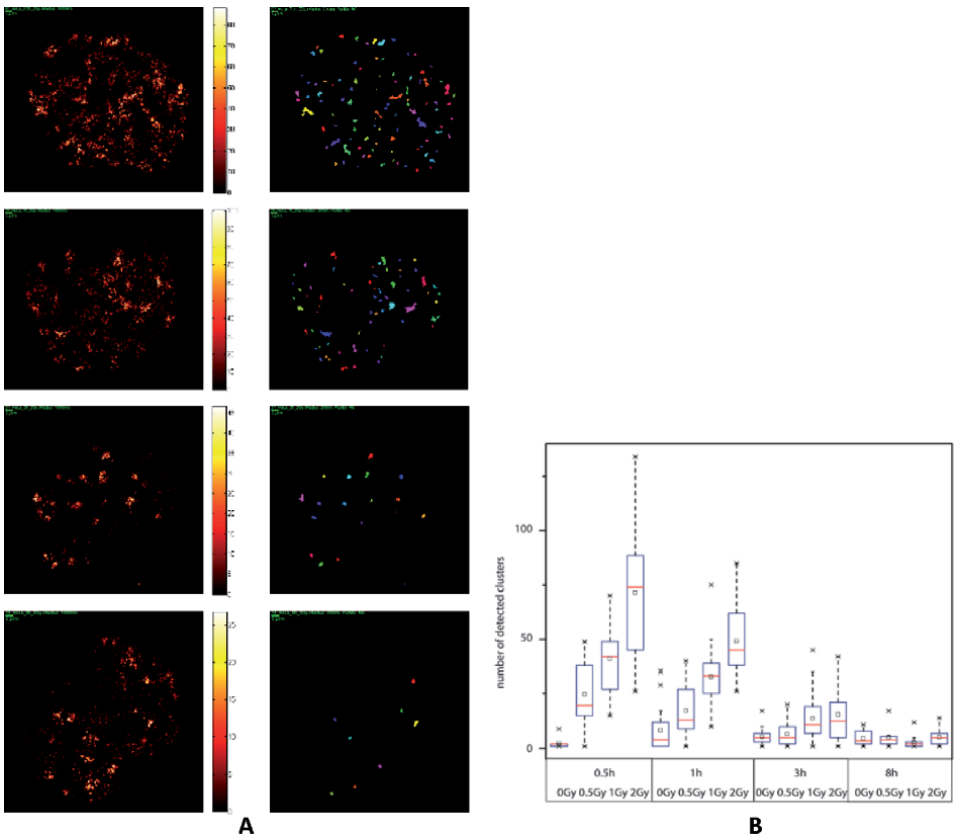

Figure 5.
(A) "Visualization of cluster formation from the SMLM image of cell nuclei after 2 Gy radiation exposure. Left column: *Density image obtained from the coordinate matrix and the next neighbor distance. The point intensity (see intensity scale bar) refers to the next neighbor frequency.* Right column: *Resulting clusters. The points belonging to a cluster are represented by a closed area (colored spots) and reflect nano-clusters within γ-H2AX foci.* Top > Bottom: *30 min, 1 h, 3 h, 8h post irradiation. (B) Numbers of γ-H2AX clusters per cell vs. dose and repair time. The boxplots show the mean cluster number per nucleus (small black square boxes), the median (red line), the lower and upper quantile (big box), and the value range within ± 2 standard deviations (dashed line). The black crosses refer to values that are differing more than 3 box lengths from the median." These figures together with the text of the relevant figure legend are reproduced from [140] with permission from the Royal Society of Chemistry.*

relaxation was independent of the dose which is in good relation to the equally sized γH2AX clusters [140, 141]. In contrast the euchromatin density increased during repair followed by a decrease after finishing the repair processes [144]. However, in total the chromatin showed an increasing clustering during repair followed by a reduction of clusters dependent on the energy of the damaging photons (unpublished). In general it can be assumed that DNA damaging by ionizing radiation does not only induce a reorganization of chromatin at the damaged sites but may also induce long range chromatin rearrangements for repair processes. Whether such chromatin rearrangements are random or directed to improve repair protein recruitment will be subject of future investigations.

5.3.2 Clustering of repair proteins at DNA damage sites

Beyond γH2AX cluster formation, foci and sub-foci clusters of repair proteins were investigated after photon or particle irradiation [141, 142, 145–147]. In the following, some typical examples are shown taken from ongoing projects:

1. 53BP1 foci were investigated in differently radio-resistant cell types, the moderately radio-resistant neonatal human dermal fibroblast cell line (NHDF) and highly radio-resistant U87 glioblastoma cell line. Specimens of both cell types were exposed to high-LET ^{15}N-ion radiation of doses of 1.3 Gy (in a 10° irradiation scheme) and 4.0 Gy (in a 90° irradiation scheme) at the particle irradiation facility of the Joint Institute for Nuclear Research, Dubna, Russia [145, 146].

   At given time points up to 24 h post irradiation, SMLM of fluorescently tagged 53BP1 molecules was performed and the coordinate data of each labeled molecule were quantitatively evaluated [137, 139, 140]. Clusters of these tags were determined as sub-units of repair foci (**Figure 6a**) and the formation and relaxation of these clusters revealed a higher ratio of 53BP1 proteins being recruited into clusters in NHDF cells (less radio-resistant) as compared to U87 cells (more radio-resistant) with different levels of distribution prior to DNA damage induction. This relation of 53BP1 inside and outside particle track clusters (**Figure 6b**) remained different for both cell types during the repair time observed. This could be seen as a measure of the "just-in-time" availability of 53BP1 proteins but did not reflect the absolute number of 53BP1 proteins available. The speed of cluster formation and relaxation differed for the two cell types (**Figure 6c**) indicating the recruitment of the existing proteins in the cell nucleus (higher in U87 cells) rather than a de novo production [147].
   A certain number of the clusters remained persistent, even longer than 24 h post irradiation (**Figure 6b**); thereby the number of these remaining clusters varied in each cell line. The heavily damaged cell nuclei maintained repair activity in order to process the complex damage patterns caused by high-LET ^{15}N-radiation. This long-standing repair activity of 53BP1 proteins was shown in both cell types and the behavior of the cells could causatively be linked to the cell-type specific radio-resistance.
   The dynamics and cluster formation of tagged 53BP1 molecules showed that these clusters were embedded within a random distribution of points. After irradiation, a fast formation of 53BP1 clusters was observed (**Figure 6c**). During the early repair time of about 30 min - 1 h after radiation exposure some clusters were dispersed while others persisted and the amount of randomly distributed proteins was growing. The latter clusters that were persistent did not disappear until the end of the repair period being studied (24 h).

2. Another study performed two-color SMLM of immunostained γH2AX and Mre11 proteins [141] and revealed significantly delayed foci formation by Mre11 compared to γH2AX. While γH2AX clusters are already established at 30 min after radiation exposure (**Figure 7**, left), Mre11 is still ubiquitously distributed in the nucleus. Mre11 cluster formation is maximal at around 180 min after irradiation with significant association to γH2AX clusters (**Figure 7**, right).

5.3.3 Topological similarities of repair clusters

The reason to apply topological analyses is to record properties of point patterns, which are invariant under certain deformations of the object. Mathematically these deformations correspond to continuous transformations of the topological space defined by the structures. Here we have considered two properties, the number of "components" (explained below), which are independent from each other in such sense that connections between points only exist within the respective components

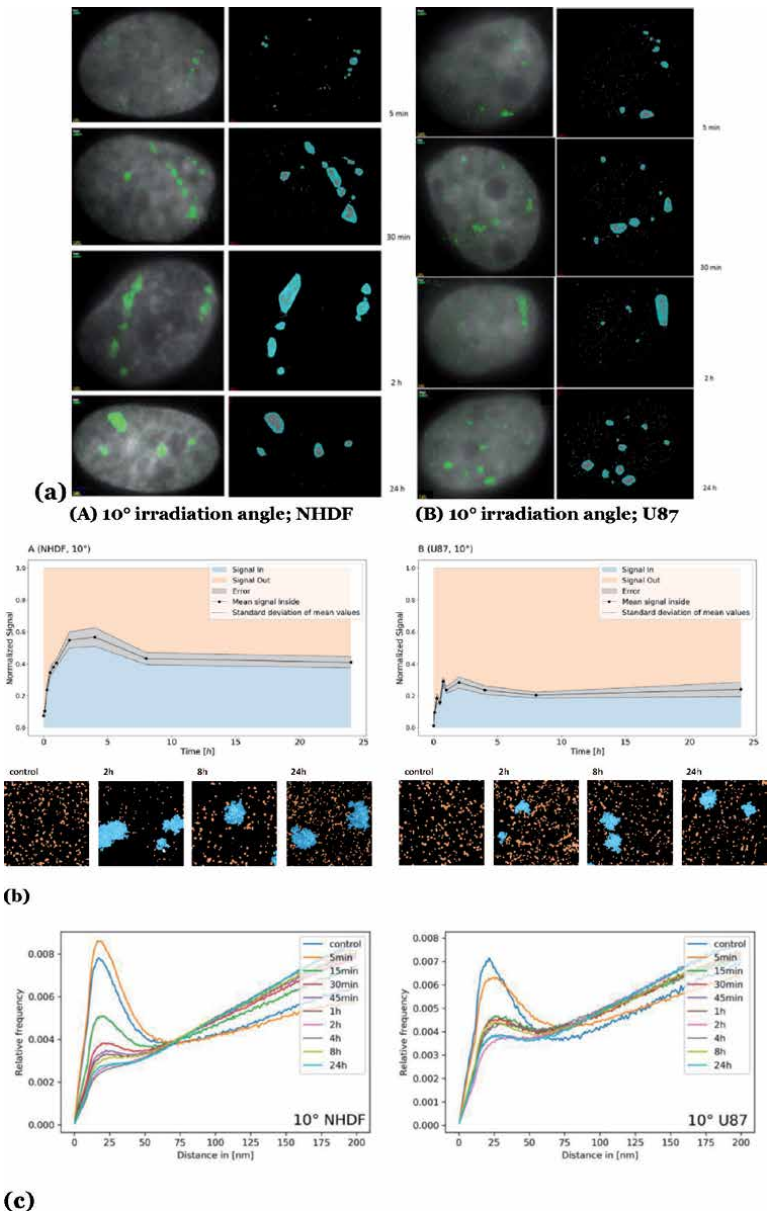

Figure 6.
(a) 2D density SMLM images of 53BP1 repair proteins. Typical examples are shown for fluorescently-labeled 53BP1 proteins in NHDF cells (a) and U87 cells (B) "after 1.3 Gy tangential ^{15}N-irradiation (10° angle between the ion beam and the cell layer). The time values indicate the period post irradiation when the samples were taken as aliquots of the same irradiated culture and fixed. For comparison, examples of non-irradiated control cells are presented. The left columns are merged images of SMLM data and wide-field images. In the right columns the SMLM images clusters and cluster areas are shown. The scale bars equal to 1 μm." (b) relative amounts of 53BP1 signals detected within (blue) and outside (orange) repair clusters. "Graphs: Mean values and margins given by the standard deviation are depicted in gray. The values are always normalized to the mean number of signals detected at a given time point. The data are presented for NHDF fibroblasts (A) and U87 cells (B) after 1.3 Gy tangential ^{15}N-irradiation (10° angle between the ion beam and the cell layer). Images: The pointillist images represent examples of sections of cell nuclei with labelling points inside (blue) and outside (orange) clusters at the given time points. The samples were taken as aliquots of the same culture at different time points (from 5 min to 24 hrs) after irradiation. For comparison, examples of non-irradiated control cells are presented (= 0 min)." (c) Ripley distance frequency analysis. The relative frequencies of pairwise distances are presented for the aliquots of the irradiated cell samples at different time points post irradiaton (color label of curves); (A) NHDF and (B) U87cells irradiated under 10° irradiation angle. Note: These figures are modified and the parts of the text written in "..." are reproduced from the original figures which were originally published under CC BY license in [146, 147].

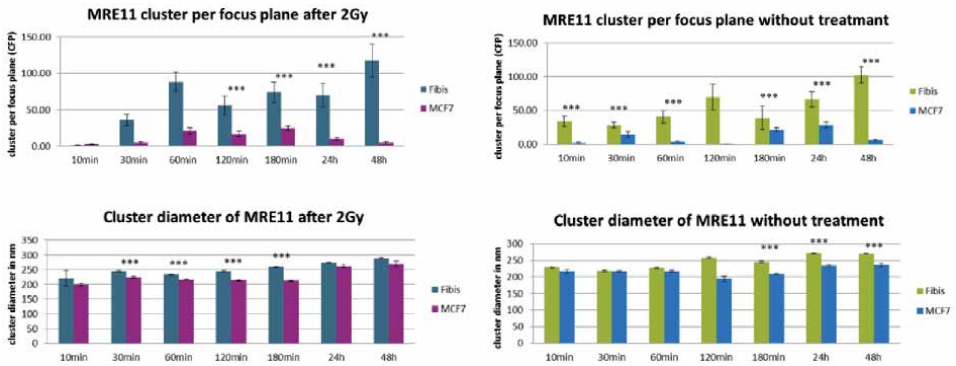

Figure 7.
*Overview of the results obtained from SMLM measurements. Left panels show the data obtained after radiation exposure for MCF-7 breast cancer cell nuclei ("MCF-7") in comparison to cell nuclei of CCD-1059SK fibroblasts ("Fibis"); right panels show the data obtained without radiation treatment, i.e., the natural occurrence of MRE11 clusters in these cells. The columns of each panel represent the mean values calculated from 20 nuclei each. The error bars on top of the column indicate the standard deviation. For each time step after the irradiation process, the data are given for cells exposed to 2 Gy ionizing radiation and for cells subjected to the same culturing procedure but not to radiation treatment. Level of significance between the corresponding values: *** = 0.1%. Note: These figures and their legends in ".." are slightly modified and were originally published under CC BY license in [141].*

and the number of "holes" of the structures inside the components (explained below, **Figure 8a**). In algebraic topology, these properties are called the Betti numbers for zero and one -dimensional simplicial complexes [148].

SMLM images as for instance of γH2AX foci/clusters are point-sets for which components and holes can be defined. A geometric relationship among the points is defined by growing spheres of radius α around each of them. Whenever two spheres mutually embed each-other's center, these centers of the growing spheres are connected and the connected points belong to the same component. With increasing radii, the number of components is reducing. At the end of the procedure, a single component is remaining, the whole γH2AX cluster. For the definition of holes, a polygon is appropriate. Whenever the edges form a closed area, a hole is counted until another line closes a triangle separated from the original hole [147, 148].

The results are presented as "barcodes" to track the formation and disappearance of components and holes with increasing α (**Figure 8**, left panel). These barcodes offer easy comparison of different sets of barcodes and their similarity can be calculated by the Jaccard index [149]. The Jaccard index results is a value between 0 and 1, where 0 is equal to no overlap of two bars and 1 describes two identical bars. Barcodes of different dimensions are defined as similar, if the averages of the individual similarity indices fulfill the Jaccard index conditions of similarity. Importantly, topological comparisons are independent of the scale so that it is possible to compare variably large clusters.

The barcode transfers the examined structures into a form of visualization that is scale invariant. The formation and dissolution of small scaled complexes is recorded alongside the lifetime of large scaled complexes. Consequently, for γH2AX clusters, the barcodes contain bars representing components and holes in the nanometer but also in the micrometer scale ranges. In **Figure 8** (right panel) a representative result of a heat map of Jaccard Indices is shown for SkBr3 breast cancer cells. 200 heterochromatin associated γH2AX clusters and 200 non-heterochromatin associated γH2AX clusters were selected by determining those with the highest and lowest heterochromatic densities in the environment and examined according to

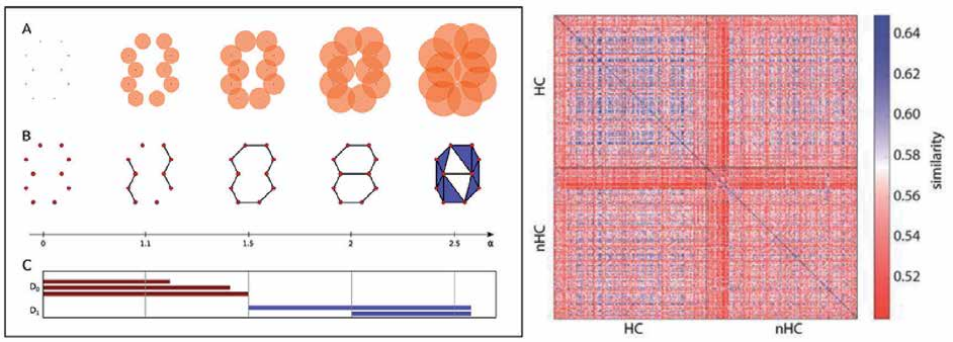

Figure 8.
Left: Illustration of the barcode data representation. (A) Continuously growing spheres, exemplarily depicted at 5 different scales α, around the point data illustrate the idea of the α-shape filtration. (B) As the growing spheres mutually embed the Centre of each-other the corresponding centres are connected by an edge. Whenever a triangle is formed, it is included in the complex as a face element. (C) Barcodes (Betti numbers) of dimension 0 (D_0) and 1 (D_1) corresponding to connected components and holes. Right: Heat map depicting the Jaccard indices averaged from components and holes for similarity of (non-)heterochromatin associated γH2AX clusters. Note: These figures are modified and were originally published under CC BY license in [138].

their topological similarity. For the average similarity for components and holes, heterochromatin associated γH2AX clusters showed a clear similarity whereas non-heterochromatin associated γH2AX clusters did not. This means that by topological analysis the heterochromatin associated γH2AX clusters could be discriminated as those clusters of high topological similarity [138]. The proximity of γH2AX clusters to heterochromatin seems to have a significant measurable impact on its structure. Interestingly, the non-heterochromatin associated γH2AX clusters and heterochromatin associated γH2AX clusters were more similar than the non-heterochromatin associated γH2AX clusters themselves. It can be clearly seen that the proximity to heterochromatin influences the structure of the clusters.

For particle irradiated NHDF cells and U87 cells, the similarity values obtained by averaging of components and holes values for each 53BP1 cluster were determined and the clusters of the 10° irradiations scheme were compared. The Jaccard indices revealed values between 0.55 and 0.82 for U87 and NHDF cells. The broad frequency distribution did not show a peak for NHDF cells whereas for U87 cells a clear peak at 0.64 was found. If the clusters of the 90° irradiation scheme were compared, the peak was located at 0.63 for U87 cells. This value was the same, if the 10° with the 90° irradiation scheme was compared. For these two comparisons (90° vs. 90°, 10° vs. 90°), NHDF cells revealed a bimodal peak distribution where one peak was located at 0.67 and the other one at 0.72 (**Figure 9**). Thus, it can be concluded that in case of the more radio-sensitive NHDF cells a higher topological similarity in 53BP1 clustering was identified than the case the less radio-sensitive U87 cells.

5.3.4 Retrotransposon Alu dosimetry

Alu short interspersed elements (SINEs) make up 11% of the human genome with over 1 million copies [150]; thereby making them ideal markers for assessing global chromatin architecture and dynamics by SMLM. Despite their involvement in many diseases of modern human [151–158] and post-transcriptional regulation [159–166], evidence grows that Alu elements are significantly regulating genome integrity and stability as a response to environmental stress. Concordantly, RNA Pol III transcriptional activation of Alu elements upon chemically and radiation induced DNA damage was observed [167] and epigenetic changes, such as DNA

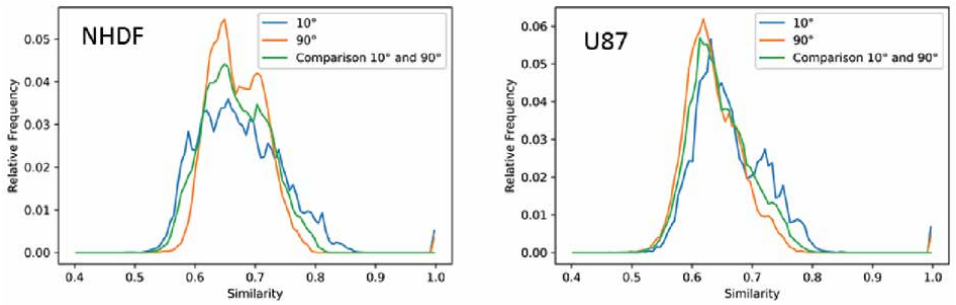

Figure 9.
"Normalized histograms of the frequencies of similarity values of barcodes (Jaccard indices) of 53BP1 clusters in NHDF and U87 cells irradiated under 10° or 90° irradiation angle and fixed 2h post irradiation. The distributions of the average similarity of dimension 0 and 1 barcodes of 53BP1 clusters in NHDF and U87 cells are shown. The similarity distributions of clusters in cells irradiated under an angle of 10° are shown in blue, the similarity distributions of clusters in cells irradiated under 90° are shown in orange, and the similarity distributions obtained when comparing clusters in cells irradiated with 10° to clusters in cells irradiated with 90° are depicted in green". Note: These figures are modified and were originally published together with the cited figure legend under CC BY license in [147].

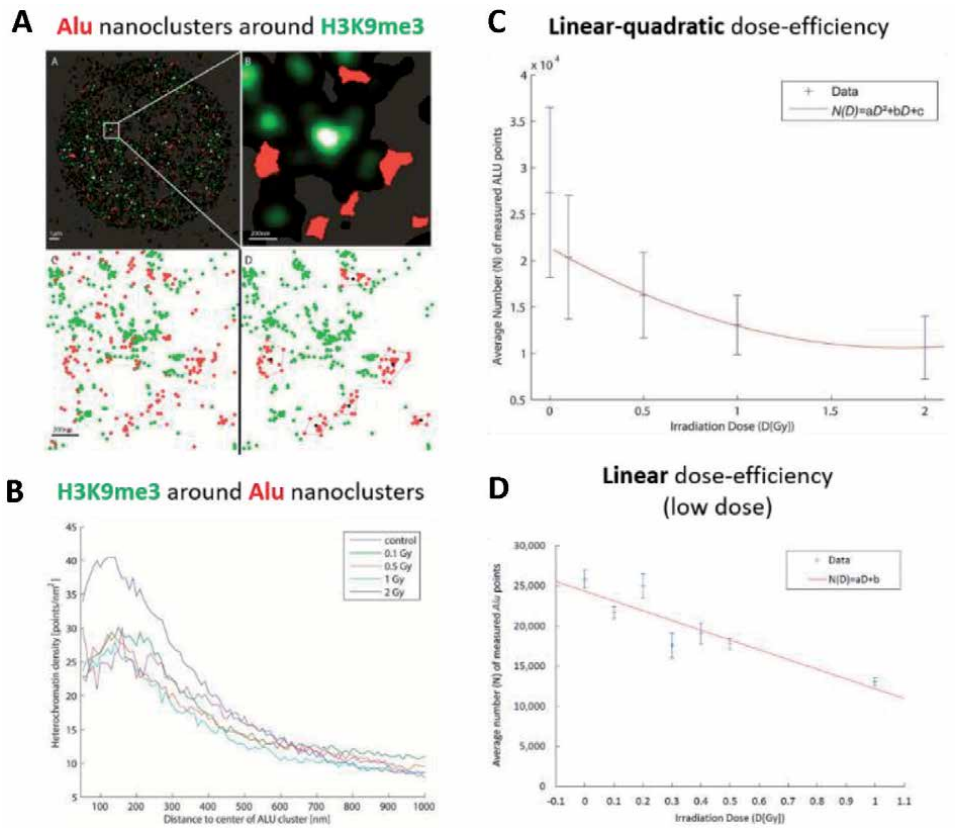

Figure 10.
(A) Single molecule localization microscopy analysis of Alu clustering and dose dependent effects of numbers of Alu labelling points after exposure to ionizing photon radiation. (B) Density distribution of heterochromatin labelling in concentric rings around the center of ALU clusters. The reduction of the density peak corresponding to heterochromatin relaxation around the Alu clusters was independent of the dose. (C) Linear quadratic dose response observed by SMLM of specific oligonucleotide nanoprobe labeling of Alu elements in SkBr3 cells after exposure to different doses of γ-radiation. (D) Linear dose response observed by SMLM of specific oligonucleotide nanoprobe labeling of Alu elements in SkBr3 cells after exposure to low doses of γ-radiation. Note: These figures are modified and were originally published under CC BY license in [16, 167].

hypomethylation, in Alu elements are differently induced in human cell lines, when exposed to different types of radiation [168].

SMLM of irradiated breast cancer cells stained by combinatorial fluorescence in situ hybridization (COMBO-FISH) [169, 170] with a unique, short 17-mer oligonucleotide specific for genomic Alu elements (**Figure 10A**) resulted in a negative linear quadratic decline of the dose efficiency curve of localization signal points in the 0.5 Gy to 4 Gy dose range (**Figure 10C**) [170]. Furthermore, differential association of Alu signals with H3K9me3 heterochromatin between irradiated and non-irradiated cells could be revealed (**Figure 10B**). The heterochromatin relaxed after irradiation. However, the extension of this relaxation was independent of the dose. Alu dosimetry was also applied to the low dose range (< 0.5 Gy) (**Figure 10D**) [16], thereby opening new paths to study the molecular mechanisms underlying the controversial low dose radiation effects [171–175], which are difficult to assess due to a lack of appropriate biomarkers for the <0.5 Gy dose range [176].

6. Conclusion and perspectives

With this article, we have addressed scientists, researchers, and clinicians working in interdisciplinary fields, which are searching for a brief introduction to current radiobiology, its fundamental principles and methodologies. We would further like to have caught the attention of radiation biologists in laboratories, clinics, and industry by demonstrating novel super-resolution microscopy techniques that have the potential to drive radiobiology to a next generation. Single molecule localization allows geometrical and topological analyses on the meso- and nano-scale at the single-cell level in situ with the advantages of easy practice and the applicability to already existing experimental methods (e.g. immunostaining, FISH). As super-resolution microscopy techniques are still not a wide-spread routine in molecular biology laboratories, the long history of fluorescence microscopy data from radiobiological studies provides a solid basis for validation. We have shown that radiobiology can be an application of SMLM based nanoscopy and its versatile data analysis method which allow the investigation of new perspectives of DNA damage induction and repair. It can even help to discover novel markers of biological dosimetry as demonstrated by our recent studies assessing dose dependent effects on retrotransposon Alu availability. Nano-scaled analysis of repair foci architecture and dynamics by assessing foci like 53BP1, Mre11, etc. will give further insight into the molecular mechanisms of DNA damage response and fate of repair pathway of individual damage sites in single cells. Indeed, evidence grows that nanostructure and function of chromatin are highly interdependent aspects that govern the fundamentals of molecular genetics, such as cell type differentiation, gene expression, DNA damage repair and reproduction. Thus, super-resolution radiobiology could serve as a general proof of principle for many other molecular biology applications in future. Finally, we believe that single-molecule localization microscopy will develop to a standard application of radiation biology and might even add to the repertoire of diagnostic technologies in clinical facilities in the future.

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Conflict of interest

There is no conflict of interest for any of the authors.

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Chapter 4

DNA Damage and Repair Mechanisms Triggered by Exposure to Bioflavonoids and Natural Compounds

Donna Goodenow, Kiran Lalwani and Christine Richardson

Abstract

Eukaryotic cells use homologous recombination (HR), classical end-joining (C-NHEJ), and alternative end-joining (Alt-EJ) to repair DNA double-strand breaks (DSBs). Repair pathway choice is controlled by the activation and activity of pathways specific proteins in eukaryotes. Activity may be regulated by cell cycle stage, tissue type, and differentiation status. Bioflavonoids and other environmental agents such as pesticides have been shown to biochemically act as inhibitors of topoisomerase II (Top2). In cells, bioflavonoids directly lead to DNA double-strand breaks through both Top2-dependent and independent mechanisms, as well as induce DNA damage response (DDR) signaling, and promote alternative end-joining and chromosome alterations. This chapter will present differences in expression and activity of proteins in major DNA repair pathways, findings of Top2 inhibition by bioflavonoids and cellular response, discuss how these compounds trigger alternative end-joining, and conclude with implications for genome instability and human disease.

Keywords: environmental compounds, bioflavonoids, DNA double-strand breaks, topoisomerase II, DNA break repair, genome instability

1. Introduction

The faithful repair of deoxyribonucleic acid (DNA) lesions is central to the maintenance of genomic integrity [1]. DNA double-strand breaks (DSBs) occur during normal developmental processes including meiosis, mating-type switching, V(D)J recombination, antigen receptor gene rearrangement, and also through normal activity of topoisomerase II (Top2) [2–5]. DSBs also result from exposure to exogenous sources such as ionizing radiation (IR), reactive oxygen species, and chemotherapeutic agents including inhibitors of Top2 [6–9]. Aberrant repair of DSBs may be mutagenic and result in cell lethality or promote oncogenic transformation. Repair of DSBs in eukaryotes occurs by either homology-dependent or homology-independent (also known as end-joining or illegitimate) mechanisms [10–13]. In yeast, homology-dependent repair predominates over end-joining [10, 14]. In mammalian cells, direct examination of repair products has demonstrated the

predominant use of end-joining [13]. The majority of studies generate targeted DSBs by endonucleases or lasers, and introduce artificial repair substrates into the system [15]. However, exposure to natural compounds can lead to multiple DSBs in a variety of chromatin regions and contexts [16–20]. Understanding how cells respond to these compounds and repair damage caused by them has important implications for genome stability.

Bioflavonoids are natural compounds in soy, fruits, vegetables, tea, coffee, and wine, and contained in energy drinks and dietary supplements [21–24]. Bioflavonoids are also in pesticides and flame retardants [25–27]. Bioflavonoids inhibit the enzyme topoisomerase II (Top2) to promote DSBs, and recent studies have elucidated the cellular mechanisms used to repair the DSBs induced by bioflavonoids [16, 28, 29]. This chapter will discuss cell type differences in expression and activity of proteins in major DNA repair pathways, summarize findings of cellular response to bioflavonoids and Top2 inhibition, discuss how these compounds trigger alternative end-joining, and conclude with implications for genome instability and human disease.

1.1 DNA double-strand break repair

There are three main repair pathways to deal with DNA double-strand breaks (DSBs) in eukaryotic cells. These include classic nonhomologous end-joining (C-NHEJ) (**Figure 1A**) that modifies and allows for ligation of ends, alternative end-joining (Alt-EJ) that generates short overhangs or exposes small regions of homology via resection to promote ligation of ends (**Figure 1B**), and homologous recombination (HR) that uses a homologous sister chromatid, chromosome, or other sequence as a template to direct repair synthesis (**Figure 1C**) [10, 30]. HR is the most accurate using a homologous template as a donor sequence. DSBs are recombination initiators in both meiotic and mitotic cells [31–33]. However, HR has the most protein involvement, is tightly regulated, largely limited to S phase, and kinetically slow. C-NHEJ is utilized throughout the cell cycle and is kinetically fast. Alt-EJ is less well characterized than the other two and considered a backup repair mechanism when HR or C-NHEJ cannot be used. For a DSB to be repaired by HR or either of the end-joining pathways, damage must first be sensed, then signal transduction pathways must be activated for the DNA damage response (DDR) to bring proteins necessary for repair to the site(s) of damage. Indirect signaling and direct repair protein levels along with histone modifications appear to direct DSB repair pathway selection [34–37]. Despite decades of extensive study of DSB repair, scientists continue to identify and characterize new factors mechanistically involved in DSB end processing, repair itself, as well as pathway choice [38].

1.2 End-joining pathway choice

Repair of DNA DSBs by C-NHEJ or Alt-EJ is characterized by ligation of two DSB ends in close proximity to each other (**Figure 1A** and **B**). Initial binding of the Ku70–80 heterodimer competes with poly(ADP-ribose) polymerase 1 (PARP1) for binding to the DSB. If Ku70–80 binds first there is minimal end processing and C-NHEJ is used [39–44]. For C-NHEJ, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited to the Ku complex. DNA-PKcs can determine if the ends are blunt, as from a nuclease cleavage or from RAG during V(D)J recombination, or if there are overhangs or protein/group adducts. If the break is clean, DNA-PKcs recruits XRCC4-XLF and LigaseIV, and these proteins work together to ligate the DNA ends [39, 42, 45]. However, if there is an overhang or proteins are attached to the break site, DNA-PKcs recruits the ARTEMIS complex for processing.

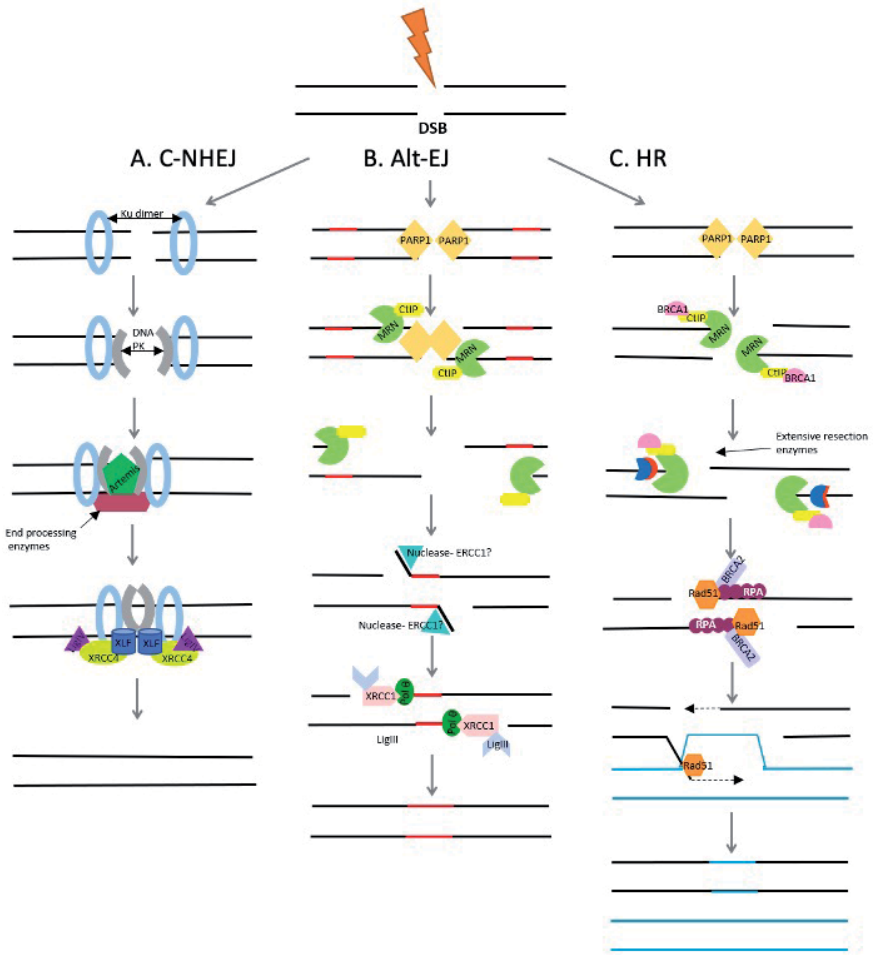

Figure 1.
The DNA double-strand breaks (DSB) are repaired by the three pathways; these are – A) non-homologous end joining (C-NHEJ) which modifies the ends and allow ligation of the broken ends to repair the DSB; B) alternate end-joining (alt-EJ of EJ) creates short DNA overhangs with small regions of homology and ligates the resected broken ends; and C) homologous recombination (HR) that uses a homologous sequence from sister chromatid or homologous chromosome or a homologous sequence within the genome.

ARTEMIS can release protein groups and with its nuclease activity to digest the DSB ends until they are blunt to facilitate ligation of the ends [46].

If PARP1 binds to the DSB before Ku70–80, it immediately adds branched poly(ADP-ribose) (PAR) groups to itself and histones in close proximity. The branched PAR recruit the Mre11-Rad50-Nbs1 (MRN) complex to process the ends and proceed by HR or Alt-EJ. Alt-EJ seems to act as a salvage repair mechanism for when HR and C-NHEJ are blocked. It is likely that Alt-EJ occurs when processing for HR has started following PARP1 binding to the break first, Ku70–80 is depleted, because the DSB ends have proteins bound to block template invasion, or the cell is in G1 phase of the cell cycle no homologous template is readily available for repair. Alt-EJ involves MRN and CtIP to resect the DSB ends in a 3′ to 5′ fashion, termed short range end resection, of 5–25 nucleotides to create short DNA overhangs with small regions of homology. Polymerase θ is utilized in Alt-EJ. After processing, XRCC1 and LigaseIII act in a complex to ligate the ends and remove the overhanging bases. Alt-EJ is more mutagenic than HR or C-NHEJ and associated with chromosomal rearrangements and translocations [44, 47–49].

1.3 Homologous recombination requires chromatin remodeling and DDR

To initiate HR (**Figure 1C**), PARP1 is recruited to the DSB first and immediately adds branched PAR groups to itself and histones in close proximity. The branched PAR recruit the MRN complex and inactive ATM kinase dimers with the acetyltransferase TIP60 attached. PARG quickly removes the PAR groups allowing the MRN complex to bind to the DSB. MRN allows ATM to bind at the DSB and activate through auto-phosphorylation and acetylation by TIP60, thereby allowing TIP60 to dissociate. Once active, ATM will phosphorylate a large number of target proteins including the MRN complex and CtIP that process DSB ends [12, 34, 42, 50].

Chromatin remodeling is extensive and required for HR-mediated DSB repair. Histone H2AX is phosphorylated by ATM as well as acetylated by TIP60. Phospho-H2AX (γ-H2AX) has some chromatin remodeling functions and acts as a signal to recruit additional proteins involved. γ-H2AX will spread away from the DSB to decorate chromatin up to 2 Mb away. MDC1, which assists with chromatin remodeling, becomes phosphorylated by ATM and recruits RNF6 dimers that have ubiquitination functions. HERC2 associates with phosphorylated RNF6 and appears to recruit PIAS4 which has SUMOlyation capabilities. RNF6 becomes SUMOlyated and mono-ubiquitinates histones in the area, which recruits RNF168, another ubiquitin ligase, that is SUMOlyated and poly-ubiquitinates nearby histones. The poly-ubiquitin trees tether BRCA1-A complexes by RAP80 mediators. These complexes cause histone modifications that bring in 53BP1, which has more histone remodeling functions and can inhibit MRN and CtIP-mediated end resection [34, 39, 43, 50].

Phosphorylation of target proteins by ATM also triggers DDR. Chk2 has protein kinase activity allowing it to phosphorylate a number of effector proteins in the cell cycle checkpoint including p53 which can be modified by either Chk2 or ATM (or ATR or Chk1). ARF protein (p14) seems to stabilize TIP60 interactions with ATM for better activation and is associated with maintaining genome stability [34].

While the histone remodeling is occurring and other proteins are being recruited, MRN and CtIP resect the DSB ends short range end resection, then Exo1 or Dna2 nucleases act in long range end bidirectional resection in a 5′ to 3′ direction away from the DSB. Exo1 has dsDNA nuclease function, while Dna2 must act with a helicase like BLM or WRN to unwind DNA for its ssDNA nuclease abilities [34, 37, 43, 51]. While long range end resection is occurring, RPA binds to the 3' ssDNA overhang to protect from nucleases. After this resection, one type of HR can occur called single strand annealing (SSA), where the two pieces of RPA coated DNA associate with one another with the help of Rad52 and if regions of homology are found they anneal to one another. Non-homologous flaps are cleaved off by enzymes like XPF-ERCC1 and ligated by LigaseIII. This type of HR can cause large deletions [12, 43, 50, 52].

Canonical HR, as well as break-induced replication (BIR) and synthesis-dependent strand annealing (SDSA) use BRCA 1 and 2 with Rad51 for homology searches that cause strand-invasion, D-loop formation and resolution/dissolution. RPA must be dissociated from the ssDNA for Rad51 binding, mediated by DSS1 and BRCA2 which displace RPA and stabilize ATP on Rad51 increasing its binding affinity for the ssDNA. Once Rad51 is loaded on the DNA and the nucleofilament has formed, it can invade neighboring DNA to search for homology with BRCA1 [34, 43, 50]. Homology less than 7 nt in length is a weak interaction and Rad51 not sufficient to initiate HR, but 7 nt or longer allows the strand to interact more strongly [50]. If significant homology is present, the ATP on Rad51 is hydrolyzed causing the dsDNA to dissociate and the nucleofilament anneals with the template strand.

RPA stabilizes this D-loop formation by binding to the displaced strand. DNA Polymerase δ or ε uses the invading strand as a primer to initiate synthesis [12, 39, 50, 53]. Resolution can happen with crossover or non-crossover products and different sets of resolvases mediate this process. For one-sided ends that utilize BIR, DNA Pol δ is used and synthesis continues until the end of the chromosome causing gene conversion that can be highly mutagenic [54].

2. Topoisomerase II, inhibitors and poisons

Topisomerase II (Top2) is a regulatory enzyme that relaxes supercoiled DNA for transcription (Top2β) and replication (Top2α). As shown in **Figure 2**, Top2 acts in a multistep cleavage and religation reaction: (1) Top2 binds to two dsDNA molecules at Top2 recognition sequences; (2) a transient DSB is generated in the first DNA helix (G-segment) creating a cleavage complex; (3) ATP hydrolysis drives a conformational change allowing the second dsDNA helix to pass through the DSB; (4) Top2 mediates religation of the DSB and the T DNA segment is released; (5) the G DNA segment is released and the enzyme returns to its original conformation (**Figure 2**). A catalytic Top2 inhibitor such as dexrazoxane acts to prevent DNA from binding to Top2 at step 1 preventing any part of the catalytic cycle [55–59].

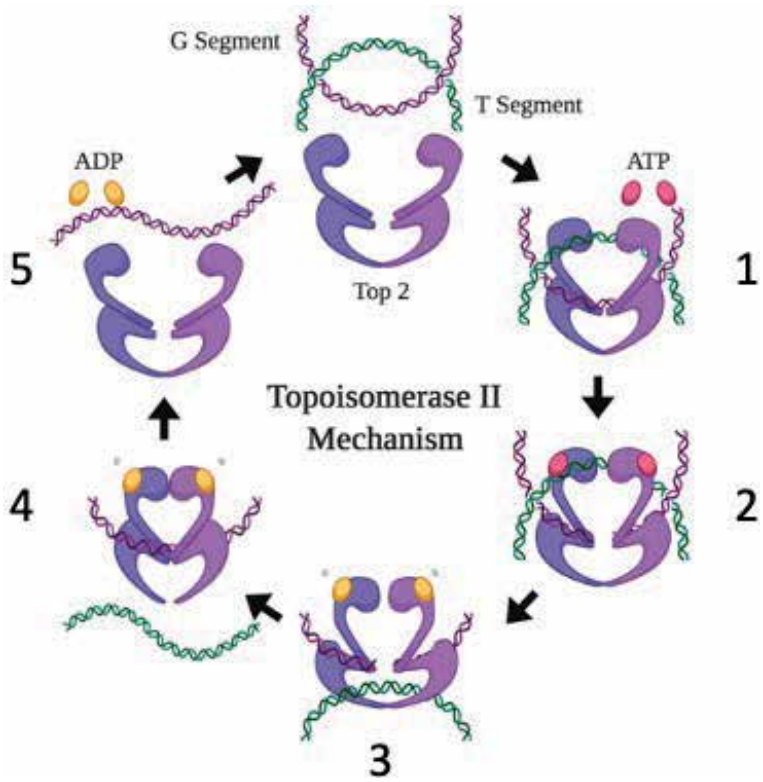

Figure 2.
Top2 acts in a multistep cleavage and religation reaction. 1) Top2 binds the G and T dsDNA molecules at Top2 recognition sequences. 2) ATP binding catalyzes the DNA DSB in the G segment, which allows the T segment to pass through the break. (3) ATP hydrolysis drives a conformational change allowing the second dsDNA helix to pass through the DSB; (4) Top2 mediates religation of the DSB and the T DNA segment is released; (5) the G DNA segment is released and the enzyme returns to its original conformation.

3. Bioflavonoids and other natural compounds as Top2 inhibitors

A class of chemical compounds called bioflavonoids are contained in soy, fruits, vegetables, tea, coffee, wine, energy drinks, and dietary supplements [21–27]. Bioflavonoids are characterized by multiple phenolic rings that are central to their ability to inhibit the enzyme Top2 in a similar manner to the chemotherapeutic drug etoposide [16, 28, 29]. Some pesticides and flame retardants also contain multiple phenolic rings and have been identified as Top2 inhibitors. Bioflavonoids are separated into 12 different sub-classes based upon their structure; however only six are contained in dietary sources: flavanols, flavonols, flavones, isoflavones, flavanones, and anthocyanidins (**Figure 3**) [60, 61].

3.1 Isoflavones

Isoflavones are polyphenolic secondary plant metabolites produced through the flavonoid-producing phenyl-propanoid synthesis pathway (**Figure 4**). In order for isoflavone production, the plant must express the isoflavone synthase enzyme which converts flavanone precursors into isoflavones. This isoflavone synthase is only expressed in legumes and a few other select species. Plants with the highest concentrations of isoflavones are soy, red clover, and kudzu. The amount of isoflavone depends upon the conditions the plants were grown, and the final concentration of isoflavones in food products (including dietary supplements) depends upon which portion of the plant is used and the processing methods. Genistein, daidzein, glycitein, formononetin, biochanin A and irilone are the main isoflavones isolated

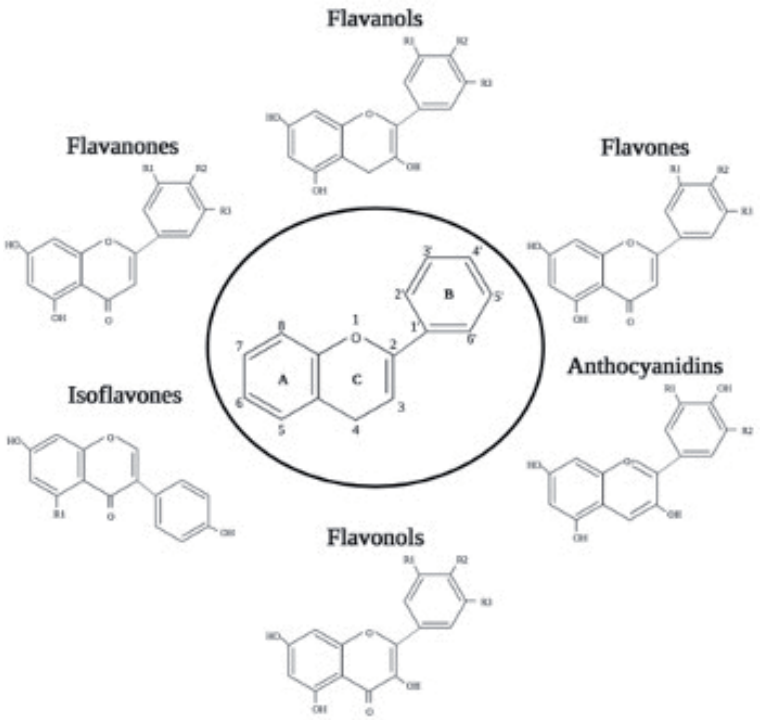

Figure 3.
Basic chemical structures of dietary bioflavonoids. The middle circled backbone represents the general bioflavonoid poly-phenol ring structure. The six structures surrounding show the general structural differences between the sub-groups.

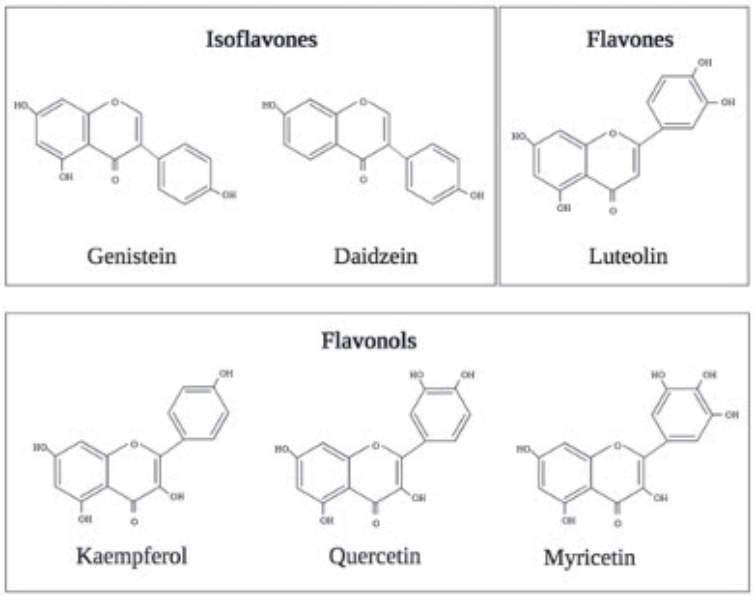

Figure 4.
Structure of commonly found bioflavonoids flavanols: Genistein and Daidzein, flavonols: Kaemferol quercetin and Myricetin, and flavones: Luteolin.

from plants [60, 62, 63]. Genistein and daidzein are of particular interest due to their high concentration in soy products [60]. Genistein is an estrogen derivative available at health food stores as dietary and menopausal supplements, and a soy phytoestrogen present in foods, particularly soybeans, and infant soy formulas [23, 64, 65].

Interest in isoflavones has spiked in the past 20 years. This is due to the attribution of consumption of isoflavone-containing products with lower occurrences of coronary heart disease, breast and prostate cancer. This hypothesis derived from observations that citizens of Asian countries have lower incidence of these diseases compared to citizens of Western countries, and that citizens in Asian countries typically ingest 8-50 mg/day of isoflavones compared to citizens in Western countries who ingest only 0.1–3.3 mg/day [66, 67] .

Due to this potential health relevance, studies examined the impact of high intake of isoflavones, but the results have been inconclusive [62]. In animal models, increased genistein intake resulted in increased rates of pituitary and mammary gland tumors and stimulated MCF-7 tumor growth. Additionally, while increased genistein intake in post-menopausal women in Asian countries decreased breast cancer risk, this decreased risk was not sustained in post-menopausal women in Western countries, including both native inhabitants and Asian immigrants. Some studies, particularly of British women, showed that increased serum genistein levels in women with early stage breast cancer had increased transcription of cell cycle progression and cell proliferation genes [62].

3.2 Flavones

Flavones are the end product of a complex multi-step synthetic pathway that occurs within a wide variety of plants (**Figure 4**). This pathway begins with phenylalanine that is converted through the generalized phenylpropanoid pathway that synthesizes most flavonoids. Subsequently, p-coumaroyl-CoA must be synthesized into chalcone with chalcone synthase. Chalcone can be isomerized into a flavanone

by chalcone isomerase. Finally, flavone synthase class I or II enzymes catalyze the synthesis of a flavone from flavanones. Flavones, similar to flavonols, can protect the plant from UV-B radiation. Flavones have the additional ability to provide protection against biological attacks from pathogenic microbes by acting as signaling molecules to activate differential gene transcription to prevent the growth of microorganisms after invasion. Additionally, flavones can be expressed to deter insects and nematodes from eating the plant or to interfere with the growth and reproduction of other plants [60].

Flavones are found across a variety of plant species, and expression of flavones appears to be widespread within the plant, from the roots to the leaves. However, though flavones are found throughout the plant kingdom, they are found much less commonly in fruits and vegetables as compared to flavonols. Apigenin and luteolin are the main flavonols contained in food sources including celery, parsley, thyme, red peppers, and fruit skins [61, 68]. In humans, flavones, much like isoflavones and flavonols, seem to have antioxidant and anti-tumor capabilities and to affect signal transduction pathways [69].

3.3 Flavonols

Flavonols are primarily in fruits, vegetables, red wine, and tea and they compose the largest portion of humans' bioflavonoid intake given their distribution across a wide number of plant species (**Figure 4**) [61]. Within plants it has been shown that flavonols have the ability to protect the plant against UV-B damage, and they protect the plants against oxidative damage with their antioxidant capability [70, 71]. Scientists and physicians want to determine ways to utilize the antioxidant capability of flavonols in human populations as a protectant against cardiovascular and neurological disease and against exercise induced oxidation in smokers and athletes [72, 73].

The most common flavonols in foods are quercetin, kaempferol, myricetin, and fiestin, with a majority of published literature focusing upon the first three. Similar to isoflavones the concentration of flavonol in the food product depends upon the plant, the growth conditions, and the part of the plant used. Flavonols are found in highest concentrations in the leaves, flowers, and fruits, which are exposed to sunlight; the exception to this being onions which grow below ground [70, 71]. The human dietary source of flavonols is dependent on culture and region. Humans residing in Asian countries typically ingest flavonols through green tea, while the Netherlands, United States and Denmark inhabitants mainly ingest them from onions, apples, and tea. Citizens of Mediterranean areas ingest flavonols from green vegetables. Within Italy, red wine is the main source of flavonols, though inhabitants of Northern villages also have a high intake from salads, soups, fruits. The prevalence of flavonols in the human diet has produced a large interest in understanding their multiple cellular effects and potential impact on human health [70].

3.4 Additional compounds as Top2 inhibitors

Additional natural compounds other than bioflavonoids may also act as inhibitors of Top2. Bakuchicin from the furanocoumarin family is present in fruits and legumes [74]. In research conducted to study DNA-polymerase inhibition activity of *Psoralea corylifolia L.* (Leguminosae), bakuchincin was found to be a weak Top2 inhibitor [75]. Additional reported naturally occurring Top1 and Top2 inhibitors are benzophenone compounds such as xanthochymol and Garcinol at effective concentrations comparable to those of etoposide (~25 – 100 μM) [76, 77]. A comparative study between the naturally occurring constituent of black seed thymoquinone

used as a spice in eastern cooking and a known Top2 inhibitor 1,4-benzoquinone showed structural and functional similarity between the two compounds and the ability to induce DNA cleavage [78].

Triterpenoids are present in plants, widely distributed within the root, stem, leaves, bark. They are components in the waxy covering of fruits and herbs such as jujube, lavender, and thyme [79]. Triterpenoids have two major components, C5 units and isopentyl diphosphate [80], and are generally present as saponins that act as defense chemicals for protection against microbes. Triterpenoids betulin lupane and oleanane from the bark of *Phyllanthus flexuosus*, derivatives of betulinc acid, and oxygenated derivatives of oleanane called celastroloids were reported to act as human Top2 inhibitors to varying degrees [81–84]. In addition, betulinic acid which is an oxidative derivative of betullin inhibits cell proliferation by inhibiting topoisomerase-DNA binding and suppressing NF-κB activation [83].

Halogenated compounds in household and baby products include polychlorinated biphenyls (PCBs), detectable in indoor carpets, and polybrominated diphenyl ethers (PBDEs), used as flame retardants, increase DNA cleavage by TopIIα in vitro and in cultured human cells [85]. Recent CRISPR-Cas9 screening against a large panel of genotoxic agents identified the synthetic small molecule pyridostatin as a Top2 inhibitor. Pyridostatin is a G-quadruplex stabilizer and this stabilization mechanism may lead to Top2 trapping on DNA [38].

4. Flavonols, flavonols, flavones, isoflavones, flavanones, and anthocyanidins act as Top2 poisons and trigger illegitimate DNA repair mechanisms

A catalytic Top2 inhibitor such as dexrazoxane acts to prevent DNA from binding to Top2 thus preventing any part of the catalytic cycle to occur [55–59]. By contrast, some chemicals including bioflavonoids act as Top2 "poisons" (**Figure 5**) [28, 86]. A Top2 poison acts on Top2 after DNA binding and prevents the normal function of Top2 (step 2 of catalytic cycle, see **Figure 2**). Top2 poisons can be further classified as covalent or traditional poisons. The potential as a covalent or traditional poison is dependent on biochemical structure. These groups are not mutually exclusive and individual bioflavonoids can act through one or both mechanisms [29, 86].

4.1 Bioflavonoids as covalent Top2 poisons

Flavanols, flavonols, flavones, flavanones, and anthocyanidins (but not isoflavones) have the potential to act as strong covalent Top2 poisons [86]. A covalent Top2 poison works in a redox-dependent manner, binding to a distal site on the Top2 enzyme and increasing its ability to cause a DSB in step 2 of the catalytic multi-step reaction through conformational changes to the enzyme. The key structural component for a covalent poison is having 3 –OH groups on the B ring of the bioflavonoid structure. However, it is likely bioflavonoids with 2 –OH groups on the B ring act as a weak covalent poison and the ability to act as a covalent poison increases with more –OH groups (**Figure 5**) [49–50]. A 4'-OH group on the B ring is necessary for binding, and 3′ and 5'-OH groups improve covalent binding strength. Thus, a strong covalent poison contains 3 –OH groups on the B ring of the bioflavonoid structure. For example, among the flavonols, structure predicts that myricetin has high activity, quercetin has intermediate activity, and kaempferol has weak activity, if at all, as a covalent Top2 poison (**Figure 4**). Cell free studies support this and show that myricetin as well as epigallocatechin-gallate (ECGC) act as strong coalvent poisons, quercetin acts as a weak traditional poison, but kaempferol does not have this activity [29, 87].

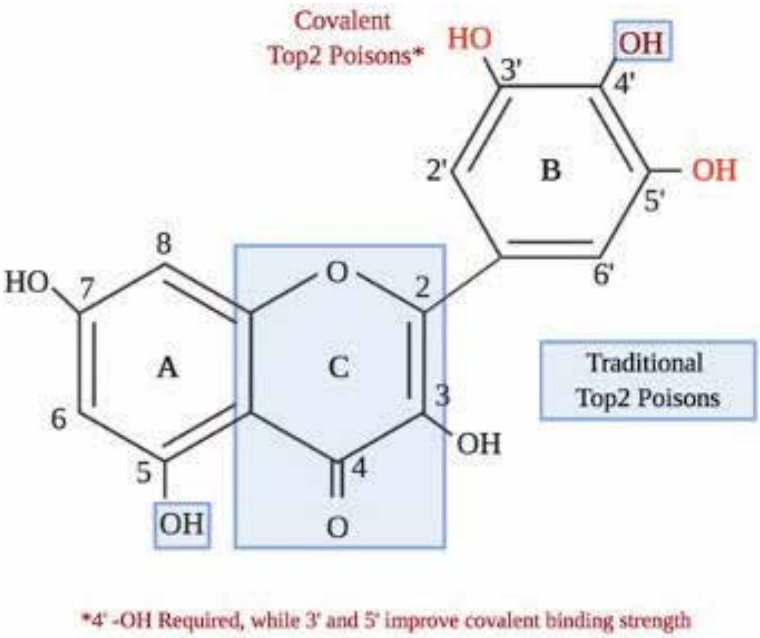

Figure 5.
Bioflavonoid classification as a covalent or traditional topoisomerase II poison. The blue boxed regions indicate required biochemical features for a traditional Top2 poison. The red 3′, 4′ and 5'-OH groups on the B ring are necessary for covalent Top2 poisons. The 4'-OH group is required for covalent binding, while the 3′, 5′ increase the binding affinity, therefore a bioflavonoid with all 3-OH groups would be a strong covalent poison.

4.2 Bioflavonoids as traditional Top2 poisons

Flavones, flavonols, isoflavones, and flavanones (but not flavanols or anthocyanidins) have the all act as traditional (or interfacial) Top2 poisons. The key structural components for a traditional Top2 poison are a 5'-OH group in the A ring, a 4'-OH group in the B ring, and a 4′ = O in the C ring (**Figure 5**). A traditional (or interfacial) Top2 poison stalls the enzyme by binding to the active site of the enzyme preventing religation, thereby resulting in the formation of a stabilized cleavage complex (SCC) [88, 89]. Flavonols are strong traditional poisons and both cell free and cell culture systems support this. Similarly, experiments in cell culture systems examining the kinetics of DSB repair following exposure to acute doses of bioflavonoids support the model that flavonols, flavones, and isoflavones including kaempferol, quercetin, myricetin, genistein, and luteolin and each act as a traditional Top2 poison. However, combinatorial activity of genistein, quercetin and luteolin together suggests they may have weak covalent poisoning capabilities when they have to compete for the traditional poisoning binding site [29].

4.3 Bioflavonoids trigger illegitimate DNA repair mechanisms

Bioflavonoids with either covalent and traditional Top2 poisoning activity induce the DSB-mediated DDR as evidenced by induction of γ-H2AX foci, ATM phosphorylation, and p53 signaling [90–92]. However, a more direct role or influence of these compounds on the repair of damage is not as clear [93]. Acute doses induce DNA damage and DDR as detected by γ-H2AX foci and phosphorylation of ATM in stem cells and CD34+ hematopoietic progenitor cells [94, 95].

Genistein and quercetin inhibit Top2 to induce DNA DSBs, and also appear to influence DSB repair pathway choice. Protein level analysis for HR, C-NHEJ, and Alt-EJ specific proteins suggests that genistein and quercetin suppress HR by reducing BRCA2 and Rad51 expression, as well as suppress C-NHEJ by suppressing levels of DNA-PKcs, Ku80, XLF and XRCC4 and trigger Alt-EJ by increasing levels of CtIP and Polymerase θ [96, 97]. DNA reporter assays suggest that quercetin interferes with DNA repair mechanisms such as HR and C-NHEJ by inhibition of PI3K/Akt signaling. In support of these studies, exposure to multiple bioflavonoids promotes the generation of chromosomal translocations in a dose-dependent manner [29, 87].

Bioflavonoids that have traditional Top2 poisoning activity lead to trapped SCCs on the DNA. Removal of SCCs is performed by the small ubiquitin-related modifier ligase ZNF45/tyrosyl-DNA phosphodiesterase 2 (ZATT/TDP2) complex. Removal of the SCC is required for DSB repair by C-NHEJ. If ZATT/TDP2 does not remove the SCC, the MRN complex or CtIP with nuclease activity may resect the DNA ends with the SCC attached to allow for DSB repair by HR or Alt-EJ [55, 94, 98–101]. Inhibition or mutation of multiple DNA repair proteins potentiates cytotoxicity of Top2 inhibitors, and MRE11 plays a direct mechanistic role in removal of Top2-DNA complexes in yeast and mammals [102, 103].

5. Pleiotropic effects of bioflavonoids

Due to their antioxidant capacity, bioflavonoids are included in dietary supplements for their presumed health benefits in protecting against inflammation, cardiovascular diseases, and cancer [87]. These beneficial health properties are due to the number of pleiotropic effects bioflavonoids have on cells by impacting signal transduction pathways, DSB repair and the cellular epigenetic landscape, which can lead to protein level changes, cell cycle stalling, and apoptosis [16, 69].

5.1 Bioflavonoids and signal transduction pathways

Bioflavonoids have antioxidant and anti-inflammatory properties. Their antioxidant properties are due to their ability to reduce reactive oxygen species of the multiple –OH groups in their chemical structure. Their anti-inflammatory properties are due to their interference with signal transduction pathways and down-regulation in the production of pro-inflammatory cytokines. Bioflavonoids decrease inflammation and immune cell recruitment through interference with the ERK/MAP kinase and NF-κB signal transduction pathways which can be beneficial to human health. NF-κB is a transcription factor that upon activation is transported into the nucleus and binds to the promoter region for a number of cytokines and apoptotic genes; therefore reduced pathway activation leads to lower pro-inflammatory cytokine production and increased cell survival [104]. Extracts from the plant *Ginkgo biloba*, rich in bioflavonoids, act as an herbal antioxidant, augment the transcription of TNF-α causing reduced activation of the NF-κB pathway. Apigenin has shown similar down regulatory effects on cytokine production likely through the modulation of NF-κB activation [105]. Quercetin and fisetin inhibit pro-inflammatory cytokine production through the suppression of NF-κB activation by decreased phosphorylation of extracellular signal-regulated (ERK) kinase and p38 mitogen-activated protein (MAP) kinase that are activators of NF-κB [106–108]. Myricetin has been shown to affect the phosphatidylinositol 3-kinase (PI3-K) pathway inducing apoptosis in pancreatic cells [109].

5.2 Bioflavonoids and epigenetic modifications

Studies in cancer cell lines demonstrate epigenetic modifications caused by bioflavonoids. Genistein, quercetin, curcumin, EGCG, hesperidin, and naringin are inhibitors of DNA methyltransferases leading to hypomethylation of DNA. In addition, many of these bioflavonoids have also been shown to act on histone acetyltransferases and histone deacetyltransferases causing cell wide alterations in histone epigenetic modification patterns [109].

Long-term epigenetic effects of bioflavonoids compounds were addressed in several mouse model studies. Exposure to genistein through maternal diet during pregnancy can have long-lasting effects on the progeny. In agouti mouse pups exposed to genistein from conception until birth, epigenetic changes were observed as altered coat color, as well as significant downregulation of genes involved in hematopoiesis of bone marrow cells, increased erythropoiesis, and a permanent signature hypermethylation of repetitive elements in hematopoietic lineages [110]. Likewise, in mice exposed to quercetin from conception until birth resulted in upregulated iron-associated cytokine expression, significantly increased iron storage in the liver, and hypermethylation of repetitive elements. Epigenetic modifications lead to long term gene expression changes of cytokines associated with inflammation in the liver of the mice in adulthood [111, 112].

6. Implications for human health

6.1 Potential anti-cancer applications

While bioflavonoids can be beneficial through intake at low or moderate doses, high doses and acute exposure of bioflavonoids may more drastically inhibit Top2 and impact genome integrity and cell survival, thus changing their overall impact on cells and human health. *In vitro* studies support the idea that bioflavonoids genistein and quercetin may act as chemo-preventive or anti-cancer agents by altering major processes within cancer cells such as apoptosis, cell cycle, angiogenesis and metastasis [113, 114]. Genistein has synergistic behavior with well-known anticancer drugs adriamycin, docetaxel, and tamoxifen, suggesting a potential role in combination cancer therapy [78]. Quercetin in combination with doxorubicin was found to be more effective in inducing apoptosis within the SKOV-3 cells [114]. A combinatory treatment with quercetin and curcumin synergistically induce anticancer activity in triple-negative breast cancer cells by modulating tumor suppressor genes in particular enhancing BRCA1 expression [115].

Several bioflavonoids have been investigated as alternate cancer therapeutics that are less genotoxic than traditional chemotherapeutics but equally effective. High concentrations of myricetin causes Top2-mediated DNA damage and apoptosis in K652 cells [116]. Fisetin interrupts the MAPK-dependent NF-κB signaling pathway in cervical cancer cells, inhibiting migration and invasion [114]. Several *in vitro* and *in vivo* studies indicate that luteolin can suppress metastasis of breast cancer by reversing epithelial-mesenchymal transition, or by acting as an antiangiogenic therapeutic inhibiting VEGF production and suppressing invasion [117, 118].

While these observations strengthen the notion that flavonoids could be useful anti-cancer agents, to date minimal clinical studies have demonstrated that these bioflavonoids retain anti-cancer properties in humans *in vivo*. A Phase I study/pharmacokinetic trial of quercetin in cancer patients intravenously injected quercetin in 11 patients with cancer at varying doses of 60–2000 mg/m^2 and identified 945 mg/m^2 as a safe and effective dose [119].

6.2 Potential inducers of infant leukemia

Aberrant repair of DNA DSBs caused by either endogenous or exogenous agents has the potential to result in DNA sequence mutations or genome rearrangements such as chromosomal translocations which can lead to disease. Negative consequences of high bioflavonoid intake can be observed most prominently in pregnant women. Epidemiological data from countries whose citizens have higher bioflavonoid intake (particularly soy products) had a 2–3 times higher incidence of infant leukemia, characterized by chromosomal translocation, suggesting maternal intake of high amounts of bioflavonoids could lead to this particular genome rearrangement and infant leukemia [120].

Infant leukemia typically occurs due to translocation events involving the mixed lineage leukemia (*MLL*) gene. Most of the *MLL* rearrangements observed in patients with infant leukemia and therapy-related leukemia (tAML) cluster together in a well-defined region of the *MLL* locus [121]. tAML is associated with treatment with Top2 poisons etoposide or doxorubicin [86, 88, 121] which has led to the hypothesis and working model that ingestion of natural Top2 poisons including bioflavonoids can lead to these translocation events and tumorigenesis [121, 122]. In support of this, boflavonoids have been shown to inhibit Top2 and induce *MLL* cleavage and translocations in hematopoietic stem cell-enriched populations [87, 121].

Foods contain multiple different bioflavonoids, and bioflavonoids are bioaccumulative which likely increases plasma concentrations [123]. Study of the potential for environmental or dietary compounds to induce infant leukemias is more relevant since they cross the placental barrier as shown with the synthetic bioflavonoid EMD-49209 [124], genistein [111, 125], quercetin [111], herbal medicines, dipyrone, and pesticides including the mosquitocidal Baygon [126, 127]. Genotoxic effects of quercetin on the human hemopoietic stem and progenitor cells (HSPCs) were shown using a genetically engineered placental barrier model from a specialized human cell line. This study showed that approximately 10% of quercetin from the maternal side is capable of crossing the placental barrier and accumulating in the fetus. Exposure *in utero* is likely more damaging due to differences in metabolic and excretion rates of mother and fetus [128] as well as rapidly developing and proliferating fetal cells that are more sensitive to Top2 inhibiting agents [129].

7. Conclusion

Bioflavonoids are prevalent in the human diet from natural sources such as fruits and vegetables, but are also found at supranatural concentrations in dietary supplements and energy drinks. These chemical compounds have numerous cellular effects including interfering with signal transduction pathways, modifying the DNA damage response and epigenetic markers, and poisoning of Top2 causing DNA DSBs and leading to aberrant repair. Given the number of cellular pathways bioflavonoids affect, and the DNA damage caused by bioflavonoid exposure, it is possible that bioflavonoids could be used as natural analogs of traditional chemotherapeutic agents. However, more research is needed to understand how these bioflavonoids cause DNA damage through Top2-dependent or -independent pathways to understand potential off-target negative effects. In addition, further research will be needed to understand the dose-dependent activities of bioflavonoids and at what doses they may be chemo-protective versus what threshold doses they may induce DNA damage that is mutagenic, and finally at what high acute doses they may induce DNA damage and apoptosis to act as effective alternative to traditional chemotherapeutic agents.

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Conflict of interest

The authors indicate no conflict of interest.

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Chapter 5

Recent Perspectives in Radiation-Mediated DNA Damage and Repair: Role of NHEJ and Alternative Pathways

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Abstract

Radiation is one of the causative agents for the induction of DNA damage in biological systems. There is various possibility of radiation exposure that might be natural, man-made, intentional, or non-intentional. Published literature indicates that radiation mediated cell death is primarily due to DNA damage that could be a single-strand break, double-strand breaks, base modification, DNA protein cross-links. The double-strand breaks are lethal damage due to the breakage of both strands of DNA. Mammalian cells are equipped with strong DNA repair pathways that cover all types of DNA damage. One of the predominant pathways that operate DNA repair is a non-homologous end-joining pathway (NHEJ) that has various integrated molecules that sense, detect, mediate, and repair the double-strand breaks. Even after a well-coordinated mechanism, there is a strong possibility of mutation due to the flexible nature in joining the DNA strands. There are alternatives to NHEJ pathways that can repair DNA damage. These pathways are alternative NHEJ pathways and single-strand annealing pathways that also displayed a role in DNA repair. These pathways are not studied extensively, and many reports are showing the relevance of these pathways in human diseases. The chapter will very briefly cover the radiation, DNA repair, and Alternative repair pathways in the mammalian system. The chapter will help the readers to understand the basic and applied knowledge of radiation mediated DNA damage and its repair in the context of extensively studied NHEJ pathways and unexplored alternative NHEJ pathways.

Keywords: Radiation, DNA damage, DNA repair, NHEJ, Alternative NHEJ

1. Introduction

Radiation is a natural part of our surroundings. Humans get exposure to natural radiation such as cosmic rays and radioactivity from earth and food. The diversified use of radiation in several technological procedures like power generation, sterilization of food products, industrial activities, therapeutics (radiotherapy), diagnosis,

nuclear weapon development etc., has increased the risk of exposure. Inadvertent accidents from nuclear power plant installations, nuclear weapon testing and illegal use of radioactive material in dirty bomb have raised an international concern for radiation safety [1–3].

Radiation therapy is the most common and deliberate exposure of high energy rays to living organisms. This exposure is mainly therapeutic for treatment of cancer but since there is no clear demarcation to protect the adjacent noncancerous cells leads to disastrous effect. The immediate exposure of high energy beam of radiation leads to destruction of cancerous cells. Whereas the adjacent normal cells are however exposed to these rays suffer adverse effects. It indirectly generates reactive oxygen species (ROS) inside the cellular system through hydrolysis of water. ROS directly targets cellular DNA and affect the cell survival by damaging macromolecules like lipid, proteins and carbohydrate. The damage induction in DNA molecules could be of various types like double strand break, single strand break, dimer formation, alteration of bases etc. Mammalian cells are equipped with very efficient DNA repair mechanism to handle these different damages [4]. Moreover among all, double strand breaks are known to be lethal damage for the cells. There are mainly two repair mechanisms that operate for repair of double strand breaks. These are 1) Homologous repair pathway (HR) 2) Non homologous end joining pathway (NHEJ). The basis on which cell decides to choose one of the two available pathways is simply on cell cycle phase, its type and damage threshold [5]. There are also exists third repair pathway i.e. Alternative non homologous end joining pathway (A-NHEJ) it is much slower than the above mentioned pathway. It comes into play when above mentioned pathway fail to repair the damage thus acting as a backup pathway. The presence of this alternative pathway has not been studied extensively but it has been speculated for its role in combinational cancer therapeutics. In this chapter, we have briefly described the various kind of DNA damage generated by radiation and role of DNA repair pathways specially NHEJ and A-NHEJ in handling the repair and their applications in progression of disease [6].

Radiation, which has particles with enough energy to rip electron from atoms or molecules is known as ionizing radiation. Radiation is the emission and propagation of energy in the form of rays or waves. The term radiation comes after the discovery of X-ray in 1895 by Wilhelm Conrad Roentgen. Henri Becquerel and Marie Curie have made significant contributions in studying the effect and application of radiation in various fields. Excitation and ionization properties are common responsible factor for radiation emitted by any radioisotopes. It has two major types: ionizing and non-ionizing radiation [6, 7].

1.1 Non-ionizing radiation

It does not carry enough energy to remove electrons from an atom or molecule. Because of their low energy, non-ionizing radiation poses a lower risk than ionizing radiation. Visible light, near ultra violet, infrared, microwave and radio waves are examples of non-ionizing radiation [5].

1.2 Ionizing radiation

Ionizing radiation (IR), as the name indicates carry sufficient energy to remove electrons from atoms or molecules. It can be in particulate or electromagnetic form. The particulate forms consist electrons, protons, neutrons, α-particles etc. and the electromagnetic form includes as cosmic rays, X-rays, gamma rays etc. [5]. Ionizing radiation exposure may cause tissue injuries to the biological system

via biochemical, cellular and molecular targets leading to cellular and molecular damages such as oxidative damage to DNA, lipids and proteins as shown in **Figure 1** which may further lead to systemic damage [6, 8].

1.3 Types of ionizing radiation

1.3.1 Alpha particles

An alpha ray consists of two protons and two neutrons. These rays have a strong nuclear force and have the ability to bind to the nucleus of any atom. Due to their charge and mass, alpha particles interact strongly with matter and only travel a few centimeters in air. Alpha particles are unable to penetrate the outer layer of dead skin cells but are capable of causing serious cell damage if an alpha emitting substance is ingested in food or air [5].

1.3.2 Beta particles

Beta particles are high-speed electron or positron emitted from the radioactive decay of an atomic nucleus such as potassium-40 during beta decay. These particles

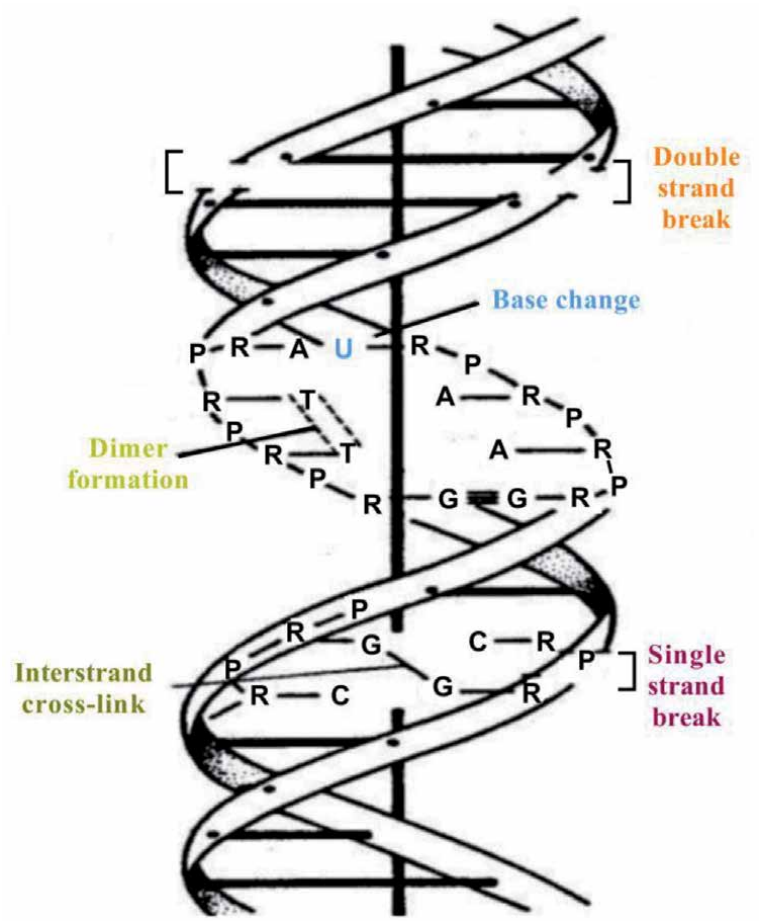

Figure 1.
Various type of DNA damage induce by radiation.

are emitted by unstable nuclei rich in neutrons, they are high energy electrons. These particles are negatively charged and have intermediate penetration power [5].

1.3.3 Gamma rays

Gamma radiation, unlike alpha or beta, does not consist of any particles; instead, they consist of a photon of energy being emitted from an unstable nucleus. These are produced by a change in the energy levels of the atomic nuclei. The wave length of this radiation varies from 0.0003 nm to 0.1 nm. Gamma rays do not have any mass or charge. It can travel at much higher speed in air than alpha or beta rays and loses only half of its energy for every 500 feet. Gamma rays can be stopped by dense and thick layer of material such as lead or depleted uranium. These materials are used as an effective shielding in radiation related work [5].

1.3.4 X-rays

X-rays are generated from electron cloud when electron moves from higher energy level to lower energy level causing excess energy to be released. It is very similar to gamma radiation [5].

2. Effects of ionizing radiation on bio-molecules

Exposure to any types of ionizing radiations, whether man-made or natural have deleterious biological effects at any dose. Primary ionization of an atom in the biological system can induce either direct or free radicals mediated indirect damage. Radiation can damage the bio-molecules by both directly and or indirectly by generating free radicals (**Table 1**) [5, 14].

Among the bio-molecules damages, DNA damage has been shown to be most important and to contribute maximally to cell death [15, 16]. Studies made on DNA irradiated *in vitro* in solution, in the dry state or *in vivo* in the biological system have revealed that radiation causes a spectrum of damages to DNA. Among them, the important ones are an alteration of purine and pyrimidine bases, single and double strand breaks, removal of bases and crosslinking of DNA with DNA or adjacent protein molecules. When a cell is exposed to radiation reactive oxygen species (ROS) is generated which targets cellular DNA for base modification, DNA adducts, DNA single strand break and double strand breaks. All these alterations

Biomolecule	Damage
DNA	Loss of nucleotide and base modification, deletion of hydrogen bonds, sugar-phosphate bonds, DNA-protein cross linking, single or double strand break, guanyl, thymidyl and sugar radicals
Proteins	Degradation and modification of amino acids, cross linkage, denaturation, molecular weight modifications and change in solubility.
Lipids	Peroxidation and carbon bond rearrangement, conjugate dieneand aldehyde formation, lipid cross-linking, increased microviscosity, cell membrane rupture.
Carbohydrates	Breakage of glycosidic Bonds and monomers, alcohol oxidation to aldehydes.
Amino acids	Generation of ammonia, CO_2, H_2S, Hydrogen molecules, Pyruvic Acid
Thiols	Redox reactions, radical formations, cross linkage.

Table 1.
Biomolecules damage by radiation exposure [9–13].

cause mutation and cell death (**Figure 1**). Endogenous genomic DNA damages are a relatively common event in the cellular life and if not repaired efficiently may lead to mutation, cancer, and cell death.

Radiation induced DNA damage can be divided into four categories [9, 10]:

1. Base damage

2. Alteration of sugar moiety

3. Cross-links formation of dimers

4. Single-strand breaks

5. Double-strand breaks

2.1 Base modification and damages

The most frequent modification is formation of hydroperoxide in the presence of oxygen. The most important one is hydroperoxidation of thymine [5].

2.2 Sugar modifications

Alteration in deoxyribose sugar is not very well understood and the alteration is (0.2–0.3 alterations of sugar per 10 SSBs. For this modification sugar is first oxidized and then hydrolysed followed by liberation of base, with or without breakage of phosphodiester bonds [5].

2.3 Cross-links and formation of dimers

Intra-strand crosslinks - between two parts of a single strand.
Inter-strand crosslinks - between the two strands.
Dimer formation - it occurs when two adjacent bases of single strands are joined by covalent bonds. It leads to the formation cyclobutane ring between them. Replication halts at the place where dimmers are formed. Thymine-thymine dimmers are most resistant and stable ones. They induce cutaneous cancers in the regions exposed to UV light [5].

There are approx 50,000 damage per day occurs inside the body due to the normal metabolic process such as maintenance of and replication of the genetic material. However, damage to DNA is native to life because its integrity is under constant attack from numerous endogenous agents such as free radicals generated during essential metabolic processes and from exogenous sources including radiation and chemicals. Endogenous damage affects the primary, rather than the secondary structure of the double helix. Four general classes of endogenous modifications can be envisaged as follows [11].

2.3.1 Oxidation

The oxidized bases formed as a byproduct due to oxygen metabolism show miscoding eg 8-oxo-7,8-dihydroguanine (8-oxoG), thymine glycol and similar oxidized bases [17]. Among these 8-oxoG is the most abundant and most dangerous one. It mispairs with adenine [18]. Strand interruptions are also generated by reactive oxygen species [19]. The spontaneous mutation rate due to single strand break is still unknown. Activation of poly ADP ribose polymerase (PARP) exerts most accurate response to single strand breaks [20].

2.3.2 Methylation

Some small molecules such as S- adenosylmethionine can methylate bases endogenously. According to recent study from almost 4000 residues generated per day 7-methylguanine (7 meG) is most important. 7-methylguanine base is relatively harmless and doesnot show any cytotoxic properties. Whereas endogenously produced 3-methyladenine (3-meA) which are few hundred in number are building block of DNA replication and should be efficiently repaired [21].

2.3.3 Hydrolysis

The base sugar bonds in DNA are relatively labile and several thousands of bases are lost each day in human cells under physiological conditions [12]. Purines are lost more easily than pyrimidines. Base loss sites probably represent the most frequent damage in human cells.

2.3.4 Mismatches

Mismatches can occur in DNA due to the incorrect incorporation by DNA polymerases, damage to the nucleotide precursors in the cellular nucleotide pool or by damage to DNA [13].

3. Single strand breaks (SSBs)

SSBs arise when diester bond between phosphate and the deoxyribose breaks. After the breakage of phosphodiester bond separation of both the strands occurs causing the water molecule to penetrate the breach. This process causes breakage of hydrogen bonds between the bases [5].

4. Double strand breaks (DSBs)

When two complementary strand of double DNA breaks in a location at a point less than 3 nucleotides is known as DNA DSBs. DSBs are considered as the most deleterious type of damage because both the complementary strands are damaged and it is very difficult for the internal repair mechanism of the cell to handle this type of damage. The factors leading to the formation of DSB include endogenous factors that are associated with physiological processes occurring in the cell and the exogenous ones [22–24].

In the presence of endogenous DNA damage, a cell can survive up to some extent, however the concentrated damages accelerated by exogenous agents such as ionizing radiations, radiomimetic drugs, ultra-violet radiations, and carcinogens can induce permanent changes. These changes lead to cancer or severely impaired cellular functioning and poor repair efficiency which may eventually cause cell death by triggering apoptosis or irreversible cell growth arrest [25]. Ionizing radiations generate ROS, which cause oxidative damage to DNA. The most important ROS are $O^{2\bullet}$ (superoxide radical), $OH^{\bullet}$ (hydroxyl radical) and H_2O_2 (hydrogen peroxide). The highly reactive hydroxyl radical ($OH^{\bullet}$) reacts with DNA and as a result, various forms of DNA damage occur. Exposure of DNA to ionizing radiations result in a number of different lesions in DNA such as base damage, single strand breaks and double strand breaks [9, 10, 26]. DNA DSBs present a major threat to the integrity of chromosomes and viability of cells. Unrepaired or incorrectly repaired DSBs

may lead to translocations or loss of chromosomes, which could result in cell death or uncontrolled cell growth. In addition, adjacent single-strand breaks in opposite strands may be converted to double strand breaks upon replication. DSBs are lethal unless repaired [27]. Ionizing radiations also induce clustered DNA damage in cells, which symbolize two, or more lesions formed within one or two helical turns of DNA and are in part responsible for the biological effects of ionizing radiation. The damage includes DSBs and non-DSB clustered damage such as SSB formed in close proximity to additional breaks or base lesions on both strands. An increase in the ionizing density of radiation increases the complexity of clustered DNA damage leading to decreased reparability of DSB in cells [28].

5. DNA DSB repair

Humans cells have two major DSBs repair mechanisms i.e. homology directed repair (HDR) and non-homologous end joining (NHEJ) [23]. However, in recent years a new mechanism called as alternative non-homologous end joining (A-NHEJ) has evolved (**Figure 2**). The selection criteria for DNA repair mechanism depends upon cell type, cell cycle phase and damage threshold. The non-dividing cells do not have the option of undergoing HDR but dividing cells can use all the three repair mechanisms with some conditions. The condition is NHEJ and A-NHEJ both can act in all the phases of cell cycle, however, the HDR is only able to act at S/G2 phase of the cell cycle [29].

5.1 Homologous recombination pathway

Homologous recombination pathway (HR) generally repairs the DNA lesions in late S or G2 phase of cell cycle. HR pathway is a series of interrelated pathways that participate in the repair of different types of DNA damages like double strands breaks (DSBs), interstrand cross links and DNA gaps. Several studies have shown

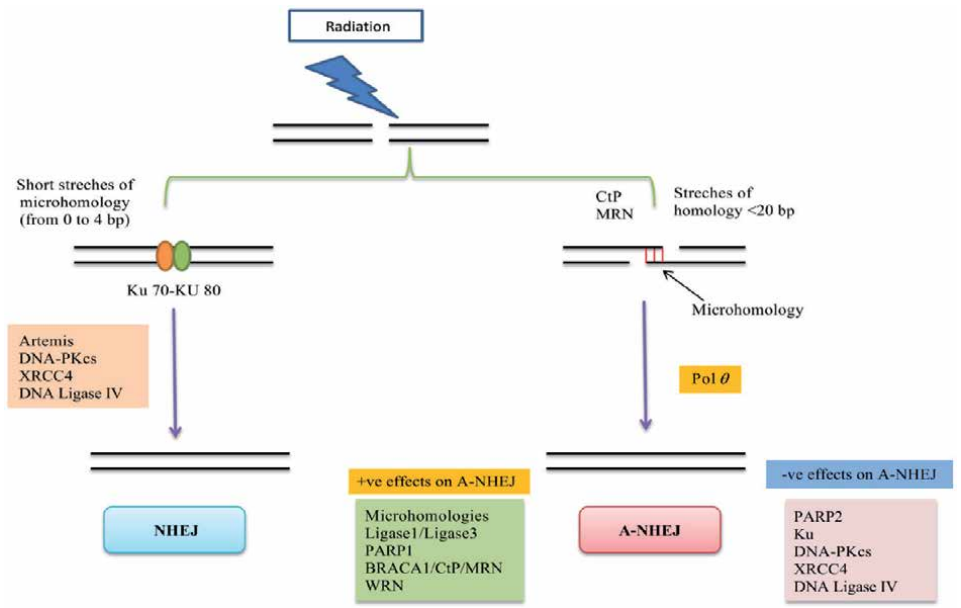

Figure 2.
Double strand break repair pathway choice.

that HR is an error-free pathway. This pathway is known as error-free because it occurs only S and G2 phases of cell cycles. In these phases of cell cycles sister chromatids are more easily available and can be used as template to synthesize new strands of DNA [30]. HR pathway is essential for cell division in higher eukaryotes to prevent recombination between non identical sequences. HR plays an important role in DNA replication for duplicating the genome and also in telomere maintenance for the recovery of broken replication fork [31–34].

HR accomplishes through following steps:

1. At the end of DSBs processing nucleolytic resection occurs to generate 3′ single-strand overhangs with 3-OH ends. This entire process makes use of MRN complex which has 3′ to 5′ exonuclease activity. 3′ single-strand overhangs are generated by this exonuclease activity [35–37].

2. Formation of a recombinase filament on the ssDNA ends: The broken DNA ends has 3′ single-stranded region which is coated with single strand binding protein, RPA. This binding of RPA removes secondary structures. After this, BRCA2 replaced RPA with the help of Rad51. Rad51 protein can interact with many ssDNA binding proteins like BRCA2, RPA, PALB2 and RAD52. Rad51 is 339 amino acid proteins that play an important role in homologous recombination of DNA during DSBs. Rad51 protein forms a helical nucleoprotein filament around DNA. The basis for Rad51 nucleoprotein filament formation to explore the homologous sequences on the sister chromatid [38–40].

3. A displacement loop (D-loop) intermediate is formed by strand invasion into homologous sequence. This invasion is prompted by Rad51, which enhances the activity of another protein Rad54B that facilitates D-loop formation by Rad51 in turn. However in meiosis the recipient DNA is similar but not identical homologous chromosome. D-loop is formed between homologous chromosome and invading 3′ overhang strand [38, 41].

4. Formation of holliday junction: The holliday junction is a biological process that can increase genetic diversity by homologous recombination, shifting gene between homologous and nonhomologous chromosome as well as site specific recombination. This process also involved in DNA DSBs repair pathways. D-loop structure is further changed into cross-shaped structure, known as holliday junction. This occurs after adding of new nitrogenous base to 3′ end of invading strand by DNA polymerase enzyme. This process ultimate leads to restoration of DNA strands on homologous chromosome. The junction is resolved after the restoration of lost sequences information and give error free repaired DNA. The double holiday junction model explained the resolution steps can be carried out by formation of two holliday junction to provide crossover and non-crossover products [42–44].

5.2 NHEJ (non-homologous end joining) repair pathway

The classical NHEJ is a pathway that repairs DSBs. This pathway is generally active in all stages of cell cycles. In NHEJ, the breaks ends are ligated without the need of homologous template. This pathway is very prominent in G0 and G1 phases of cell cycles to repair up to 85% DSBs formed by IR. These breaks formed by IR are very complex and contain non ligatable end groups [45–48].

NHEJ pathway carried out in following steps.

5.2.1 Detection of the DSBs and tethering of the DNA ends

The first step of NHEJ is detection of DSBs site by Ku70/80 proteins. Ku70 (69.8 kDa) & Ku80 (82.7 kDa) is an important heterodimeric complex involved in NHEJ pathway [49]. This dimer is a central DNA binding core and helps in binding of broken ends of DNA with higher affinity. This binding leads to the formation of a bridge between two proximal DNA ends which may help in tethering of the broken ends of damaged DNA [50]. This heterodimer has toroid shape with large central ring to accommodate duplex DNA ends [51, 52]. The inner portion of the central ring is lined with positively charged amino acids. These positively charged amino acids interact with phosphodiester backbone of DNA ends in order to safeguard it from nucleolytic degradation. Ku70 and Ku80 contain unique amino (N) and carboxy (C) terminal regions. The N terminus is phosphorylated by DNA-PKcs and last 12 amino acids of carboxy terminal region of Ku80 is required for interaction of DNA-PKcs with Ku heterodimer [53]. The Ku70 proteins is mandatory for chromosomal organization. The carboxy terminal region of Ku70 is involved in chromosomal organization. The carboxy terminus of Ku70 proteins contains SAP domain (SAF-A/B, Acinus, and PIAS) [54, 55]. The binding of Ku protein with DNA leads to the conformational change in the C terminal region of Ku70 and Ku80. This conformational change facilitates interaction of Ku proteins to other proteins such as XLF, DNA-PKcs, Ligase IV complex, XRCC4 and DNA polymerase μ etc. [55–60]. Thus Ku proteins considered as the corner stone of this pathway. The first protein to interact with Ku is DNA-PKcs. It is also involved in tethering of DNA ends at DSBs which further facilitate the recruitment of other repair proteins [61]. The molecular weight of DNA-PKcs is 469 kDa and contains 4128 amino acids and it is largest protein kinase which is specifically activated by binding to duplex DNA [62]. The conserved region in the extreme C -terminus of Ku80 mediates interaction with C-terminus region of DNA-PKcs. The interaction between DNA-PKcs.Ku further allows DNA-PKcs to interact across the DSB by the formation of (DNAPKcs- Ku-DSB complex or DNA-PK) Synaptic complex which serves to tether the broken ends of the DNA [50].

DNA-PKcs has weak serine threonine kinase activity and it is enhanced by DSB ends and Ku proteins. DNA-PKcs has weak serine threonine kinase activity and it is enhanced by DSB ends and Ku proteins. DNA-PKcs are when autophosphorylated leads to the liberation of DNA ends for processing and ligation. There are sixteen site that has been reported as autophosphorylation sites in DNA PKcs [63, 64]. Autophosphorylation of threonine 2609 and serine 2056 cluster play major roles in NHEJ process. It has been reported that radiosensitivity increases when phosphorylation of entire serine 2056 is inhibited whereas DNA ends processing is accelerated when there is phosphorylation at threonine 2609 [60, 63, 65–67]. The endonucleolytic activity of Artemis and ligation function of Ligase IV also supported by DNA PKcs [68].

5.2.2 Processing of DNA ends to remove damaged/non-ligatable groups

The next step after the detection of DNA ends in NHEJ is processing of the DNA termini to remove non ligatable end groups along with other lesions. Breaks in the DNA induces by IR are complex and depending on the nature of breaks require different processing enzymes like Artemis, DNA polymerase μ/λ, PNK etc. [69].

Artemis has 5′-3′ exonuclease activity however upon complex formation with DNA –PK, it acquires endonuclease activity as well. This acquisition of endonuclease activity helps in opening DNA hair pins during V(D)J recombination [70, 71]. In the processing in DNA the gaps induce by IR are filled by DNA polymerase. The enzyme that plays pivotal role in NHEJ are DNA polymerase μ and λ. These are recruited at DSBs sites by complexation with Ku proteins. Both polymerases

are recruited to the DSBs site only when they interact with Ku proteins. They both carry out reactions for gap filling and the only difference in them is requirement of template DNA. Polymerase λ is template dependent whereas polymerase μ is not so much dependent on template DNA. After gap filling proteins such as APLF, PNK, WRN etc. remove non-ligatable ends. The APLF removes non-ligatable ends by exonuclease and endonuclease activities. Whereas PNK removes non-ligatable DNA ends by its 3-DNA phosphatase and 5'-DNA kinase activities. WRN is a member of RecQ helicase family and removes non-ligatable DNA ends by DNA dependent ATPase, 3′-5' DNA helicase and 3′-5′ exonuclease activities [72].

5.2.3 Rejoining of the broken ends of DNA

For the completion of DNA repair process, the broken ends of the processed DNA must be rejoined. In NHEJ pathway, the rejoining and ligation step is carried out by Ligase IV, an ATP dependent enzyme. Ligase IV forms phosphodiester bonds between broken ends of DNA and catalyzing the ligation step. After hydrolysis of ATP, covalent linkage of AMP moiety occurs at specific lysine residue in the active site of DNA ligase. After linkage there is release of pyrophosphate [73]. This process releases AMP. Ligase IV has two C-terminal BRCT domains and is separated by a linker region. The linker region of Ligase IV interacts with the alpha helical region of XRCC4 to form an extremely stable complex. Till date, there are no published data on enzymatic activity of XRCC4 in DNA repairing process. XRCC4 is an important mediator for the recruitment of various NHEJ factors to the site of DNA damage and accelerate the process of DNA repair. Previous studies on NHEJ pathway in DNA repair process support the role of XRCC4 in stabilization and enhancement of DNA Ligase IV enzymatic activity [73–75]. DNA Ligase IV rejoins one strand of DNA at a time and simultaneously recruits and activates other repairing proteins that responsible for ligation of opposite strand of DNA.

6. Alternative NHEJ pathways

Recent studies identified an alternative repair pathway for DNA DSBs and also known as alternative NHEJ (A-NHEJ). The drawback of alternative pathway is that, it is very slow as compare to C-NHEJ [29]. This pathway is only activate when all other repairing pathways fails to repair DSBs. Because of this, A-NHEJ pathway is also considered as backup pathway for NHEJ (B-NHEJ). In A-NHEJ, the broken ends of DNA are ligated by Ligase III and Ligase I [76, 77]. In 2011, Odell ID et al. explored the effectiveness of Ligase III in repairing DSBs. Ligase III is more effective than Ligase I because Ligase III interact with ERCC1 and PARP1. XRCC1 promote efficient base excision repair and PARP involved in base excision and single strands breaks repair [78]. Apart from this, XRCC1 and PARP1 can also be used as biomarkers to sense the repairing process by A-NHEJ pathway [75]. A recent study illustrates the compromising of A-NHEJ pathway by existing by C-NHEJ factors like Ku proteins etc. A-NHEJ is basically a backup pathway which is activated when NHEJ pathway is compromised. NHEJ pathway is compromised due to absence of one or more core component such as DNA Ligase IV, Ku70/Ku80 heterodimer. A-NHEJ requires single stranded DNA at the ends so certain recombination proteins such as MRE 11A and CtIP act in this pathway [79]. Mutation in NHEJ pathway is extremely rare which makes it difficult to understand whether a-NHEJ is standing pathway or the components involved in this pathway also have its utility in replication, recombination or repair. a-NHEJ require pol θ along with poly (ADP-ribose) polymerase I(PARP), MRN complex and CtIP [79]. A-NHEJ starts when

phosphorylated CtIP stimulates MRN complex for its endonuclease activity which generates 15-100nucleotide 3′ overhangs. NHEJ requires short microhomolgy of 0–4 bp whereas A-NHEJ requires microhomology of <20 bp. The annealing of the two 3'overhangs is stabilized by pol θ that is sealed by DNA ligase I or DNA ligase III. Apart from these functions pol θ also has transferase activity to add nucleotide to provide microhomology that is absent. Insertion of short templates are not necessarily involved with microhomology but also in human lymphoid translocation around (20–50%) [80]. There have been certain evidences that show pol θ activity when long 3'ssDNA tails generated by the process of extensive resection embeds annealed microhomologies. This process generates non-homologous 3'ssDNA tail that is needed to be removed before extension by pol θ [81]. So during A-NHEJ pathway there may be requirement of nuclease activity from other pathways as well as seen in mammalian system in xeroderma pigmentosunm group F (XPF). XPF uses ERCC1 nuclease complex, APLF or Artemis-DNA –PKcs. Therefore it may be noted that proteins required for A-NHEJ are PARP1, the MRN complex and its partner CtIP and for end joining either LIG1 or LIG3 [25].

There is a possibility that A-NHEJ is slower than NHEJ as seen in class immunoglobulin class switch recombination where missing DNA ligaseIV can be replaced with DNA ligaseI or DNA ligaseIII. This substitution occurs but with tenfold slower kinetics [82, 83]. This substitution of DNA ligaseIV with DNA ligaseI or DNA ligaseIII suggests presence of backup components of such important enzymes but of lower repair efficiency and with slower kinetics. Future work is needed to identify all the differences between NHEJ and A-NHEJ and also the component of A-NHEJ. Not only distinction but also the repair kinetics is also an important point to be taken into consideration. The fine balance between NHEJ and A-NHEJ is also mediated by ataxia telangiectasia mutated - mediated DNA damage response. In the absence of ATM NHEJ is favored. There is extremely rare and lethal for mammals that lack components for NHEJ [84]. Therefore it should be noted that components for A-NHEJ i.e. its enzymes and proteins may have other functions as well apart from being a substitute. So according to Dueva and Iliakis 2013 there are two models through which A-NHEJ is activated. The first one states that A-NHEJ comes into play when NHEJ or HRR which were engaged for the repair of double strand break but failed to complete the process. According to the second model states that A-NHEJ comes into action when either of the process NHEJ or HRR attempted for the repair mechanism but somehow failed [29]. Basically A-NHEJ comes into play as a backup process for NHEJ or HRR with slight differences. When A-NHEJ back up the failure of NHEJ it can occur throughout the cell cycle as NHEJ is active throughout the cell cycle. But when it backs up the shortcomings of HRR it can only occur in S- and G2- phase of the cell cycle. This type of repair pathway contributes to 10–20% of radiation induced DSBs [34, 85]. A-NHEJ basically operates on resected end that inactivates NHEJ and paves way for HRR which truly justifies the dependences of A-NHEJ on certain proteins such as MRN complex, CtIP, BRCA1 [86, 87].

6.1 Role of A-NHEJ in leukemia progression

Leukemia and lymphoma are the type of cancer that shows translocation of chromosomes with involvement of A-NHEJ [88, 89]. There is an availability of evidences that show A-NHEJ play active role in erroneous repair of programmed DSBs during V(D)J AND Class Switch Recombination(CSR) [29]. Severe combined immunodeficiency syndrome (SCID) is a disease which occurs due to mutation in DNA repair proteins [90]. SCID like phenotype is observed in murine models that lack RAG proteins [91–93]. These murine models are also seen to develop tumors because of the translocation in Ig locus due to A-NHEJ. A model was also proposed

which suggests A-NHEJ mediated genomic instability was suppressed with the help of RAG1/2 proteins and NHEJ factors [94, 95]. RAG complex formed post cleavage shunts the broken ends of DNA to NHEJ thus suppressing recombination events. It is seen that RAG mediated DSB repair during CSR is not compromised in cells lacking NHEJ but is shifted to A-NHEJ [82, 96, 97]. There is effect of absence of DNA-PKcs and it uses Lig1 or Lig3.XRCC1 which acts together with Lig3 is not necessary for A-NHEJ during CSR. Infect the absence of these components increases CSR efficiency [98, 99]. PARP1 and PARP2 is nonessential component during CSR but PARP1 favors A-NHEJ whereas PARP2 suppress translocation during CSR [100].
It is very interesting to note that in chronic myelogenous leukemia (CML) there is increased production of ROS due to increased cell division which is facilitated by BCR-ABL tyrosine kinase. Increased ROS inside the cells leads to DNA damages especially DSB. This leads to the up-regulation of A-NHEJ [101–103]. The cells which are BCR-ABL positive CML shows up regulation of key proteins for A-NHEJ i.e. Lig3α and WRN whereas down regulation of key proteins of NHEJ Artemis and Lig4. Therefore A-NHEJ enables the cells of CML to repair ROS induced DSB and survive. Though this repair pathway of A-NHEJ is error prone the price the cells pay for survival is genomic instability [104].

In acute myeloid leukemia (AML) mutation that occurs are internal tandem duplication (ITD) of FMS-like tyrosine kinase3 (FLT3) receptor. FLT3-ITD is type of cancer which utilizes microhomology mediated A-NHEJ to repair double strand breaks. It causes increased number of deletion. The cells expressing FLT3-ITD has increased protein level of Lig3α but decreased level of Ku protein required for NHEJ. This causes shift towards the A-NHEJ for DSB repair [105].

6.2 Targets for cancer therapy

PARP1 inhibitors could act as therapeutics for cancer in BRCAness (**Table 2**). Certain therapeutic strategy involves the use of DNA ligase as targets [106]. In BCR-ABL-positive CML it is treated with tyrosine kinase inhibitor Imatinib, this strategy immense hope for targeting A-NHEJ factors for therapeutics. Tobin et al. reported that BCR-ABL-positive CML resistant to Imatinib were sensitive to combinational treatment of Ligase and PARP inhibitors which correlates with hyperactive A-NHEJ [109]. This therapy was effective in therapy resistant breast cancer cell lines as it became

Cancer	Genetic Background	Drug targets	Altered Repair pathway	References
MCF7 breast cancer	Reduced DNA LIG4, Enhanced DNA LIG3a and PARP1	PARP1 with DNA ligase inhibitors	NHEJ	[25, 106, 107]
Chronic Myeloid leukemia	BCR-ABL, enhanced expression of LIG3a, PARP1, and WRN	PARP1 with DNA ligase inhibitors	HR	[72, 102, 108]
Breast, Ovarian	BRCA 1 deficient BRCA 2 deficient	PARP 1 PARP 1	HR	[38, 106]
Non-BRCA1/2 breast cancer	XRCC4 deficient	unknown	NHEJ	[56–59, 73, 74]
Leukemia, proB-cell lymphoma	KU, P53 deficient	Unknown	NHEJ	[49, 53, 58, 79, 105]

Table 2.
Disease, impaired repair pathway along with their therapeutic targets.

sensitive to DNA ligase and PARP inhibitors [107]. Many PARP inhibitors obstruct DNA replication by trapping PARP [108]. Lig3α or PARP inhibitors are also included in novel therapeutic strategies for AML associated with FLT3 mutations [105].

Therefore there is an immense possibility of treatment of cancer with A-NHEJ inhibitors which involves tumors with increased A-NHEJ. And it will be interesting to see if there is possibility of protecting an organism from carcinogenesis by limiting the function of A-NHEJ.

7. Conclusion

Radiation and other assaults that cause DNA damage leading to double strand break are dealt by the mammalian system by relying on tightly regulated repair pathways that are end-joining or recombination-based repair pathways. These are highly regulated repair pathways and results in accurate restoration of the genome. Error prone double strand break repair is still prevalent despite of its mutagenic potential. We must also understand that it is not simply a backup mechanism that comes into play when accurate repair pathway is not possible. The various factors that regulate it are cell cycle stage, local sequence context (homology), and genome structure. So the error prone repair pathway is also very important as it prevents major genome catastrophe. Detailed survey of literature puts forward the fact that error prone pathway paves way for genome evolution in somatic tissues in context of cancer. It is apparent that clear understanding of how A-NHEJ operates and is regulated inside the cell after double strand break will have important therapeutic implication in context of cancer treatment and cure.

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Conflicts of interest

The authors declare there are no conflicts of interest.

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Chapter 6

Interstrand Crosslink Repair: New Horizons of DNA Damage Repair

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Abstract

Since the dawn of civilization, living organisms are unceasingly exposed to myriads of DNA damaging agents that can temper the ailments and negatively influence the well-being. DNA interstrand crosslinks (ICLs) are spawned by various endogenous and chemotherapeutic agents, thus posing a somber menace to genome solidity and cell endurance. However, the robust techniques of damage repair including Fanconi anemia pathway, translesion synthesis, nucleotide excision and homologous recombination repair faithfully protect the DNA by removing or tolerating damage to ensure the overall survival. Aberrations in such repair mechanisms adverse the pathophysiological states of several hereditary disorders i.e. Fanconi Anemia, xeroderma pigmentosum, cerebro-oculo-facio-skeletal syndrome and cockayne syndrome etc. Although, the recognition of ICL lesions during interphase have opened the new horizons of research in the field of genetics but still the detailed analysis of conditions in which repair should occur is largely elusive.

Keywords: DNA damage repair, Interstrand cross links (ICLs), Homologous Recombination Repair, Translesion synthesis, Non-homologous end-joining repair, FA pathway

1. Introduction

There is an amalgam of various environmental, endogenous as well as chemotherapeutic agents that are continuously having a contact with the genetic material in living beings and making it a point of real concern throughout the globe. The attack of reactive oxygen as well as nitrogen species on DNA have contributed towards a large amount of defects and complex chemical structures that take place in DNA [1]. These damages give rise to a series of simple and bulky base modifications that distort the helical structure, abasic sites, the breaks in phosphodiester linkages along with the interstrand crosslinks (ICLs). These lead to various mutagenic changes in the genetic blueprint and become a reason of inhibition of the transcriptional or replicative machinery that induce activate apoptotic divisions or necrosis [2].

Interstrand cross-links (ICLs) are the anomaly that link the complementary strands of DNA by the covalent linkage between the bases. These are formed by the chemicals along with the two reactive electrophilic groups. It is a highly sequence-dependent reaction in which the two nucleophilic groups on the opposite strands are aligned geometrically and enable the dual reaction of the bifunctional cross-linking agent with it. This complex chemical reaction give rise to ICLs,

mono-adducts, intrastrand cross links as well as DNA-protein cross-links [3]. The ICLs are made with the help of reactive endogenous chemicals such as lipid peroxidation product known as malondialdehyde or aided with the reactive aldehyde group of an unpromptedly formed or the enzyme-derived abasic site in the DNA molecule with a normal base on the complementary strand [4].

A large amount of anticancer and chemotherapeutic agents such as mitomycin C (MMC), cisplatin, nitrosoureas and nitrogen mustards are notorious for introducing formidable blocks in the normal metabolic processes of DNA with ICLs and need repair for cell sustenance. ICLs are also caused by various antitumor agents that defects DNA through radical processes like C-1027, neocarzinostatin [5]. With the passage of time, the organisms have developed various complex mechanisms to alleviate these deleterious defects from the genome. The failure to remediate the defect can contribute towards cell death that can occur either through a mitotic catastrophe or the p53-dependent apoptotic pathway. In the mammalian cells, the repair mechanisms for ICLs repair are still ambiguous [6]. According to an estimation, about 40ICls that form in a mammalian genome can destroy a defective cell that lacks ability to be repaired.

The in vivo study gives an overview of the elimination of the ICLs in cellular DNA of both prokaryotes and eukaryotes. The model organisms are used for the clear understanding of the repair mechanisms. These include *E. coli* and yeast. The ICLs repair mechanisms in bacteria and yeast are replication dependent and independent while in vertebrates, they follow repairment pathway during replication of DNA [7]. Moreover, the recent study suggests the operation of replication independent ICL repair pathway in vertebrates.

The ICL repair pathway have been deduced from the relative sensitivity of the DNA repair defective cell lines to the cross linking agents. Pathways of ICL repair have mostly been inferred from the sensitivities of DNA repair defective cell lines to crosslinking agents. During the S phase of the cell division in vertebrates, the ICL repair is induced by the help of impeded replication forks. The process of ICL repair needs a nexus of multiple factors along with the structure specific endonucleases, for example TLS and HR. If a disturbance occurs during the repair, the genomic instability results that bring forth the birth of Fanconi anemia, a cancer prone ailment [8]. There is another ICL repair pathway that takes place in the G0/1 phase during the cell cycle which is a replication and recombination independent pathway [9]. In addition, the tolerance of ICLs in G1 as compared to S phase makes it an underappreciated pathway because there, the stalled replication fork possesses high toxicity. Contrarily, the toxicity of ICL in G1 can be depicted when it terminates the transcription of a gene playing a vital role.

The latest studies have proposed the role of NER proteins (as they cut one side of ICL) [7], Homologous recombination along translesion synthesis polymerases (Polζ, Rev1) that are involved in filling the gap for both type of cells undergoing replication as well as non-replicating ones [10]. The proteins involve in the ICL repair have a vital role in the pathophysiology of several hereditary diseases Proteins implicated in the repair of ICLs have a critical role in the pathophysiology of several hereditary disorders. In addition, cells deficient in the Fanconi Anemia (FA) pathway are highly sensitive to ICLs [11] and this pathway has been suggested to play an important role in mammalian ICL repair at replication forks promoting homologous recombination. There has been a series of continuous research on ICL lesions in the past decade and it covered the various aspects of ICLs be it as their identification, detection methods or their development along with the repair mechanisms and the exploitation of cross linkers in the laboratory. These have paved the way towards the better and more reliable understanding of ICLs in the complex biological samples. This chapter foregrounds the multiple aspects of the interstrand cross-link repairs with a reference to their pathophysiology and lesion repair mechanisms.

2. Basic biochemistry of ICL-generating agents

A large variety of natural and synthetic chemicals are notorious for bringing ICLs on the front and are regarded as the ICL inducers or inducing agents. In the same way, the metabolic byproducts formed in the cell also contribute towards ICLs formation. Their structure and function vary greatly but ICLs inducers are known for their bifunctional reactivity with both of the strands of DNA. The endogenous as well as exogenous sources of ICLs are summarized as follows:

2.1 Endogenous sources of interstrand cross links

The endogenous sources of ICLs comprises of the reactive aldehydes that are generated as a result of lipid peroxidation along with base excision repair (BER) [12]. There are other endogenous by products of lipid peroxidation, the α, β-unsaturated aldehydes or enals namely crotonaldehyde, acrolein, along with the 4-hydroxynonenal (4-HNE). These are formed as a result of oxidative stress [13]. Moreover, there are exogenous contributors as well namely cigarette smoke and automobile exhaust to expose with acrolein and croton-aldehyde. The DNA nucleobases interact with enals to give rise to exocyclic adducts. These adducts then interact with proteins. The incorporation of enals to dG is done with the help of Michael addition in which addition of N^2 -amine occurs to generate N^2-(3-oxopropyl)-dG adducts. The next stage is cyclization of N1 with the aldehyde, giving rise to N^2 -γ-hydroxypropano-dG adducts [14]. These products are also genotoxic to human beings. Shapiro and Leonard are famous for their earlier study of nucleosides reactions with glyoxal, chloroacetaldehyde, malondialdehyd along with related bis-electrophiles [14, 15]. The in vitro formation of ICL is attributed to the opening of the exocyclic 1, N^2-dG product that minimizes the steric hindrance and forms ICL on exposure towards an aldehyde [16].

Moreover, there are DNA lesions that are formed as a result of accumulated acetaldehyde in the cells. The acetaldehyde is produced as a result of alcohol metabolism with aldehyde dehydrogenase 2 (ALDH2) as a biocatalyst. The drug disulfiram if used, blocks the enzyme ALDH2 and accumulates the acetaldehyde in the cells. The lesions produced are DNA adducts, breaks in single or double-strands of DNA (DSBs), sister chromatid exchanges (SCEs), point mutations, along with crosslinks in DNA [17]. The DNA adducts like N^2-ethylidene-2′-deoxyguanosine, N^2-propano-2′-deoxyguanosine, N^2-ethyl-2′-deoxyguanosine, along with N^2-etheno-2′-deoxyguanosine are vital DNA damage agents that follow the accumulation of acetaldehyde in the cells. The acetaldehyde reacts with guanine and forms a crosslink precursor known as N^2 -propanoguanine (PdG) which in turn reacts with N2 amine of guanine in 5′-CpG sequence consequently forming acetaldehyde interstrand crosslinks (AA-ICL). In Asian continent, the irreparable detoxification of acetaldehyde is found more often and is linked with alcohol mediated cancers [18]. Moreover, cells in *Saccharomyces cerevisiae* don't have ability to repair ICLs and are acetaldehyde sensitive thus gives validation of acetaldehyde mediated ICLs [19].

The intestinal pathogens in human beings known as *Enterobacteriaceae* and other bacteria play a vital role in the progression of colorectal cancer. They produce colibactins that are genotoxic in nature and bring harm to human beings. With their structural chemistry still unknown, colibactins produce ICL dependent DNA double-strand breaks (DSBs) and activates the ICL repair pathways [20]. *Cellulo* also depicts another picture of the DNA damaging mechanism in which colibactin producing bacterial exposure towards the genomic DNA of cultured human cells made it susceptible to interstrand cross links. There are different changes observed in the intoxicated cells including the replication stress, the activation of

ICL AGENTS	Source	Adducts formed/ Target DNA sequences	Clinical benefits	Elimination half-life of drug	Metabolism	References
Platinums						
Carboplatin	Synthetic (made from cisplatin)	Adducts: G-Pt-G and Pt-GG DNA sequence: 5'-GC	Treatment of ovarian cancer	1–2 hours	Kidney	[28]
Oxaliplatin			Treatment of colorectal cancer	26 hours or 20 hours	Kidney	[29, 30]
Mitomycin						
Mitomycin C	*Streptomyces caespitosis*	5′-CG-3'	Treatment of Esophagal and bladder carcinoma	Alpha-half-life of 8.2 mins, beta-half-life of 51.8 mins	Hepatic	[10, 31]
Azinomycin						
Azinomycin B	Streptomyces sahachiroi	5′-GNC or 5′-GNT sequences	having antitumor activity against P388 leukemia in mice	N.A	N.A	[32]
Chloroethylnitrosoureas						
Carmustine	Synthetic (nitrogen mustard)	G-C base pair	Treatment of multiple myelomas	70 minutes	Liver	[33]
Nitrogen mustards						
Cyclophosphamide	Synthetic	5′ -GNC	Treatment of lymphoma, multiple myeloma and ovarian cancer	**3 to 12 hours**.	Liver	[34]
Ifosfamide			Treatment of sarcomas and organ cancers	60–80% in 72 hours	Kidney	[35]
Chlorambucil			Treatment of Chronic lymphocytic leukemia, Hodgkins lymphoma and Non-hodgkin lymphoma	1.5 hours	Liver	[36]

Table 1.
Exogenous agents of Interstrand cross-link lesions.

ataxia-telangiectasia along with Rad3-related kinase (ATR), as well as the retrieval of Fanconi anemia protein D2 (FANCD2). Contrarily, FANCD2 knockdown or ATR inhibition decreases the survival capability of cells having an exposure towards colibactins. The evidence ensures that collectins mediated DNA defects in infected cells favors DNA ICLs [21].

2.2 Exogenous sources of ICLs

The other sources of ICLs are exogenous in nature. They have the same mechanism of bifunctional alkylating agents but differ in their preferences for sequences, topologically restrict the DNA and need certain processing within the cell to form functioning ICL inducers [9]. In spite of the fact that they have a history of damaging DNA, their innovative uses also aid in understanding the mechanisms they follow to contribute in various therapeutic applications.

These include psoralens that belong to the family of furocoumarins, being mutagenic are still a matter of contention with their photochemotherapeutic applications in inflammatory skin diseases like psoriasis, vitiligo and eczema [22]. The Psoralens generates adducts on interaction with pyrimidines, most often with thymine and give rise to ICLs at the sequences made up of d(TpA):d(TpA) residues [23]. The several derivatives of psoralen form multiple changes in the DNA helical structural framework and exhibit their toxic nature. The DNA duplex adducted with 4′- (aminomethyl)-4,5′,8-trimethylpsoralen (AMT) exhibited 561 unwinding and 531 bending into its major groove [24].

Another chemotherapeutic agent known as cis-platinum diamminedichloride i-e CDDP, cisplatin also induces ICLs. It makes an adduct with purines, most often at the N7 position of the guanines, hence ICL forms at d(GpC): d(GpC) sequences. This is employed in various head and neck cancers, esophageal, epithelial lung, colon, gastric, bladder along with ovarian and testicular tumors. About 90% of the total defects are formed by 1,2-IaCL and 1,3-IaCL along ICL making only 5% of the total DNA lesions [23].

Apart from these anticancer agents, one of prime importance is Adriamycin which is also termed as doxorubicin. It generates a great response against a range of tumors be it as breast tumors, acute leukemia, lymphomas, stomach, sarcomas, multiple myelomas or bone tumors. It is employed as a singly or in combined form [25]. The interaction of Adriamycin is clearly understood with the help of the *in vitro* transcription assays that demonstrates the drug-induced DNA adducts at the GpC sites [26]. The electrospray mass spectral analysis revealed details of GpC drug binding regions and gives the information that the cross links are favored by formaldehyde under the certain conditions [27]**. Table 1** illustrates the exogenous agents of Interstrand crosslink lesions.

3. ICL Repair genes and human disorders

The proteins involved in the repair of ICLs have vital role in pathophysiology of various hereditary disorders for example xeroderma pigmentosum (XP), cerebro-oculo-facio-skeletal syndrome (COFS), Fanconi Anaemia (FA), trichothyodistrophy as well as Cockayne syndrome (CS) [37]. FA is associated with aplastic anemia, cancers (often acute myelogenous leukemia) and bone marrow failure. The mutational changes in any *FANC* genes contribute towards genomic instability and the sensitivity against the ICL agents [38]. According to an estimate 18 genes are involved in FA and the products of genes collaborate for ICL repair during the S phase [39]. Apart from these, the defective NER pathways also result in several rare

autosomal-recessive diseases like XP, CS, TTD and COFS syndrome [40]. Moreover, there are 11 genes that are associated with NER pathways and the defect in these occur due to the mutations in these genes. XP is associated with pigmentation, photosensitivity as well as cancerous skin diseases. Another inherited syndrome known as CS is present in which there are several problems arises namely ocular defects, mental deficiency, extensive demyelination, short stature, photosensitivity, large hands, feet, as well as ears [37]. There are wide ranging clinical spectrum of CS and the patients acutely affected are categorized under COFS syndrome patients. TTD is associated with neuro-ectodermal symptoms and clear sulfur-deficient brittle hair [41]. These NER diseases are different from each other with respect to their physical characteristics involving cutaneous ailments.

Keeping in view the various DNA repair factors, ICL genes has found to be having a strong link with cancer. There are several genes that are revealed by next-generation sequencing and play a part in hereditary breast cancer as well as ovarian cancer syndrome (HBOC). These genes are *BRCA1, BRCA2, PALB2, BRIP1 and RAD51C* exhibiting a close link with HBOC in the ICL repair pathways [42]. The preventive medication strategy requires the early detection of the mutations happening in BRCA1 and BRCA2 genes to help in process of recovery.

4. Recognition of ICL lesions in mammalian cells

During the course of ICL damage, the UHRF1 protein comes to rescue at the site within a fraction of seconds [43]. These proteins identify ICLs with the help of its SET and RING finger associated (SRA) domain, the same domain notable for its recognition ability for the hemi-methylated DNA and employment of DNMT1 to ensure the maintenance of methylation signature in the cells of mammals [44]. The relative affinity of UHRF1 protein in response to hemi-methylated DNA as well as ICLs are somewhat similar and proposed that UHRF1 interacted with both of them through related mechanisms. The UHRF1 proteins are employed preceding the incorporation of FANCD2 to ICLs [43]. About 10 minutes are lagged between the assembling of UHRF1 and FANCD2 to ICLs. This strengthens the assumption of other proteins being employed or the other PTM events that might occur during this time interval. The proper mechanism of UHRF1 mediated FANCD2 repair is not clear but implicate a direct protein–protein interaction. There has also been a proposed role of UHRF1 in a nuclease scaffold [45]. It is also proposed that the rapid incorporation of UHRF1 to the ICLs paves the way for FA mediated repair of lesion later on. As ICLs vary in their structural framework, there is a probability that in addition to UHRF1, other ICL sensor proteins do exist in the same way.

5. Factors involved in ICL repair pathway

There are several proteins that take part in the ICL repair. Along with these, included 15 proteins that are not only specific to FA genes (A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, and P) but also to other repair pathways [46]. The important recombination factors like RAD51, the structure-specific endonucleases like MUS81/EME1 and XPF/ERCC1, translesion DNA polymerases and Holliday junction processing factors all contribute towards the repair of ICLs.

A rare human genetic disease known as FA, which is associated with pancytopenia, various developmental abnormalities and a high cancer risk [47]. The cells procured from FA patients depict the large amount of chromosomal breakage as well as the formation of radial chromosomes [48] that bring strength to the idea of

high genomic stability in the ICL repair-deficient cells. The classical FA pathway has FA core complex (consisting of A, G, FAAP20, C, E, F, B, L, and FAAP100), an E3 ubiquitin ligase activity and the catalytic activity dedicated to the RING domain comprising FANCL protein. The core complex also acts on monoubiquitination of FANC1/D2 complex and is stimulated by damaged DNA [49]. The next step is the utilization of other downstream effectors that are attracted by the activated complex. These comprises nucleases, homologous recombination factors and translesion polymerases to remediate the lesions [50]. Whereas the exact function of monoubiquinated FANCD2 is still ambiguous.

An ATP dependent DEAH domain helicase namely FANCM exhibit a DNA translocase activity. It combines with FAAP24 and forms a complex structure comprising a histone-fold complex i-e MHF1/MHF2. It is a significant part of activated FA pathway [51]. The biochemical analysis also proposed that FANCM/FAAP24 complex is responsible for stabilizing and remodeling the stopped replication forks of DNA [52]. The complex of FAAP24 plays a vital part in the checkpoint activation that also need ATR to begin its function [53]. However, FANCM takes part in recombination independent ICL remediation by stimulating ubiquitination of PCNA thus promotes the incorporation of other NER incision factors to the sites with ICLs [51].

The group of genes associated with FA comprises of FANCD1 (BRCA2), FANCJ, FANCN, as well as FANCO are the recombination factors that forms a connection with susceptibility for breast or ovarian cancer. The downstream processing of ICL require the employment of recombination factors, mostly when there are the double strand breaks in the DNA. The paralogous gene of FANCO (RAD51C) is RAD51 [54]. FANCO forms complex structures on interaction with RAD51B, RAD51D, XRCC2, as well as XRCC3. Another significance of these paralogs is the utilization of the recombinase RAD51 while managing a single stranded DNA [55]. RAD51 and its paralogs are vital to cells tolerant against ICLs and vice versa because they provide the homologous recombination in response to ICLs as well as the double strand breaks [56].

The endonucleases also pay a part in ICLs repair. Three important heterodimeric structure-specific endonucleases are MUS81/EME1, SLX1/SLX4 and XPF/ERCC1. SLX4 is often mutated in the complementation group consisting of FANCP [57]. The combination of SLX4 and SLX1 make up a heterodimeric nuclease. Its function is to resolve the Holliday junction formed during the remediation of ICls [58]. During the process, SLX4 act as a scaffold protein that combines the multi-activity nuclease complex comprising MUS81/EME1 as well as XPF/ERCC1. The latter acts in either of the NER pathway as well as ICL repair. The studies proposed that NER works independent of SLX4 with XPF/ERCC1 complex and the analysis of FANCP patients further strengthens the idea as they were resistant against the UV radiations [59]. Further studies suggest that XPF/ERCC1 activity requiring SLX4 involves the complete detaching in ICL repair. It is a replication dependent remediation of ICLs [60]. Digesting nuclease (SNM1A) then follows and digest the detached oligonucleotides [61]. This step is a better alternative as compared to the bypass step used for synthesis.

Moreover, the lately discovered nuclease FAN1 also has a significant part in remediation of ICL. The ubiquinated FANCD2 aids in employing FAN to ICL regions. This step is mediated with the ubiquitin-binding zinc finger domain that is present in FAN1 [62]. Another important domain of FAN1 exhibit 5′-3′ exonuclease activity as well as structure-specific endonuclease activity at 5′ [63]. FAN1 thus cuts the exposed ends of DNA along with DNA replication structures that hinders the process.

Other important participants in ICL repair are the translesion DNA polymerases. The blockage of normal replicative DNA polymerases is done before reaching the ICL regions. Other translesion polymerases in *Xenopus laevis* include Y-family polymerase Rev1 as well as B-family polymerase Pol ζ (Rev3/Rev7) have a significant part in complete removal of ICLs. These models also use replisome remodeling

machinery so that the extension of stalled DNA strand occur on one base before the ICL region [64]. On unwinding, Rev1's deoxycytidyl transferase of Rev1 incorporates cytosine on the complementary strand across the ICL region [65]. This is then succeeded by Pol ζ that extends the unpaired strand.

6. ICL lesion removal in quiescent G0/G1 phase

The comprehension of ICL repair is a difficult task because it has an implication on both strands of DNA. The cells in G0/G1 phase do not require homologous recombination for ICL repair [66]. Moreover, all eukaryotic organisms ranging from *Saccharomyces cerevisiae* to the human beings, require NER for the incisions of ICL. The single stranded gap is produced at the first step of NER by the oligonucleotide on ICL lesion. This can be bypassed with the help of translesion DNA polymerases REV1 just like the DNA polymerases (η, ι, κ, and ζ,). Both the DNA polymerases κ, and ζ, as well as REV1 are vital for this stage of NER [67].

7. ICL recognition and repair in proliferating S-phase

The repair of ICL faces several complications during the S phase. The data exhibits the formation of double stranded breaks by interaction with ICL causing agents [59]. The ICL induced Double stranded breaks can be repaired by HR rather than non-homologous end joining (NHEJ) method [68]. This brings to the conclusion that ICL-induced DSBs are linked with DNA replication forks. NER indicates ICLs in *S. cerevisiae* and NER function is important for ICL repair. So, all NER-mutants exhibit hyper sensitivity to the ICL causative agents. Contrarily, the cells deficient in *XPF*- as well as *ERCC1*- show immense hypersensitivity to the ICL agents (mitomycin C & nitrogen mustard) in mammals. The product of *XPF* as well as *ERCC1* make up an endonuclease which is hetero-dimeric in nature identifies and incise the single stranded branched structures [69]. Moreover, MUS81-EME1 along with XPF-ERCC1, the homologous structure specific endonucleases are also keen in repairing the ICL lesions [70]. MUS81-EME1 is notable for its binding with the double-stranded branched structures, flaps at 3′ end, as well as Holliday junctions [71]. Either of the two XPF-ERCC1 and MUS81-EME1 are responsible for ICL-induced double strand formation. Since, a multitude of nucleases are recognized recently being the key players in ICLs incision, the mechanism underlying the process need to be explored. We abridge the current knowledge about the ICL repair mechanism in S phase. HR repairs the ICLs induced DSBs. An experiment conducted in *S. cerevisiae*, gives an outline of hypersensitivity against ICL causative agents in *rad51*, *rad52, rad54*, *rad59*, as well as *mre11* mutants but not in case of *yku70* mutants. The hypersensitivity of *rad52 yku70* double mutants to ICLs is at par with that of rad52 mutants [72]. The HR deficient strains show the increase in accumulated DSBs successively on treating with ICL inducers as there lacks an ability to cure DSB which means that NHEJ is not a pre-requisite to remediate DSBs stimulated by ICLs. The mammals follow the same process in their cells. The HR deficient cells depict hypersensitivity against ICLs like cells having mutated paralogs of *RAD51*, *RAD54*, *RAD54B*, along with *BRCA2*, while it is not observed in cells deficient in NHEJ [73]. It significantly highlights the role of HR in repairing DSBs and re-initiating the halted replication forks of DNA. Fanconi anemia (FA) genes are key players in the remediation of ICL in eukaryotes. The proper role of FA gene products in biochemical reactions are still not identified properly, but are notable for their control of HR at the replication forks of DNA [74].

8. Interstrand crosslinks lesion repair mechanisms

Lesions in interstrand crosslinks epitomize an arduous challenge in genome maintenance pathways due to the compromise of genomic information present on both strands. Therefore, an application of non-damaged strand as a template for accurate repair in straightforward cut and patch mechanism is not feasible. In this regard, ICL repair employs the concerted and synchronized interaction of dynamics from numerous mechanisms of DNA damage repair, including NER, homologous recombination, mismatch repair, translesion synthesis, ataxia telangiectasia, Rad3 related and Fanconi anemia pathway. **Figure 1** illustrates the schematic mechanism of ICL repair [75].

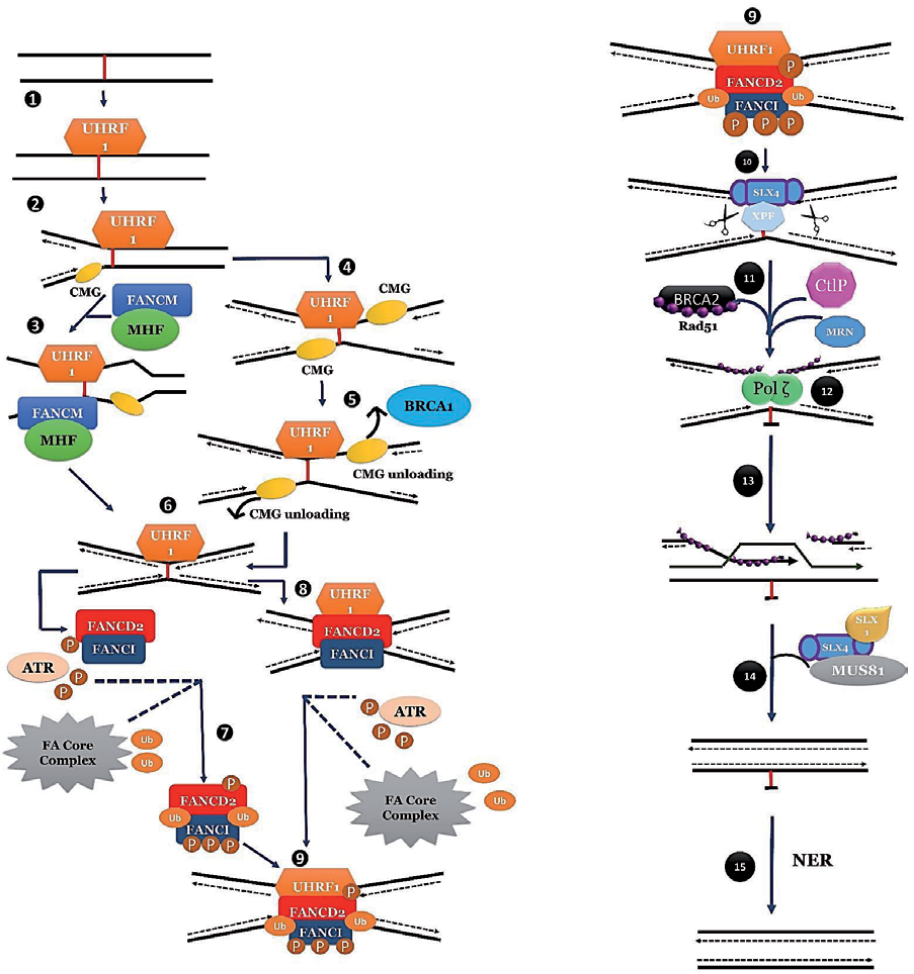

Figure 1.
Schematic of ICL repair mechanism. (1) After the formation of ICLsin the cells, UHRF1 is recruited through its SRA domain immediately. (2) Single replication fork reaches at ICL. (3) Then Replication machinery is transversed through ICL by the help of FANCM/MHF complex and allowes the ICL for later repair. (4), (5) On an alternate basis FANCS or BRCA1 allows the unloading of CMG helicase complex, when second replication fork arrives at ICL. (6) Then replicative polymerase reaches at −1 position of ICL, leaving X shaped similar to the transverse mechanism. (7) Then ATR allows the phosphorylation of FANCD2/FANCI complex at multiple sites and meanwhile FA core complex mono-ubiquitinate at FANCD2/FANCI complex at K561 and K523 respectively. (8) The complex is then recruited to ICL at the replication fork. (9), (10) This ubiquitinated complex recruits SLX4/XPF on ICL in order to unhook the ICL. (11) Afterwards, CtlP an MRN complex resect the double strand breaks and BRCA2 facilitates the formation of RAD51 filament on single stranded DNA generated by resection. (12) Then Polζ carry out the polymerization step through the unhooked ICL. (13) Rad51 then facilitates the invasion of strand with subsequent extension of the other strand. (14) Lastly SLX4 and nucleases resolve the Holliday junction (15) and NER repair proteins remove the damaged nucleotides.

8.1 Role of homologous recombination in ICL repair

The phenomenon of homologous recombination repair (HRR) employs homologous DNA sequences as template for repair and tolerance of DNA lesions that obstruct DNA replication in S-phase. Homologous recombination usually encompasses four step (i) double strand break recognition tailed by nucleolytic processing to produce 3′ single stranded ends of DNA, (ii) protein-mediated strand invasion of single-stranded DNA with homologous chromosome (iii) synthesis of DNA which regenerates degraded DNA using undamaged homologous chromosome as a template and (iv) resolution of Holliday junction intermediates. Usually the platinum drugs drive fruitful results in the treatment of BRCA1- and BRCA2- associated ovarian cancers [76]. However, the protein products of these two genes give rise to HR-mediated repair of DNA damage. A dynamic combination of BRCA1 and associated RING domain protein 1 (BARD1) exhibits ubiquitin ligase activity that is essential for the proper localization of RAD51, which is a central player in Homologous Recombination repair. Through BRCA2 mediated interaction with RAD51, it is specifically targeted to sites where recombination is initiated [77]. However, RAD51-deficient cells represent hypersensitivity towards ICL-inducing agents.

In this regard, the model organism, *Escherichia coli* has provided deep insights in the mechanisms involved in HRR of bacteria. Usually, RecA of bacteria has proven to be an effective protein in all major aspects of HRR due to its ability of forming nucleoprotein filament with both single and double stranded DNA. In *E. coli*, RecBCD complex- combination of nuclease/helicase, initiates the phenomenon of recombination by creating 3′-terminal single-stranded DNA substrate for the activity of RecA protein. RecBCD complex usually binds to the end of linear double stranded DNA and RecA in combination with single-stranded binding proteins (SSBP) allows an incessant formation of presynaptic filament on DNA. This nucleoprotein complex allows a rapid and efficient search for homology within the double-stranded DNA recipient, with subsequent formation of a joint molecule. After the formation of joint molecule, DNA PolI regenerates the sequence and the resultant Holliday junction is resolved by the action of RuvC protein that acts in concert with RuvAB proteins to coordinate the steps of branch migration and Holliday junction resolution [78].

In *Saccharomyces cerevisiae*, the incision of DNA is carried out by an anonymous nuclease. A yeast homologue of RecA, Rad51 works in conjunction with Rad52 dislocates the single-stranded DNA that is ostensibly covered by RPA. The subsequent nucleofilament works with Rad54 and Rad55/57 in DNA unwinding and strand annealing between donor DNA and incoming Rad51 nucleoprotein. The resolution of subsequent recombination intermediates is frequently carried out by assorted set of mechanisms including mus81-mms4 nuclease and Resolvase A [79].

8.2 Translesion DNA synthesis in DNA interstrand crosslinks

Translesion DNA Synthesis polymerases are considered essential for ICL repair in both S/G2 and G1 to bypass an ICL unhooked from one of the two cross-linked strands. The phenomenon of Translesion synthesis encompasses multiple polymerases with a dynamic ability to carry out an insertion of nucleotide across the lesion and others carrying out further extension. Based on genetic and biochemical studies, an assortment of polymerases has been implied in repair of ICLs. Usually translesion synthesis is a threefold step: (i) release of replicative polymerase after an interruption of normal bidirectional DNA with lesion, (ii) release of specialized translesion polymerase onto a site and starts the replication at a short distance past the lesion, (iii) the replacement of translesion polymerase with replicative DNA polymerase which continues the normal process of replication [80].

For HR-mediated repair of replication-dependent DSB and excision of ICL from the genome, this is vital to generate an intact template. In this regard, an assortment of polymerases allows the bypass of unhooked ICLs in vitro by using cross-linked DNA substrate model. In *Escherichia coli,* PolIV can easily bypass the unhooked ICLs of N^2-N^2-guanine in a non-mutagenic manner [81]. A set of human TLS polymerases entail Pol η, Pol ι, Pol κ, REV1, and Pol *ν* that tend to insert the complementary bases or evade anatomically varied ICLs. Competencies of such polymerase-catalyzed reactions is contingent upon the structure of ICL and the amount of double-stranded DNA around ICL.

The role of TLS polymerases in ICL repair is strongly supported by the study of genetics. In yeast, mutations in genes encoding subunits of Polζ i.e. Rev3, Rev7 or REV1 render cells hypersensitive to cross-linking agents [72]. Polζ is majorly important for the cross-linking resistance of non-replicating cells. However, to date *in vitro* studies have not been able to show bypass of ICL damage by Pol ζ-REV1, thus suggesting the other factors involved in lesion bypass. However, Pol η mutants are not sensitive for cross-linking agents [82].

In mammals, Pol ζ (comprising of REV3 and REV7 subunits) and REV1 are significant factors in ICL repair. However, the cells deficient in any of the aforementioned genes are highly sensitive to various cross linking agents [83]. REV1 act as TLS polymerase scaffold and thus facilitates the polymerase exchange with additional deoxycytidyl transferase activity that is involved in insertion of dCMP residues opposite to ICLs.

8.3 FA proteins and ICL repair

All Fanconi Anemia patients usually indicate hypersensitivity to cross-linking agents, signifying that FA pathway plays an indispensable role in distinguishing, beckoning or repair of lesions generated by agents. However, the precise role of FA proteins in response to ICLs is still in its infancy. FA pathway tends to participate in both replication-dependent and independent pathways of ICL repair. After an exposure of FA cells withy cross-linking agents, they accumulate chromosomal breaks and radial chromosomes [84] which is an outcome of defects in cellular responses to ICLs.

After recognition of ICL and signaling cell cycle arrest, FA pathways function to coordinate the repair of ICL. Approximately, thirteen Fanconi anemia proteins are essential for resistance against ICLs and the clampdown of chromosomal stability. Eight FA proteins tend to form a nuclear protein complex in order to mono-ubiquitylate FancD2 and FancI. This event is crucial for the cellular resistance to ICL agents. Disruption in FA core complex and ID complex tend to decrease ICL repair efficiency [85]. The depletion of FANCD2 prevents identification of post-incision product i.e. double-strand breaks (DSB). The programmed DSB that is promoted by FANCI-FANCD2 complex majorly leads to the formation of Rad51 filaments and thus allows subsequent repair via Homologous recombination. Notably, FA pathway has been associated with proteins involved in HDR, TLS and Nucleotide excision repair. However, the exact role of FA proteins in HDR provides a vague notion. Though, there exists an interaction between the conduits of FA-BRCA, as FANCD1 exhibits homology with BRCA2 and for this reason, numerous proteins of FA pathway unswervingly interact with BRCA1 and BRCA2. In this way, it is believed that FA pathway donot play a significant role in all Homology Directed repair mechanisms (HDR), because of having a role in the recruitment of repair proteins in ICL damage. Certainly, *in vitro* analysis recommend that FANCD1/BRCA2 play a momentous role in ICL repair [86]. FANCD2 allies with the Mre11-Rad50-Nbs1 (MRN) complex, that is considerably crucial for incision of DNA strands during double-strand breaks (DSBs), a preliminary step of all homology dependent processes [87].

In response to cross-linking agents, FANCD2 has been exposed to co-localize with Nucleotide Excision Repair component, XPF that affects the solidity of ubiquitylated FANCD2. After replication arrest, FANCD2 has also been shown to co-localize with Rev1 [88]and core complex components of FA i.e. FANCA and FANCG have been shown to be required for Rev1 foci formation [89]. Because of a dynamic ability to play an indecisive role in HDR and upstream process of TLS and NER, FA pathway orchestrates and regulate such repair mechanisms for a suitable removal of ICL damage. In this way, inactivation of FANCD2 affect both nucleolytic incision and translesion synthesis [90]. Recent investigations have examined the role of FA pathway in ICL repair by means of DNA substrates carrying site-specific ICLs in the supernatants of Xenopus.

Having a DNA substrate containing MMC-like ICL adducts significantly distorts DNA helix. The other study has stated that ICL repair can proceed through replication dependent and independent mechanisms [85]. In nutshell, ICL repair could take place in an absence of DNA replication in *Xenopus* extracts and upon transfection of an ICL- containing plasmid in G1-arrested mammalian cells is consistent with accumulating evidence for ICL repair in G1.

8.3.1 RUNX poly(ADP-ribosyl)ation and BLM interaction facilitate the Fanconi anemia pathway of DNA repair

Fanconi anemia is considered as a universal genome maintenance network that orchestrates the repair of DNA interstrand crosslinks (ICL). The tumor suppressors RUNX1 and RUNX3 have been shown to regulate the FA pathway independent of their canonical transcription activities, by controlling the DNA damage dependent chromatin association of FANCD2. RUNX3 usually modifies by PARP-dependent poly(ADP-ribosyl) ation which in turn allows RUNX binding to DNA repair structures lacking transcription-related RUNX consensus motifs. After DNA gets damage, the increased interaction between RUNX3 and BLM facilitates the efficient FANCD2 chromatin localization. The mutations of RUNX-Walker motif in breast cancers have been impaired for DNA damage-inducible PARylation, thus unveiling an impending mechanism for FA pathway inactivation in cancers [91].

8.4 Suppression of NHEJ reduces ICL sensitivity

Even though Homologous Recombination promotes repair of double strand break in S-phase, an alternative mechanism, Non-homologous end joining (NHEJ) also exist to repair damaged DNA in all phases of the cell cycle. The phenomenon of NHEJ employs a simplest mechanism of splicing to rejoin the free end of DNA. The process involves the binding of KU70-KU80 heterodimers to the free double-stranded ends of DNA, thus allows the binding of DNA-dependent kinase subunit (DNA-PKcs) and initiates the activation of downstream steps [92]. DNA is processed to remove 5′-or 3’-ssDNA tails and the subsequent ends are directly rejoined by the activity of DNA ligase IV-XRCC4. Unlike HRR, in which homologous sequences proofread the repair process, NHEJ generates deletions, insertions and translocations in case of joining of incorrect ends.

In past, researches on mice and yeast has stated the notion that human cell lines defective in factors of Non-homologous end joining i.e. KU70, KU80, Ligase, DNA-PKcs or XRCC4, donot exhibit hypersensitivity towards ICL-inducing agents [93]. However, recent analysis has indicated that inhibition of NHEJ pathway in cell lines of FA patients can reduce the toxicity of ICL-inducing agents. For instance, in a knockout model of chicken or nematode, specific FA-like defects can be salvaged by the co-deletion of ligase IV or KU70. Moreover, through simultaneous inhibition

of NHEJ by PKcs inhibitor, NU7036 in FANCA- and FANCD2- deficient human cell lines, the high sensitivity to MMC can be rescued easily. Through analysis of mitotic spreads in these cell lines, a rare sight of uncharacteristic radial chromosomes was observed. These annotations direct that a key purpose of the FA conduit in repair of Interstrand crosslink lesions, is to subdue the forged ligation of ICL-induced Double Strand breaks amid non-homologous chromosomes.

HR and NHEJ pathway provides the complementary functions in the repair of *de novo* double strand breaks and the co-inhibition of these repair pathways leads to increased cell death [94]. However, Fanconi Anemia cells are not defective in HR per se, so the inhibition of NHEJ in FA cells still allows them to proliferate and repair double strand breaks. This is mainly due to the reason that FA pathway mainly endorses HR at stalled replication forks through stabilization of intermediate that is a prerequisite for unhooking and TLS. If still the replication fork is not stabilized, HR can befall but the generated free end of DNA likes to bound by KU70-KU80, as it has a very high affinity for the structures [95]. By inhibition of NHEJ pathway, the less active and less toxic FA-independent HR pathway can re-establish the replication fork.

9. Conclusion

The development of interstrand cross-links play a chief role in the mechanism of significant chemotherapeutic agents. Emerging evidences suggest that these ICL lesions may also be formed by environmental agents and unwanted byproducts of metabolic processes. A better understanding of these lesions could lead to the improvement of supplementary therapeutic agents and strategies. However, despite the efforts of considerable investigations, the mechanism of ICL repair is still an enigma. At the transcriptomic level, proteins involved in a number of repair pathways have been identified. However, the detailed analysis of conditions in which repair should occur is largely elusive. What's clear is that a repair of interstrand-cross links in eukaryotes involves multiple factors from NER and HRR pathways. Given the state of activities, it is ostensible that diverse experiments need to be done before we get a vivid picture of this important repair mechanism.

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Section 3

DNA Repair: Cancers and Diseases

Chapter 7

DNA Repair Defects in Sarcomas

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Abstract

DNA repair pathway is considered to be one of the most important mechanisms that protect cells from intrinsic and extrinsic stresses. It has been established that DNA repair activity has a crucial role in the way that cancer cells respond to treatment. Sarcomas are a group of tumors with mesenchymal origin in which their association with DNA repair aberrations has been reported in numerous studies. Special attention has been focused on exploiting these alterations to improve the patient's overall survival and overcome drug resistance in cancer. While there is a large degree of heterogeneity among different types of sarcomas, DNA repair alteration is found to be a common defect in the majority of patients. In this chapter, we will introduce and review some of the most important dysregulated components involved in the DNA repair system, and discuss their association with tumorigenesis, cancer aggressiveness, drug resistance, and overall prognosis in the patients with sarcomas.

Keywords: DNA repair, Sarcoma, drug resistance, gene alteration

1. Introduction

Sarcomas represent a divergent and heterogeneous group of malignancies comprising more than 70 subtypes, with a common characteristic of being derived from mesenchymal lineages such as bone, muscle, cartilage, and fat [1]. Sarcomas are rare, accounting for less than 1% of adult cancers and approximately 15% of childhood malignancies [2]. They occur in all ages with an extensive intertumoral and intratumoral biological heterogeneity and widely varied clinical prognosis [3]. The primary standard of care approach for treatment of sarcoma patients is consist of surgery, radiation, and chemotherapy-based strategies [4]. Although the cure rate for the patients with localized sarcoma is generally more than 70%, the survival rate of metastatic and relapsed patients is still less than 30% and has not been changed in the last decades [1, 2, 5, 6]. Based on the tissue type of primary manifestation, sarcoma tumors could be categorized into two main groups: soft-tissue sarcomas (STS) and bone sarcomas. STS are more common with the incidence of approximately 13,000 reported cases versus 3000 cases of bone sarcomas each year in the United States [4]. Among STS, liposarcoma, leiomyosarcomas, and undifferentiated pleomorphic sarcomas are the most common types in adults, whereas rhabdomyosarcoma is the most common type

seen in pediatric age [7]. Osteosarcoma has the highest prevalence among bone sarcomas with a bimodal age distribution; an initial peak between the age of 10 to 20 and a second peak in incidence above the age of 60 [8]. Based on genetic criteria sarcomas can also be classified into two main groups: sarcomas with low level of genomic alterations and fairly normal karyotypes, and sarcomas with high level of genomic alterations and complex karyotypes [5]. The sarcomas found in the first group have chromosomal translocations as illustrated in **Table 1**; whereas osteosarcoma, chondrosarcoma, and liposarcoma are more genetically complex and have broader range of dysregulations resulted from copy number variations, mutations, etc. (**Table 1**) [5, 9–11].

It is well established that DNA damage response (DDR) system has a major impact on prognosis and clinical response to treatment in cancer patients [12–14]. Studies have investigated the dysregulation of different DDR pathways in various types of sarcomas and provided possible prognostic and therapeutic potentials among DDR components in order to overcome drug resistance and improve overall survival of these patients. In this chapter, we review some of the most important dysregulated DDR components which are involved in five different pathways (base excision repair (BER), nucleotide excision repair (NER), DNA mismatch repair (MMR), homologous recombination (HR), Non-homologous end joining (NHEJ), and DNA damage sensors (ATR and CHK1)) in sarcoma, and discuss the therapeutic developments and prognostic potentials in this area (**Figure 1**).

Sarcoma type	Gene translocation/inversion
Ewing sarcoma	EWSR1-FL1
	EWSR1-ERG
	EWSR1-ETV1
	EWSR1-E1AF
	EWSR1-FEV
	TLS-ERG
	EWSR1-ZSG
Synovial sarcoma	SSX-SS18
Chondrosarcoma	HEY1-NCOA2
	EWSR1-NR4A3
	TAF15-NR4A3
	TCF12-CHN
Liposarcoma	FUS-DDIT3
Rhabdomyosarcoma	PAX3-FOXO1
	PAX7-FOXO1
Fibrosarcoma	ETV6-NTRK3
	COL1A-PFGFB
	FUS-CREB3L1

References: [9, 12–14].

Table 1.
Most common chromosomal aberrations in sarcomas.

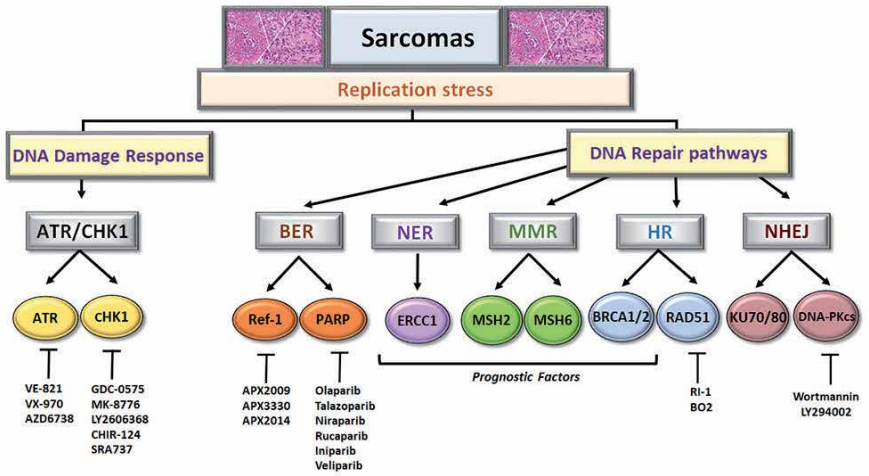

Figure 1.
Schematic summary of the most important DDR components and their respective inhibitors in sarcomas.

2. DNA repair machinery in Sarcoma

2.1 Base excision repair (BER) pathway

Base excision repair (BER) is a repair mechanism responsible for repairing single-strand DNA breaks (SSBs) or different types of damages including oxidization, deamination, and alkylation on a single base that do not induce significant distortion to the DNA helix [15]. Among several proteins that are involved in this pathway, APE1/Ref-1 and Poly (ADP-ribose) polymerase (PARP) are considered as the most important players in cancer progression and drug resistance [16–19].

2.1.1 APE1/Ref-1

One of the most important components of BER pathway is APE1/Ref-1 (apurinic/apyrimidinic endonuclease 1/redox factor-1). APE1/Ref-1 is a multifunctional protein involved in response to oxidative stress, cell cycle regulation, transcriptional activation, protein stability, apoptosis, and cell survival [16, 20]. The different functions of this protein can be categorized into two main activities: apurinic/apyrimidinic endonuclease activity and reduction–oxidation (redox) activity. The endonuclease activity allows APE1/Ref-1 protein to function as a DDR component in BER pathway by recognizing and cleavage of the abasic site [21]. The redox activity of APE1/Ref-1 gives it a critical transcriptional regulatory role in which enhances the activity of numerous transcription factors, including STAT3, NF-kB, HIF-1, and AP-1 [21, 22]. APE1/Ref-1 has a crucial role in maintaining cancer cells in a survival state through its DNA repair properties [23, 24]. Also, its redox function increases the activity of signaling pathways that are involved in promoting growth, migration, and survival in tumor cells as well as inflammation and angiogenesis in the tumor microenvironment [23, 25]. The overexpression of APE1/Ref-1 has been reported in many tumor types, and that change is associated with drug resistance, metastasis, cancer aggressiveness, and overall poor prognosis [16].

Several studies have shown that APE1/Ref-1 protein is overexpressed in sarcoma patients and is correlated with metastasis and lower survival rates [26–30].

The correlation between angiogenesis, as an important factor in tumor growth and metastasis, and APE1/Ref-1 in osteosarcoma was elucidated by the series of studies conducted by Wang et al. [26, 31–33]. They showed that transforming growth factor beta (TGFβ) is directly regulated by APE1/Ref-1 and its expression level was significantly reduced in APE1/Ref-1 deficient osteosarcoma cells [31]. TGFβ increases the chances of cancer metastasis through multiple mechanisms including immunosuppression, invasion, and angiogenesis [34]. They demonstrated that knocking down APE1/Ref-1 using specific siRNA in osteosarcoma led to down-regulation of TGFβ expression and suppression of angiogenesis *in vitro* based on human umbilical vein endothelial cells (HUVECs) in transwell and matrigel tube formation assays [31]. In addition, siRNA-mediated silencing of APE1/Ref-1 significantly suppressed tumor growth in xenograft mice models [31]. These experimental data indicated that APE1/Ref-1 promotes angiogenesis in osteosarcoma through a TGFβ-dependent pathway [31]. Additionally, they showed that the expression levels of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are also regulated by APE1/Ref-1 [32]. However, the suppression of angiogenesis in APE1/Ref-1 knockdown cells is not dependent on their transcriptional activity [32, 33]. Wang et al., also used siRNA against APE1/Ref-1 protein to investigate its inhibition in osteosarcoma [26]. They demonstrated that the siRNA-mediated inhibition of APE1/Ref-1 sensitized the osteosarcoma cells to DNA damaging agents: methyl methanesulfonate, H2O2, ionizing radiation, and chemotherapeutic agents [26]. Another study conducted by Xiao et al., investigated the association of APE1/Ref-1 polymorphisms with osteosarcoma [35]. They performed a 2-stage case–control study in a total of 378 osteosarcoma patients and 616 normal controls and concluded that the patients who have certain APE1/Ref-1 polymorphisms have lower APE1/Ref-1 expression and higher survival rates [35]. Over the past few years, small molecule inhibitors targeting APE1/Ref-1 have been developed and showed remarkable anti-tumor effects with limited toxicity in a variety of cancers, in both *in vitro* and *in vivo* models [36–40]. However, the efficacy of these inhibitors still needs to be investigated in sarcomas.

2.1.2 PARP

Poly (ADP-ribose) polymerase (PARP) is another important DDR protein involved in cell proliferation, differentiation, and transformation [41]. PARP has the ability to covalently add poly ADP-ribose (PAR) chains to target proteins and alter their functions [42]. This enzymatic activity gives PARP the capability of being involved in diverse set of cellular processes including DNA damage repair [41, 42]. PARP inhibitors have drawn a lot of attention in cancer research community based on their remarkable anti-tumor effects in HR-deficient cancers [43]. Studies on PARP function in DNA repair system have led to development of numbers of FDA-approved inhibitors for treatment of various solid tumors [44–46].

PARP protein is found to be playing a key role in sarcoma as well, highlighted by several preclinical and clinical studies conducted in recent years. A study designed by Park et al., underscored the association between PARP activity and poor prognosis in osteosarcoma patients and showed the efficacy of PARP inhibition in combination with chemotherapy in this disease [47]. They evaluated the expression level of DNA damage molecules in 35 osteosarcoma patients and found that the expression levels of PARP1, γH2AX, and The Breast Cancer Susceptibility genes (BRCA1 and BRCA2) are accompanied with shorter overall survival in these patients [47]. *In vitro* experiments on osteosarcoma cell lines demonstrated that the PARP inhibitor olaparib as a single agent could inhibit cell proliferation in a dose- and time-dependent manner [47]. Moreover, the combination of olaparib with doxorubicin

showed significant synergistic effects in osteosarcoma cells [47]. The *in vivo* experiments also validated the growth-suppressive effects of individual and co-treatment of olaparib and doxorubicin in orthotopic osteosarcoma mice models [47]. In osteosarcoma cells treated with the combination treatment of olaparib plus doxorubicin, flow cytometry analysis showed increased apoptosis as evident by increased expression levels of cleaved caspase 3, cleaved PARP1, BAX, and decreased levels of BCL2 [47]. The sensitivity of HR-deficient osteosarcoma cells to PARP inhibition is demonstrated in a study performed by Engert et al. [48]. They treated a panel of osteosarcoma cell lines with PARP inhibitor talazoparib alone and in combination with chemotherapeutic drugs (temozolomide (TMZ), SN-38, doxorubicin, cisplatin, methotrexate (MTX), etoposide/carboplatin) [48]. They found a direct correlation between HR repair deficiency and increased sensitivity of osteosarcoma cells to PARP inhibition [48]. All osteosarcoma cell lines harboring BRCA1/2 mutation for both alleles (so-called "BRCAness") have shown a significant reduction in cell growth following treatment with talazoparib (MG63, ZK-58, Saos-2, and MNNG-HOS) [48]. However, U2OS (osteosarcoma cells) that are heterozygous for BRCA2 mutation and carry one intact allele were resistant to PARP inhibition [48]. Furthermore, TMZ showed the highest anti-proliferative synergistic effect with the PARP inhibitor among other chemotherapeutic drugs, and this effect induced through apoptosis pathway as indicated by caspase activation, increased expression level of BAX and BAK, DNA fragmentation, and loss of mitochondrial membrane potential [48]. These findings suggested a promising potential for development of novel therapeutic strategies using PARP inhibitors in combination with conventional treatments in osteosarcoma patients with features of BRCAness (more discussed in Section 3.4.1). Likewise, a large number of preclinical studies on other types of sarcomas including Ewing sarcoma, chondrosarcoma, rhabdomyosarcoma, and other STS emphasized the effectiveness of PARP inhibition in combination with chemotherapy as a promising therapeutic strategy for treatment of sarcoma patients [49–53]. In a study conducted by Laroche et al., the anti-tumor effect of PARP inhibitor rucaparib in combination with a chemotherapy drug trabectedin was explored in a panel of STS cell lines and a mouse model of liposarcoma [54]. The data obtained from this study demonstrated that the combination of rucaparib and trabectedin synergistically inhibited cell growth and induced G2M cell cycle arrest, γH2AX intranuclear accumulation, and apoptosis *in vitro* [54]. They also carried out *in vivo* experiments and showed that this combination significantly suppressed tumor growth, increased the progression-free survival, and elevated the percentage of tumor necrosis in the xenograft mice model [54].

Although preclinical studies have presented PARP inhibition as an effective treatment option in sarcoma, clinical trials have failed to demonstrate a promising clinical outcome in patients so far [55, 56]. Schafer et al. conducted a phase I/II clinical trial of PARP inhibitor talazoparib in combination with low-dose temozolomide in patients with refractory/recurrent solid tumors including sarcoma [55]. From April 2014 to January 2018, 40 patients (including 15 Ewing sarcoma, 4 osteosarcomas, 2 synovial sarcomas, and one rhabdomyosarcoma) were enrolled in this study and treated with talazoparib and temozolomide [55]. The data showed that this combination therapy was well tolerated; reversible neutropenia and thrombocytopenia were the primary dose-limiting toxicities (DLTs) [55]. However, no significant anti-tumor activity was observed in sarcoma patients [55]. Similarly, a phase II clinical trial of PARP inhibitor (olaparib) in refractory Ewing sarcoma patients has also failed to demonstrate a promising clinical outcome [56]. One possible explanation for this direct contrast between preclinical and clinical studies is that PARP inhibition could induce anti-tumor effects in *de novo* Ewing sarcoma but not in pretreated, chemoresistant patients [55, 56]. However, the limited number of

completed clinical trials of PARP inhibitors in sarcoma compared to other tumors hinders us from making a definite conclusion (**Table 2**). Future preclinical and clinical studies will shed more lights on the effectiveness of PARP inhibition as a possible treatment approach for sarcoma patients.

2.2 Nucleotide excision repair (NER) pathway

NER is a DDR pathway responsible for repairing bulky DNA lesions induced by ultraviolet irradiation, carcinogens, and some chemotherapeutic agents such as cisplatin [57]. The involvement of NER pathway in DNA damage induced by chemotherapeutic drugs attracted researchers to investigate the association of NER activity with the response to these cytotoxic agents in various cancers. Although there are some controversies regarding the role of NER pathway in cancer, some studies showed direct correlations between NER activity and increased response to chemotherapy [15, 57]. Recent efforts in whole-genome sequencing and data analysis of The Cancer Genome Atlas have led to a better understanding of the roles of the molecules involved in this pathway and introduced NER genes as prognostic biomarkers of response to various DNA damaging chemotherapeutic in different types of cancers [15, 57–60].

2.2.1 ERCC1

ERCC1 is the key component of NER pathway that has been investigated in a large number of studies due to its prognostic properties in cancer treatment [61–63]. The association between the expression of ERCC1 and response to trabectedin in STS was investigated in a recent translational study designed by Moura et al. [64]. Expression levels were evaluated using qRT-PCR in 66 patients with advanced STS who were treated with trabectedin. The results showed that the expression level of ERCC1 is correlated with patients' progression-free survival [PFS (the length of time during and after treatment that the disease does not get worse)] and overall survival. Patients who had higher expression levels of ERCC1 showed better responses to the trabectedin and had longer PFS rates [64]. Similarly, ERCC1 expression has reported to be associated with treatment response in other sarcomas such as osteosarcoma and leiomyosarcoma, highlighting the importance of this key NER protein as a predictable biomarker in sarcoma [65, 66]. Polymorphism of NER genes and the relation of different alleles with the treatment response has also been investigated in osteosarcoma, indicating the association of some polymorphisms with a higher risk of osteosarcoma development [67]. A study conducted by Obiedat et al., investigated the relationship between polymorphisms of ERCC1 and ERCC2 and response to cisplatin-based chemotherapy and clinical outcomes in osteosarcoma patients [68]. They analyzed the association between ERCC1

Compound	Phase	Cancer type and trial details	Clinical trail identifier
Olaparib	II	Adult participants with recurrent/metastatic Ewing sarcoma	NCT01583543
Talazoparib	I	Advanced or recurrent solid tumors (including Ewing sarcoma)	NCT01286987
Iniparib	II	Advanced, persistent, or recurrent uterine carcinosarcoma	NCT00687687

Table 2.
Sarcoma clinical trials of PARP inhibitors.

(C118T (rs11615) and C8092A (rs3212986)) and ERCC2 (A751C (rs171140) and G312A (rs1799793)) polymorphisms and clinical parameters including event-free survival (EFS) (the length of time after treatment that a patient lives without any complications or event that the treatment intended to prevent or delay) rates in 44 patients with osteosarcoma who were treated with cisplatin-based neoadjuvant chemotherapy [68]. The findings illustrated that there is a significant positive correlation between ERCC1 C8092 A genotypes and median EFS rate. In other words, the patients who carried allele C (CC & CA) had longer EFS rates than patients with AA genotype, highlighting the importance of ERCC1 polymorphism in osteosarcoma [68]. Taken together, these studies suggested that ERCC1 could be considered as a reliable predictive factor of the effectiveness of some DNA-damaging chemotherapeutic drugs in sarcoma patients, and different polymorphisms could be used as prognostic biomarkers for designing the best treatment strategy.

2.3 DNA mismatch repair (MMR) pathway

2.3.1 MSH2-MSH6 (MutSα)

MMR pathway is responsible for repairing base mismatches, insertions and deletions arise from DNA replication, genomic recombination, and other error-prone DNA repair systems [69]. MSH2-MSH6 (MutSα) complex plays an important role in this pathway by recognizing the mismatched bases and starting the MMR process [69]. Different polymorphisms and expression levels of MMR components have shown to be associated with prognosis and survival in cancer patients [70–72].

A study conducted by Li et al., emphasized the importance of MMR pathway in Ewing sarcoma and showed that the expression levels of MSH2 and MSH6 is correlated with an increased chance of metastasis and poor prognosis in these patients [73]. They used the GEO database to investigate the correlation of the key dysregulated genes and pathways with prognosis information and metastasis status of the Ewing sarcoma patients [73]. The findings highlighted the MMR pathway as the most significantly enriched KEGG pathway in EWS patients [73]. The expression levels of key MMR components including MSH2 and MSH6 are found to be significantly associated with metastasis, shorter EFS, and overall poor prognosis in Ewing sarcoma patients [73]. Several studies have investigated the role of MMR pathway in osteosarcoma. Liu et al., investigated the growth-suppressive effects of MSH6 gene silencing in combination with cisplatin in osteosarcoma [74]. Microarray-based gene expression analysis of samples obtained from 67 osteosarcoma patients along with 24 normal patients demonstrated that MSH6 is significantly up-regulated in osteosarcoma patients [74]. Then, they evaluated cell proliferation, cell cycle distribution, gene and protein expression, and apoptosis of osteosarcoma cell line MG63 after co-treatment with cisplatin and siRNA targeting MSH6 [74]. The data showed that silencing MSH6 in combination with cisplatin reduced expression levels c-Myc, cyclin D1, Bcl-2, Stathmin, and PCNA and increased BAX expression in osteosarcoma cells [74]. This combination treatment also induced significant anti-proliferative effects, indicating that MSH6 could be considered as a potential therapeutic target for treatment of osteosarcoma patients [74]. In another study, proteomic analysis for identification of proteins that are differentially expressed between osteosarcoma and normal osteoblastic cells revealed that chromosome segregation 1-like (CSE1L) protein is significantly associated with the growth of osteosarcoma cells [75]. Co-immunoprecipitation and RNA-seq analysis in this study showed that CSE1L acts as a positive regulator of MSH6 in osteosarcoma cells [75]. In addition, they knocked down CSE1L protein in osteosarcoma cells and found significant growth suppression [75]. Furthermore, to investigate the role of MSH6, they overexpressed MSH6 in

CSE1L-knockdown osteosarcoma cells [75]. The results showed that overexpression of MSH6 significantly increased cell proliferation rate and reversed the anti-tumor effects observed in CSE1L-knockdown cells, indicating that CSE1L activity is dependent on MSH6 expression [75]. Moreover, down-regulation of MSH6 resulted in suppression of cell growth in both *in vitro* and *in vivo* experiments [75]. The prognostic potential of MSH6 and CSE1L was also explored by evaluation of the MSH6 and CSE1L expression levels in tumor samples [75]. They found a significant correlation between the expression of these two proteins and overall poor prognosis in osteosarcoma patients [75]. Similarly, another study on osteosarcoma patients showed that overexpression of MSH2 and MSH6 is significantly associated with shorter survival time, lower sensitivity to chemotherapy, and higher chances of metastasis [76]. These studies underscored the significance of MMR proteins as both prognostic biomarkers and possible therapeutic targets in sarcoma.

2.4 Homologous recombination (HR) pathway

2.4.1 BRCA1/BRCA2

HR is the major DDR mechanism responsible for repairing double-strand DNA breaks (DBSs) [69]. HR repairs DSBs in an error-free manner by using homologous sequence of sister chromatid as an undamaged template [15]. BRCA1, BRCA2 and RAD51 are the key factors involved in this DDR pathway which have shown to be dysregulated in various types of cancers [15, 77–79]. Inherited mutations of the BRCA genes predispose individuals to develop tumors in various organs including breast and ovary [80]. Moreover, the chance of developing cancer significantly increases by acquiring BRCA mutations, and these mutations are commonly seen in patients with breast and ovarian cancers [80]. However, it has been reported that BRCA mutation has potential for inducing synthetic lethality in the cancer cells [81]. PARP inhibition in BRCA-mutated cancer cells (HR-deficient) induces synthetic lethality and cell death and provides a promising opportunity to eliminate cancerous cells (**Figure 2**). Several PARP inhibitors have been approved as monotherapies in HR-deficient ovarian and metastatic breast cancers [81].

As we discussed earlier in this chapter (Section 3.1.2), studies on osteosarcoma demonstrated that BRCA is frequently mutated in osteosarcoma and PARP inhibition either as a monotherapy or in combination with chemotherapy could induce significant anti-tumor effects in BRCA-mutated osteosarcoma cells [48, 82]. Although the significance of BRCA mutation status as a prognostic factor in sarcoma has been reported in a numerous studies [83–85], more clinical trials are warranted to determine the efficacy of PARP inhibitors in BRCA-mutated sarcomas. The importance of BRCA status in sensitivity to the chemotherapeutic drug trabectedin in STS is emphasized in a review paper gathered by Monk et al., and presented that BRCA mutations are significantly associated with favorable clinical response to trabectedin [83]. The frequency of BRCA mutation in soft-tissue sarcoma though, has not found to be significantly high in a study conducted by Seligson et al. [86]. They performed DNA sequencing analysis on 1236 STS patients as well as an additional 1312 leiomyosarcoma patients [86]. The unselected STS analysis revealed that only 1% of patients had BRCA2 mutation [86]. However, subset analysis showed that BRCA2 mutation could be found in 10% of leiomyosarcoma patients [86]. The frequency of BRCA1 mutation was not significant in either analysis [86]. Furthermore, they showed that PARP inhibition demonstrates effective clinical outcomes in BRCA2 deficient leiomyosarcoma patients [86]. Consistently, another study demonstrated a significant correlation between the overexpression of BRCA1,

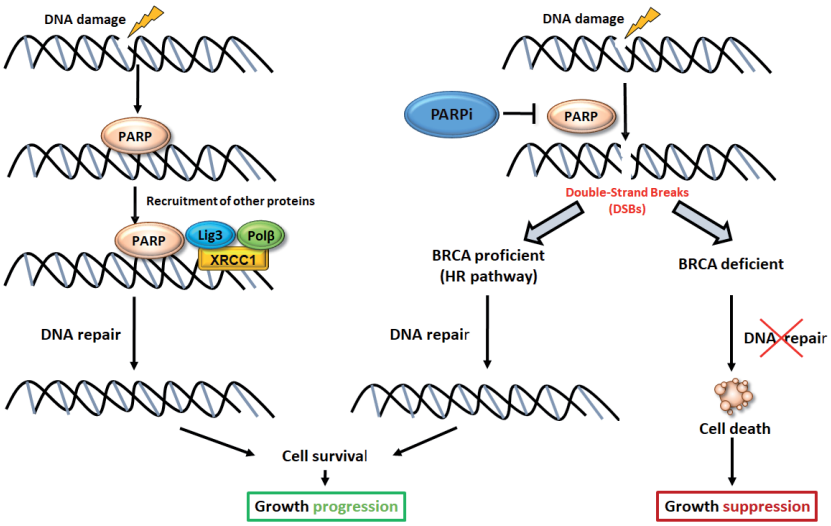

Figure 2.
Schematic role of PARP and PARP inhibition in synthetic lethality. (A) After binding to damaged DNA, PARP undergoes conformational change and poly(ADP-ribosyl)ation which results in recruitment of other DNA Damage Response (DDR) proteins, like DNA ligase 3 (Lig3), DNA polymerase β (polβ) and X-ray repair cross-complementing protein 1 (XRCC1), leading to DNA repair and cell survival. (B) PARP inhibitors block PARP activity, leading to double-strand break (DSB). The cells that have normal homologous recombination (HR) pathway are able to repair the DSBs in a error-free manner, leading to cell survival. However, BRCA-mutated cancer cells (HR-deficient) are unable to efficiently repair DSBs which ultimately results in cell death.

BRCA2, PARP, and γH2AX and higher tumor stage, higher chances of metastasis, lower survival rates, and overall poor prognosis in STS patients [87]. These studies highlighted the significance of BRCA status in sarcoma and underscored the fact that HR mutations should be considered as predictive factors for increasing the overall survival of patients by choosing the best treatment strategy.

2.4.2 RAD51

RAD51 is another key protein in the HR pathway that is also associated with prognosis and treatment response in various cancers. A growing number of studies demonstrate that RAD51 protein is overexpressed in many cancers including breast, prostate, bladder, pancreas, and lung, and this overexpression can up-regulate HR activity and result in resistance to DNA-damaging drugs [88–91]. Increased expression of RAD51 has also been reported in sarcoma patients [92, 93]. Du et al. conducted a study to explore the relationship between RAD51 expression and resistance to radio- or chemotherapy in osteosarcoma [93]. They suppressed the expression of RAD51 using shRNA and found increased sensitivity to chemotherapy and radiation in osteosarcoma cell lines through induction of cell cycle arrest and apoptosis [93]. Hannay et al., investigated the association between RAD51 expression and resistance to chemotherapy in STS patients [92]. They evaluated the RAD51 expression in 62 human primary recurrent and metastatic STS samples [92]. Only 3 tumor samples showed no RAD51 expression, while most of them had overexpressed RAD51 expression levels [92]. They showed that siRNA-mediated RAD51 targeting resulted in STS sensitivity to doxorubicin [92]. Overall, these studies highlighted the significance of RAD51 in chemoresistance and suggested that RAD51 could be considered as a prognostic factor or even a therapeutic target for treatment of sarcoma patients.

2.5 Non-homologous end joining (NHEJ) pathway

The NHEJ pathway is another important pathway responsible for repairing DSBs [94]. Unlike HR, NHEJ directly re-ligates two broken DNA strands without requiring a homologous sequence as an undamaged template, which makes this pathway prone to making errors [94]. NHEJ is initiated by binding of KU70/80 proteins, followed by recruitment of other key factors such as DNA-dependent protein kinases (DNA-PKcs), XRCC4, XLF, LIG4, and PAXX (a newly identified NHEJ component) [95] to complete the repair process [96]. Loss of the key factors involved in this pathway is positively correlated with increased genomic instability and sensitivity to DNA damaging chemotherapy drugs [94]. However, the over-activation of NHEJ has also been reported to be associated with increased genomic instability and tumorigenesis due to error-prone and inappropriate repair [94]. Thereby, both loss and over-activation of NHEJ factors have found to be associated with increased cancer incidence [94]. Moreover, a large number of studies have shown that differential expression of key NHEJ factors has significant impacts on the treatment response and overall prognosis in different types of cancers [94, 97–101].

Several studies have shown the significance of NHEJ components in sarcomas. For example, in a study on Ewing sarcoma patients, Kyriazoglou et al., has reported that NHEJ and HR genes are significantly up-regulated in comparison with healthy blood donors [102]. They analyzed the expression levels of 15 genes in 32 cases of Ewing sarcoma using Real-time PCR. XRCC5, XRCC6, Polm, LIG4 from the NHEJ pathway and RAD51, RAD52, RAD54, BRCA2, and FRANCD from the HR pathways have found to be significantly up-regulated in Ewing sarcoma patients [102]. In another study, Ma et al. investigated the role of PAXX protein in chemoresistance in osteosarcoma [96]. They found a significant positive correlation between enhanced PAXX-KU70 interaction and NHEJ efficiency and resistance to doxorubicin and cisplatin [96]. They also showed that PAXX deficiency re-sensitizes osteosarcoma cells to the chemotherapy drugs, which provides evidence that PAXX protein could be considered as a target for treatment of chemoresistant osteosarcoma patients [96]. Additionally study conducted by Hu et al., demonstrated the significance of KU80 expression in radiosensitivity of osteosarcoma cells [103]. They have shown that shRNA-mediated suppression of KU80 protein sensitized U2OS osteosarcoma cells to radiation through shortening of telomere length [103]. Taken together, key NHEJ factors have important roles in cancer progression, drug resistance, and patient's prognosis [94], which makes them interesting for further research regarding their prognostic and therapeutic potential in sarcomas. However, targeting NHEJ remains challenging as little is known about the inhibitors. Several PI3K inhibitors such as wortmannin and LY94002 (**Figure 1**) are being used for therapeutic intervention of DNA-PKcs in the NHEJ pathway [104].

2.6 ATR/CHK1 DNA damage sensors in DNA repair pathways

2.6.1 ATR

Ataxia-telangiectasia and Rad3-related protein (ATR) is one of the most upstream DDR kinases and belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) protein family [105]. ATR is a serine/threonine-protein kinase activated in response to a broad spectrum of DNA damage, including DSBs and various DNA lesions that interfere with replication [106]. In response to DNA damage, several proteins are phosphorylated at Ser/Thr-Glu motifs and additional sites in response to DNA damage by ATR [107]. ATR phosphorylates its major downstream effector checkpoint kinase 1 (CHK1) and prevents the entry of cells with damaged

or incompletely replicated DNA into mitosis from the G2 phase of the cell cycle [108]. This regulation is particularly apparent in cells with a defective G1 checkpoint, a common cancer cell feature because of p53 mutations [109, 110]. ATR also suppresses replication stress (RS) by inhibition of extra origin firing, particularly in cells with activated oncogenes [111]. Therefore, ATR could be an ideal therapeutic target in cancer. Currently, ATR inhibitors have been developed and are used either as single agents or in combination with radiotherapy or chemotherapy in both preclinical and clinical studies [112].

Several preclinical studies demonstrate that ATR could be a therapeutic target in sarcomas [113–117]. Laroche-clary et al., designed a study to investigate the anti-tumor effects of ATR inhibition in STS [113]. They treated STS cell lines with ATR inhibitor VE-822 either as a single agent or in combination with gemcitabine as a chemotherapeutic drug [113]. The data demonstrated significant synergist effects between these two drugs [113]. They found considerable cell growth suppression, apoptosis induction, and increased γH2AX expression after combined treatment of STS cells with VE-822 and gemcitabine in a higher efficacy than either agent alone [113]. Furthermore, they performed *in vivo* experiments on a patient-derived xenografts (PDXs) of undifferentiated pleomorphic sarcoma and found significant tumor growth suppression and increased PFS after treatment with combination of VE-822 and gemcitabine [113]. Taken together, this study highlighted the importance of ATR in STS and showed that ATR inhibition in combination with chemotherapy is efficacious in pre-clinical models. In another study, a series of parallel high-throughput siRNA screens were performed by Jones et al., in synovial sarcoma tumor cells and the results were compared with more than 130 non-synovial sarcoma tumor cells to get better insights into genetic dependencies and potential therapeutic targets in synovial sarcoma [114]. The analysis revealed a significant reliance of synovial sarcoma tumor cells on ATR protein kinase activity [114]. Furthermore, they showed that ATR inhibition will result in significant anti-tumor effects in synovial sarcoma *in vitro* and *in vivo* [114]. They also performed combination treatments with cisplatin and PARP inhibitors and found higher tumor-suppressive effects than either agent alone [114]. In summary, this study presented ATR protein alteration as a key factor in synovial sarcoma progression and proposed a novel therapeutic potential for synovial sarcoma patients [114]. The role of ATR protein was also demonstrated in Ewing sarcoma [115]. Nieto-Soler et al., designed a study to investigate the importance of ATR pathway in Ewing sarcoma [115]. They showed that Ewing sarcoma tumors that have high levels of RS are significantly dependent on ATR pathway [115]. Furthermore, they treated Ewing sarcoma cell lines and mice models with two independent ATR inhibitors and found considerable anti-tumor effects both *in vitro* and *in vivo* [115]. Collectively, this study highlighted the dependency of Ewing sarcoma to ATR pathway and identified ATR inhibition as a promising therapeutic strategy in Ewing sarcoma with high levels of RS [115]. Future preclinical studies and subsequent clinical trials will provide with additional reliable data on the effectiveness of ATR inhibition in sarcoma to translate this therapeutic approach into clinic as a possible treatment approach for sarcoma patients.

2.6.2 CHK1

As mentioned above, checkpoint kinase 1 (CHK1) is the major downstream effector of ATR [108]. CHK1, a serine/threonine-specific protein kinase, plays an essential role in preventing cell cycle progression when damaged DNA is being repaired [118, 119]. DNA damage is sensed by ATR, activated ATR phosphorylates and activates CHK1. CHK1 has several targets which all act to regulate cell cycle

arrest [118]. Phosphorylation of the CDC25 dual specificity phosphatase family mediated by CHK1 causes phosphatase degradation, resulting in increased phosphorylation and inhibition of multiple cyclin dependent kinase (CDK) proteins, positive regulators of the cell cycle [119]. In addition to CDC25 phosphatases, WEE1 kinase is phosphorylated and activated by CHK1, subsequently leading to the inhibitory phosphorylation of CDK1 [120]. It is therefore logical that inhibitors of CHK1 in cancer treatment could facilitate cell cycle progression with damaged DNA and induce apoptosis [118].

Several preclinical studies and a few clinical studies demonstrate that CHK1 could be a therapeutic target in sarcoma treatment [115, 116, 121–124]. Larocheclary et al., conducted a study to investigate the role of CHK1 protein kinase in p53-mutant and wild-type STS [122]. They performed a systematic screening of a panel of 10 STS cell lines after combination treatment of CHK1 inhibitor (GDC-0575) with gemcitabine [122]. They showed that GDC-0575 induced apoptosis by abrogating DNA damage-induced S and G2–M checkpoints [122]. Moreover, they observed a synergistic or additive effect of GDC-0575 in combination with gemcitabine *in vitro* and *in vivo* in TP53-proficient but not in TP53-deficient sarcoma models [122]. Before conducting the mentioned study, they had analyzed the expression profile of a series of 339 complex genomics sarcomas and 108 translocation-related sarcomas, they showed that CHK1 expression is significantly associated with poor prognosis in sarcoma patients [125]. Moreover, they evaluated the efficacy of CHK1 inhibition in STS patients in a phase 1 clinical study with 3 STS patients (two with p53 mutation and one without p53 mutation) [122]. Two STS patients who had p53 mutation demonstrated promising response to the combination of gemcitabine and GDC-0575, while the other patient displayed no clinical benefit [122]. In conclusion, they provided pre-clinical and clinical evidence of the significance of CHK1 activity in STS and revealed that combination of CHK1 inhibitors with chemotherapy could be a promising treatment strategy for p53-mutant STS patients [122, 125]. There are also numbers of studies which have highlighted the important role of CHK1 activity in osteosarcoma progression and drug resistance and showed that CHK1 inhibitors either as a single agent or in combination with other drugs could be considered as a promising therapeutic target for treatment of osteosarcoma patients [126–129]. Regarding the role of CHK1 in Ewing sarcoma progression, some studies demonstrated that CHK1 protein is over-activated in Ewing sarcoma and showed that Ewing sarcoma cells are sensitive to CHK1 inhibitors either as a single agent or in combination with other drugs *in vivo* and *in vitro* [116, 121, 130, 131]. Further clinical investigations are needed to confirm whether treatment of sarcoma with CHK1 inhibition is efficacious therapeutic approach to improve sarcoma patient outcomes at a higher level of evidence.

3. Implications/conclusions

Collectively, the studies summarized in this chapter indicate that it will likely take more than just targeting a particular dysregulated DNA repair pathway in the context of chemotherapy to cure many relapsed and aggressive sarcomas. As mentioned above, targeting the dysregulated process of replication stress and genomic instability which promotes tumorigenicity in many cancers such as sarcoma is an area of intense interest [132]. The use of small molecule inhibitors that block not only DNA repair mechanisms but other global networks that may be connected to or independent of DNA repair mechanisms may be key to improving clinical outcomes. As such, our group used a systems biology approach to discover risk signatures and potential biomarkers of therapeutic response in pediatric adolescent and young

adults with aggressive osteosarcoma. We found that the MYC-RAD21 copy number gain correlated with poor overall survival and was a potential marker of replication stress. We demonstrated that an increase in replication stress via a combination therapy consisting of BET and CHK1 inhibitors in xenograft models of pediatric and AYA osteosarcomas that have copy number gains of MYC and RAD21, was efficacious and well tolerated [126]. Furthermore, to obtain insight into other potential treatments where DNA repair inhibitors can be combined, numerous efforts have focused on investigating and understanding of the cross-talk between the various DNA damage-repair pathways as well as with the tumor microenvironment so that novel therapeutic combinations can be identified [133]. For instance, it has reported that hypoxic conditions within the tumor microenvironment impairs the fidelity of DNA repair pathways [133]. Furthermore, increased immune response to tumor neoepitopes have been observed in cancer with impaired/dysregulated DNA repair pathways [133]. Therefore, preclinical and clinical validation of using DNA repair inhibitors in combination with anti-hypoxic or immunomodulatory therapies warrants additional investigation. Notably, DNA repair mechanisms clearly contribute to tumor resistance [134]. In fact, one mechanism, by which tumor resistance is regulated involves cancer stem cells (CSC) which have increased DNA repair capacity [134]. Additionally, it has been reported that chromatin structure (euchromatin vs. heterochromatin) impacts the efficacy of DNA repair [135]. Thus, combination therapy targeting DNA repair pathways with agents targeting CSC or epigenetic proteins that regulate chromatin also require further evaluation. Several studies have shown associations between DNA repair pathways. With advancements in next-generation sequencing and use of precision genomics one clinical implication is that it may be possible to identify germline and/or somatic mutations involved in DNA repair proteins that could help delineate subsets of sarcoma patient-population that are predisposed to factors such as likelihood of getting the disease, onset of relapse/metastasis/recurrence, or possibility of therapeutic resistance to certain treatments [136]. Furthermore, within the patient population genetic polymorphisms associated with efficacy for DNA repair also become evident [137].

With progress in scientific technology, characterizing and profiling key components of the repair pathways is now more feasible. This results in increased preclinical validation studies using DNA repair inhibitors to improve therapeutic outcomes for otherwise therapeutically plateaued cancers like sarcomas. Development and implementation of novel therapeutic interventions involving DNA-repair proteins in combination with other targeted therapies and/or standard-of-care agents may help improve clinical outcomes in the patients. Furthermore, the role of DNA repair proteins and damaged cellular DNA are not only relevant in sarcomas but are pertinent to other cancers as well as contributing to the pathogenesis of many other diseases [138]. Therefore, identification of novel therapeutic combination involving DNA repair proteins is of high clinical value as it may be applicable for treating other human ailments.

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to fund new childhood cancer treatments and pediatric cancer research. For more information on how to help, please contact Curing Kids Cancer at 1-866-933-CURE (2873) or visit curingkidscancer.org<http://curingkidscancer.org/> to learn more.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

Epigenetics and DNA Repair in Cancer

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Abstract

Cells can use chemical modifications in chromatin to regulate accessibility to DNA to the repair complexes and to prevent transcription in case of damage. We analyzed the relationship between repair systems and epigenetic mechanisms in DNA and RNA. We searched the PubMed database for genes involved in DNA damage response (DDR) and methylation in mRNA and DNA repair, in cancer. Epigenetic modifications, particularly histone modifications and nucleosome remodeling, trigger a signaling cascade of kinases in DNA damage response (DDR) toward efficient repair. SWI/SNF remodelers promote the recruitment of repair factors in DNA, such as DNA double-strand breaks (DSBs) that activate kinases in DDR. RNA methylation via m6A has recently attracted attention as a possible alternative pathway for repairing DNA damage. m6A is a dynamic methylation mark on mRNA that accumulates after UV irradiation and regulates transcription to facilitate DNA repair. Currently, studies seek to understand how signaling pathways activate proteins in the early response to damage. The repair maintains DNA integrity, which is a challenge in cancer because this process also represents a potential barrier to anticancer agents. The impact that epigenetic regulation can have on DNA repair is beginning to be understood.

Keywords: nucleosome remodeling, SWI/SNF complex, m6A, methylation, cancer

1. Introduction

Cells are exposed to a vast amount of exogenous genotoxic agents, such as ionizing radiation or UV light, or endogenous agents, including reactive oxygen species (ROS), derived from oxidative respiration or replication processes that can cause errors in the nucleotide chains. This damage interferes with different biological or metabolic processes, e.g., replication and transcription [1]. If these alterations are not properly repaired, then mutations, chromosomal aberrations, genomic instabilities, and other harmful effects can occur, triggering alterations such as carcinogenesis. Cells developed complex systems to deal with these problems, as they have repair systems that are activated in response to checkpoints in the cell cycle to prevent cycle progression to eliminate damage or send cells into apoptosis, when repair is no longer possible. In cancer, the response to damage is mainly activated by genotoxic agents, double-strand breaking (DSBs) repaired by homologous recombination (HR) or non-homologous end joining repair (NHEJ). Cells have signaling networks to supervise the integrity and fidelity of the major

events of the cell cycle (checkpoints) until they recognize and respond to DNA structure damage and repair. This damage response cascade is known as DNA damage response and is responsible for control of genome stability after DSBs' formation [2, 3].

Epigenetic modifications are alterations at the DNA level that do not cause permanent change in the sequence but might also cause conformational modifications. Here, chromatin plays an essential role, such that various damage response factors can gain access to the DNA sequence. Chromatin can be modified by histone changes, ATP-dependent nucleosome remodelers, and non-histone proteins, including chaperones or a high mobility group (HMG). It demonstrates that reorganization of the dynamic chromatin structure is an intrinsic component of efficient DNA repair and DDR [4–6]. Epigenetic modification has gained relevance in recent years, which involves a change in RNA. In addition, 6-methyladenosine (m6A) methylation is one of the most common RNA modifications, and is visible in eukaryotic species, such as yeast to mammals and prokaryotes and bacteria and mycoplasma. There has recently been substantial progress in m6A epitranscriptomics in its role in the initiation and progress of cancer. Studies on links between m6A and cancer yield different results in diverse tumors, suggesting that the effect of m6A modification can be variable: it affects proliferation, growth, invasion, and metastasis, but the involved pathways are just beginning to be unveiled [7, 8].

In this chapter, we will address chromatin modifications on DDR and how they function as therapeutic targets in cancer. Pathways that repair also create extraordinary work in maintaining DNA integrity, but in cancer, they are a challenge, as they represent a potential barrier to anticancer agents.

2. Chromatin dynamic in the DNA damage response

The damage response triggers the rapid recruitment of repair proteins and checkpoint activation at the site of injury in DNA structure, a crucial step for DDR signaling pathway initiation. Cancer cells are characterized by deregulation in the signaling pathways that control checkpoint homeostasis, when genes associated with DDR suffer mutations, as the injury cannot be repaired correctly and is accumulated in the genome, triggering cellular transformation [9, 10]. Signaling pathways most affected will be those of apoptosis, cell cycle, and repair, contributing to harmful effects on genome integrity, thus increasing the risk of cancer [11]. Loss of function by germlines or somatic mutations of DDR-associated genes can trigger the inability of the repair single-strand break (SSBs) or DSBs, causing cell death [12]: this is the most deleterious type of DNA damage (since a single unrepaired DSB can be lethal).

The cell develops different repair mechanisms, such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), NHEJ and homologous recombination (HR) as well as homologous recombination repair (HRR) [2]. The specific type of DNA repair will be activated according to the lesion, the cell cycle phase, the genomic location, and the chromatin environment [3]. However, for this to occur, the processes associated with DNA repair pathways must overcome the physical chromatin condensation barrier and packaging to gain access, detect, and repair the damage. Cooperation with different histone modifications and nucleosome remodelers are involved in DNA repair [13]. The chromatin structure functions as part of the machinery regulating genome stability and provides necessary tools to carry out basic cellular processes for genetic information integrity.

Chromatin remodelers can alter or modify the chromatin structure, catalyzing the disruption of DNA-histone contacts and displacing or evicting nucleosomes

with ATP hydrolysis to gain access to DNA. They can then regulate the stiffness, flexibility, and mobility of chromatin within the nucleus [14] or facilitate the accessibility of TFs to functional DNA elements, such as promoters or enhancers. Several ATP-dependent chromatin remodeling complexes have been directly implicated in DSB response. In yeast, INO80, SWR1, switch/sucrose non-fermenting (SWI/SNF), and remodeling the structure of chromatin (RSC) complexes are recruited to the DSB and reconfigure the nucleosomes around it so as to facilitate DNA repair and/or to modulate checkpoint activation. HMG B family is specifically involved in DSBs' repair while promoting end joining in NHEJ *in vitro* [4, 5, 15, 16].

The SWI/SNF complex regulates the correct recruitment of repair factors for NHEJ and HRR and, signaling of DDR generated by DSBs [6]. In different cancers, up to 20% of the genes, mutated or altered, belong to the SWI/SNF complex [17]. Two subunits differ according to their composition, called Brahma-related gene 1 (BRG1)-associated factor (BAF) and BRG1 polybromo-associated factor (PBAF). Further, two ATPases BRM (SMARCA2) or BRG1 (SMARCA4) are mutually exclusive but structurally related [18, 19]. On the other hand, ARID1A/1B/2, PHF10, DPF1/2/3, PBRM1 (BAF180), beta-actin, SMARCE1 (BAF57), BCL7A/B/C, BCL11A/B, SS18, and BRD9, are subunits found in mammals, so it is probable that these proteins' function are related to evolutive strategies in chromatin regulation associated with greater complexity and/or specificity to the SWI/SNF complex focalization [17]. In particular, the subunits SMARCA4, SMARCB1, ARID1A/B (BAF250A/B), PBRM1, and ARID2 have tumor suppressor function [20, 21]. this lack or silencing of a single protein, belonging to each subunit, can affect the interaction with other components of the SWI/SNF complex. The response to DNA damage was observed as nucleosome remodelers that interact through bromodomain with histone modifications and epigenetic marks (**Figure 1**).

Some functions for SWI/SNF subunits make it possible to understand the importance of this complex in cancer. Erket et al. [22] identified SMARCB1 as often lost or altered in malignant rhabdoid-type tumors. In addition, Agaimy et al. [23] and Nombiraan et al. [24] identified how alterations in SMACA4 compromise patients with non-small cell lung cancer, which clarified the prognosis, diagnosis, and personalized therapeutic potential in patients with mutated or altered SMARCA4. In the study by Yoshida et al. [25], SMARCA4 loss of function is related to thoracic sarcomas. However, Herpel et al. [26] suggest that SMARCA4 and SMARCA2 subunits, catalytic centers of SWI/SNF, should be added to diagnostic evaluation panels for lung adenocarcinomas, this is supported by results obtained in their work on protein expression by IHC, in more than 300 patients with non-small cell lung cancer. As earlier described, the proteins of the SWI/SNF complex are involved in multiple mechanisms of DDR and DSB, while NER stands out, involved with other epigenetic modifications. This is demonstrated by Lee et al. [27], who observed that Brg1 subunit interacts with acetylated H3 in addition with H2AX in early stages of DNA damage, facilitating signaling, or DDR. Particularly in DNA repair, Ribeiro et al. [28] showed that both BRM and BRG1 promote normal TFIIH (ERCC2) function in transcription and NER by regulating the expression of the GTF2H1 gene and found that cells with permanent BRM or BRG1 loss can restore GTF2H1 expression levels. Therefore, DNA damage sensitivity of BRM or BRG1 deficient cells correlates with GTF2H1 protein levels and can be used to select SWI/SNF-deficient cancers that are more sensitive to platinum drug chemotherapy. In studies carried out by Decristofaro et al. [29] and Watanabe et al. [30] in breast and lung cancer cell lines, respectively, they identified that expression of ARID1A or ARID1B was decreased or absent. They also found similar behavior in other SWI/SNF subunits (BRG1, BRM, BAF60a, BAF60c, BAF53a, and

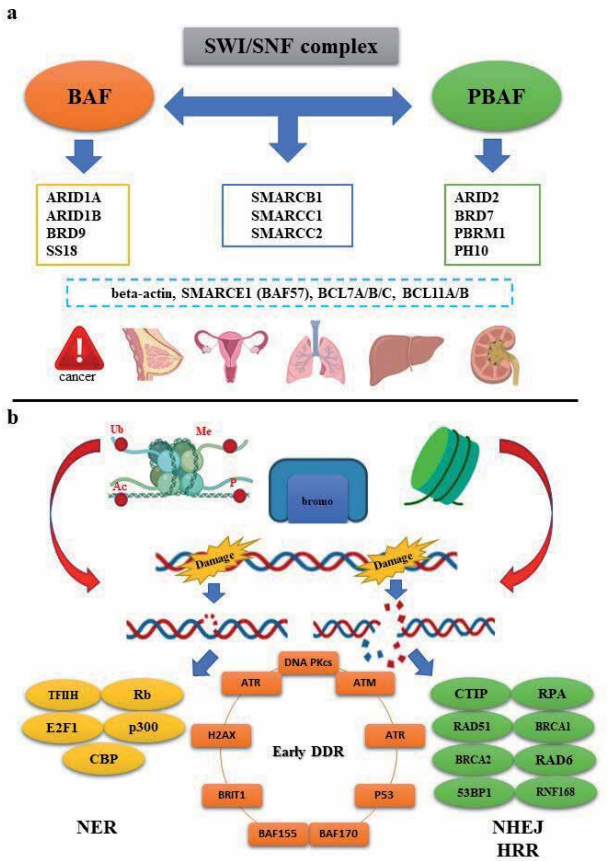

Figure 1.
The SWI/SNF complex in DNA damage response (DDR). (a) The biochemical characteristics of the BAF and PBAF subunits make the SWI/SNF complex a very heterogeneous family with exclusive properties in mammals. Alterations in this group of nucleosome remodelers give rise to cancer. (b) During DDR, SWI/SNF subunits interact through their bromodomains with histone modifications and epigenetic labels. For DNA repair to occur, it is necessary to activate the kinase cascades by ATM, responsible for recruiting and phosphorylating different repair genes such as E2F1, BRCA1 or 53BP1 and thus activating NER (nucleotide excision repair), HRR (homologous recombination repair)or NHEJ (non-homologous end joining), Ub: ubiquitination, Me: methylation, Ac: acetylation, P: phosphorylation, bromo: bromodomain.

SMARCB1). Duan et al. [31] identified that expression of ARID2 was significantly downregulated in hepatocellular carcinoma compared to adjacent nontumor tissue. Their research revealed that ARID2 inhibits cell cycle progression and tumor growth by interacting with the Rb-E2F signaling pathway. The relationship of E2F with epigenetic modifiers has become clear. Manickavinayaham et al. [32] demonstrated that the bromodomains of related acetyltransferases, p300 and CBP, specifically bind to the acetylated motif of E2F1; they found that interaction with acetylated E2F1 is critical for p300/CBP recruitment of DSBs and induction of histone acetylation at the sites of damage. They also demonstrated that in nucleosomes flanking DSBs, p300 and CBP mediate acetylation of multiple lysine residues on H3, including H3K18 and H3K56. Biswat et al. [33] and Guo et al. [34] demonstrated that E2F2 induction of histone acetylation and chromatin decondensation in response to UV radiation promote efficient NER. Lin et al. [35] shows that ATR creates induction of E2F1 in DNA damage. ATM/ATR is known to activate P53 in response to damage and plays a central role in DDR activation. ATM plays an important role in DDR and DSBs, as it regulates several pathways of cancer and epigenetic modifications.

3. Chromatin modifications in DSB repair

DSB repair can activate two repair pathways in mammalian cells: the HRR and NHEJ. First it is active throughout the cell cycle, and second it is active in S and G2 phases [36]. Usually, NHEJ is initiated by DSB recruitment of Ku70/80 or XRCC6 XRCC5 heterodimer. Ku70/80 is DBS sensor and facilitates the downstream factors recruitment, including DNA-PKcs, PAXX, XLF, XRCC4, and ligase IV. Recruitment of downstream factors helps complete DNA repair. However, HRR involves MRE11-RAD50-NBS1 (MRN) complex to activate ATM protein kinase [37]. Also, HRR can be influenced by PKcs and act in concert with the MRN complex and the recruitment of this complex to DNA DSB by hSSB1 (single-stranded binding protein), as well as the activities of 53BP1/RIF1 and BRCA1/CtIP [38]. Cells start a cascade of phosphorylation events in response to DNA damage, mediated by three phosphatidylinositol-3-related kinases: ATM, ATR, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The kinases activate a rapid reaction of abundant sensors for DSBs to check injuries, recruit effectors, and generate a coordinated response to maintain the genome integrity [37, 39].

Different epigenetic modifications are related to these kinases, while H2AX is an important chromatin-based substrate for phosphatidylinositol-3-related kinases, when it is phosphorylated on S139, and is named γ-H2AX. This phosphorylated form serves as a checkpoint for HR and NHEJ. The foci formation of γH2A.X is the most often observed epigenetic modification triggered by DNA damage; together with the response to DSB and the SWI/SNF complex, it promotes phosphorylation of S139 in its C-terminal region through ATM in human cells. Domains of γ-H2AX are established by contact with the DSB site. In fact, the break site defines the densities and spread of γ-H2AX [40–42]. In addition, PBAF functions in the ATM pathway silence transcription in *cis* on DSB by promoting mono-ubiquitination of H2A on K119 [43] and efficient early repair on exposure to ionizing radiation (IR), mediated by DSB and NHEJ [44]. H2AX induction by UV depends on ATR, but the formation of DSB in late stages contributes to ATM activation and the increase of H2AX. DNA-PKcs, after induction of DSB, is responsible for H2AX and chromatin remodeling factor (KAP1) phosphorylation. Furthermore, DNA-PKcs is required for chromatin remodeling in early postirradiation stages and promotes the rapid recruitment of DDR initiation proteins at DSBs sites [37]. An early modulator is BRIT, a chromatin-binding protein that forms irradiation-induced nuclear foci (IRIF) and works as a proximal factor at checkpoints in DNA, controls multiple damage sensors, and early mediators to DDR. BRIT1 colocalizes with γ-H2AX, using ATM/ATR to form the BRIT1-SWI/SNF interaction through its BAF170 and BAF155 subunits, potentiating the response to damage [45, 46].

Moreover, γ-H2AX recruits MDC in the chromatin, is phosphorylated by ATM, functioning as the protein coupling site of checkpoints and repair, such as 53BP1 and the UBC13-RNF8-BRCA1 complex. The protein 53BP1 is phosphorylated by ATM and helps in the BRCA1 recruitment: together with the post-translational modifications in the damage site, they help promote other repair proteins. ATM and RNF8 facilitate the repair of DNA DSBs in the phases G1 and G2 of the cell cycle, regardless of the repair pathway used [47, 48]. Similarly, ubiquitination in the DSB regulates the repair protein BRCA1 and P53, as well as BARD1 recruitment by 53BP1 [49]. BRCA1 and 53BP1 are necessary to regulate downstream histones phosphorylation and ubiquitination to direct the repair on NHEJ and HR. Thus, BRCA1 repairs DNA damage via HR, and 53BP1 plays a crucial role in the NHEJ repair pathway, ensuring DNA DSBs are repaired correctly [50, 51]. BRIT1 is required for DNA damage-induced intra-S and G2/M checkpoints, as regulation of the BRCA1 and Chk1 expression [52]. The loss of function of either of these two genes can alter

the HRR pathway, resulting in genetic instability and an increased risk of breast or ovarian cancer in BRCA1/2 germline mutation carriers [53, 54]. Cruz et al. [55] analyzed the RAD51 foci in 20 samples from patients with breast cancer, 10 in germinal-BRCA1, and 10 in germinal-BRCA2. The results provide new evidence of HRR restoration functionality as a frequent mechanism of PARPi resistance and demonstrate the potential of functional biomarkers to discriminate against tumors that will fail PARPi monotherapy. This emphasizes the major role played by ATM and DBSs-associated repair pathways in breast cancer treatment, whether mediated by IR or chemotherapy.

Qi et al. [56, 57] found that BRG1 decreases the nucleosomes stability at DSBs and creates an open and relaxed chromatin structure in SW13 and U2OS cells. It shows that BRG1 is crucial in early damage repair by remodeling the chromatin structure near DNA damage sites. They show that the BRG1 domain facilitates the RPA replacement with RAD51 at the DSB site in the HR, interacting with the RAD52 mediator and regulating its recruitment into the DSBs. de Castro et al. [58] demonstrated for the first time that Arid2 expression is important for HR. They found that Baf200 and Brg1 are required for efficient recruitment of Rad51 to a subset of DSBs, repaired by HR, where Rad51 and Arid2 are part of the same complex. Haokip et al. [59] demonstrated that SMARCAL1 and BRG1 regulate each other in HeLa cells with DNA damage-inducing agent doxorubicin, resulting in an increase in the SMARCAL1 transcription and protein. They found that BRG1 is present at the Enh1 and Enh2 region of SMARCAL1 promoter in untreated HeLa cells. Experiments showed how BRG1 is present on SMARCAL1 promoter, with protein occupancy increasing when DNA is damaged, indicating that BRG1 can positively regulate SMARCAL1, creating a regulatory loop. Regarding the relationship with proteins of initial DDR pathway, Keka et al. [60] showed that the loss of Smarcal1 reduces the XRCC4 recruitment to DSB sites several times and suggests that Smarcal1 is required for DNA-PKcs/Ku70/Ku80 complex to the correct functions. Diplas et al. [61] found that loss of SMARCAL1 in glioblastoma cells can induce alternative lengthening of telomere (ALT) phenotypes, in the same way as ATRX. ATRX mutations are the most prevalent abnormality in glioma, as Han et al. [62] identified that knockout ATRX inhibited glioma cell growth, tumor invasion, and a decrease in H3K9me3 availability, which can inhibit the ATM acetylation resulting in increased glioma cell chemosensitivity. They found that ATRX is involved in DNA damage repair by regulating the ATM pathway, suggesting a good prognostic marker in predicting temozolomide (TMZ) chemosensitivity.

Although little is known about the role of H1.2 in response to damage and interaction with the repair machinery proteins, its function has become clearer. Kim et al. [63] reported that p300-mediated acetylation of p53 and DNA-PK-mediated phosphorylation of H1.2 alter the p53-H1.2 interaction, thus alleviating the repressive effects of H1.2 on the transactivation of p53. After DNA damage, p53 and H1.2 undergo modifications in an orderly fashion, with H1.2 phosphorylation at T146 followed by p53 acetylation. p53-H1.2 interaction is essential to enhance p53 function, and point mutations that mimic its constitutive modifications induce efficient growth inhibition and apoptosis. Li et al. [64] described a new mechanism to H1.2, without other H1 isoforms, to regulate the DNA damage response and repair through repression of ATM recruitment and activation. Moreover, H1.2 functions as a molecular brake for ATM binding to MRN, whereas DNA damage-induced ATM activation requires both the MRN complex assembly and H1.2 release. As such, these authors revealed a new link between chromatin disturbances, destabilization of H1.2, and ATM activation. For the first time, it was found that the RNF168/RAD6 complex can promote mono-ubiquitination of histone H1.2 *in vitro*, and the H1.2 mono-ubiquitination can be induced after IR treatment. They concluded that

H1.2 mono-ubiquitination *in vitro* and *in vivo* by RNF168/RAD6 is evidence that mono-ubiquitination can establish a suitable microenvironment for other E2/E3 complexes to catalyze polyubiquitination or multi-mono-ubiquitination of H2A and H2AX over H2A and H2AX ubiquitination, which is dependent on RNF8/Ubc13 [65]. Thorslund et al. [66] proposed that linker H1 represents a key chromatin-associated RNF8 substrate, whose UBC13-dependent K63-bound ubiquitylation at DSB-containing chromatin provides a scaffold to RNF168 binding through its UDM1 module. RNF168 ubiquitinates H2A into K13/K15 and possibly other proteins to trigger repair factor recruitment in DSB. Giné et al. [67] implicated H1.2 as a valuable protein for apoptosis induction in chronic lymphocytic leukemia cells, and its release pattern is correlated with deletions of 17p and treatment response; they emphasized that histone H1.2 could be an essential apoptotic signal induced by agents acting independently of p53.

4. Methylation in DNA damage and repair

DNA methylation is considered a post-replication modification by the methyl group (–CH3) addition at carbon 5 cytosine, known as 5-methylcytosine (5mC), primarily in dinucleotides of CpG [68]. This enzymatic reaction is catalyzed by three DNA methyltransferases (DNMTs) (DNMT1, DNMT3A, and DNMT3B). These modifications are commonly found in promoter regions, the CpG islands, as their main function is transcription silencing, decreasing, or repressing the gene function. Methylation is the epigenetic modification most studied in cancer, as it is well-known that DNA hypermethylation can transcriptionally silence tumor suppressors and DNA repair genes, giving neoplastic cells survival advantages [69]. Various genes related to cell cycle regulation, tumor cell invasion, cell signaling, apoptosis, and chromatin remodeling are hypermethylated and silenced in almost all tumors. As demonstrated by Pal et al. [70] for the first time, CpG sites in the promoter of H2AX, RNF8, and CYCS are methylated; they show the collaborative participation of hypermethylation of DR5, DCR1, DCR2, CASP8, CYCS, BRCA1, BRCA2 and H2AX and the hypomethylation of DR4, FLIP, and RNF8 in sporadic breast cancer; the authors proposed that promoter methylation of these apoptotic and DDR genes is not due to a random phenomenon, as the progressive modification of aberrant epigenetic alterations are associated with tumor advancement, which generates the dysregulation of the DDR-apoptotic pathway, promoting tumor development. Hinrichsen et al. [71] found that increased methylation in the promoters of MLH1, MSH2, PMS2, and p16 genes are correlated to an advanced stage in hepatocellular carcinoma. Epigenetic marks and DDR are crucial points to understand if changes in DNA methylation can contribute to resistance of cancer treatment, particularly by radiotherapy [72].

Various epigenetic marks have been associated with the response to damage, primarily the methylation of lysine 4 and 79 in H3 (H3K4me and H3K79me, respectively) that contribute to DDR and DNA repair [73]. The marks on H3K4me1/2/3 regulate the repair of DSBs through chromatin accessibility [74, 75]. Furthermore, H3K36 methylation is associated with "open" euchromatin and helps RNA pol II activate transcription [76]. Chang et al. [77] demonstrated that the tumor suppressor PHRF1 can move on the DSB due to H3K36me2/me3 and NBS1, then ubiquitinate PARP1, and trigger the subsequent repair by NHEJ. Yet, it is known that DOT1L methyltransferase catalyzes the H3K79 mono-di-trimethylation through its non-SET domain. Recently, in the study by Kari et al. [78], the depletion or inhibition of DOT1L activity was shown to result in altered DNA damage response, indicated by decreased levels of γH2AX, but with increased KAP1 phosphorylation. Loss

of DOT1L function leads to faulty HRR-mediated DSB repair without affecting NHEJ. Highlighting DOT1L-mediated H3K79me3 importance in the early response to DNA damage and DSB repair, its inhibition also increases radiation sensitivity and chemotherapeutic agents in colorectal cancer patients' treatment. Dot1L and H3K79 methylation was previously associated with the role of 53Bp1 in response to DNA damage [79]. FitzGerald et al. [80] corroborated that not only H3K79me recruits 53BP1 to DNA damage sites, but also H4K20me. This indicates that H4K20me concentration is essential for the repair pathway related to 53BP1 and BRCA1, in cooperation with the 53BP1-RIF1-MAD2L2 complex. The histone post-translational modification of H3K27me3 and H4K20me is a diagnostic indicator in melanoma [81].

There is DNA methyltransferase, in which expression is regulated by epigenetic modification during DNA damage. MGMT is an O^6-methylguanine-DNA methyltransferase responsible for the repair of damaged guanine, without other cofactors transferring the methyl at 06-meG to cysteine residues, removing adducts in a single step, thus protecting chromosomes from mutations, carcinogenesis, and alkylating agents [82, 83]. MGMT can do this only once, after its DNA-binding domain change by alkylating, which is detached from DNA and targeted for degradation by ubiquitination [84]. Loss of MGMT expression is due to promoter methylation [85], used as an advantage for good prognosis in a glioma, which can predict whether alkylating agents can benefit treatment [86]. MGMT can interfere with TMZ response on tumor cells and is responsible for efficient repair of TMZ and induced toxic DNA adducts, reducing treatment efficacy. Targeting MGMT seems to overcome chemoresistance in gliomas, but the prognostic value of MGMT methylation is controversial, as genomic rearrangements result in MGMT overexpression, independent of its promoter methylation, contributing to resistance [87].

5. Genome damage repair via m6A participation

Modified nucleotides on DNA create a vast list, shown by Fragou et al. [88], but methylation by methyltransferases is an epigenetic modification that occurs on DNA and RNA. In fact, RNA contains more modified bases than DNA, as these RNA modifications are present in rRNA, tRNA, snRNA, miRNA, or mRNA, giving rise to the emergence of a wide chemical diversity on its side chains. One of these modifications on mRNA has been studied since its discovery in 1974 [89, 90], the methylation of adenosine residues form N6-methyladenosine (m6A) is the most abundant post-transcriptional mRNA modification, and is detected in approximately 25% of mRNAs [91]. The m6A modification mechanism of action functions like methylation on DNA and histones, i.e., its presence or absence on mRNA determines the fate of the transcription. The addition of a methyl group to the N6 site of A, occurring in the RRACH sequence [92, 93], has a distinctive position in the vicinity of stop codons, internal exons, in 5'UTRs which could promote 5'cap-independent translation when found on the first nucleotide adjacent to the 7-methylguanosine cap; this gives protection from decapping [7, 94, 95] and in 3'UTRs regulates the affinity of RNA binding proteins [96]. This is known as m6A modification, which is involved in a variety of cellular processes including gene expression through regulating RNA metabolism, such as mRNA translation, degradation, alternative splicing, export and folding [97, 98], control of protein translation [94], and others. All these effects are globally known as RNA epitranscriptomics.

For some time, m6A modification was considered static and unalterable, but is now well-known as reversible and dynamic. It is difficult to examine gene regulation at the RNA level without appropriate methods; genome-wide sequencing became a

major tool for many years, but had limitations with the m6A assay. The sensibility of the methodologies used was a critical point, because until recently, techniques had a detection limit of about 0.01%, in contrast to the content of m6A, less than 0.001% is sufficient to regulate biological processes [99]. Antibody-based, high-throughput sequencing technology allowed us for the past few years to locate the specific m6A sites and explore their biological significance. Linder et al. [91] solved the problem of distinguishing between m6A and adenosine by using incubation of an m6A-antibody to induce a specific mutational signature of m6A residues after UV light-induced antibody-RNA crosslinking and reverse transcription.

This modification is added and eliminated by proteins called "writers" and "erasers," respectively; these proteins regulate the abundance, prevalence, and distribution of m6A; this exerts its biological function and is modulated by protein "readers." First, m6A is added to the nucleus by the methyltransferase complex, which is formed by two core proteins, METTL3 and METTL4 [92]. METTL3/METTL4 form a functional heterodimer, METTL3, which is the catalytic subunit which uses S-adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) as donors for methyl transfer in adenosine within the consensus motif G(G/A) ACU [100]. METTL4 has the active site blocked, and lacks amino acid residues to form hydrogen bonds with the ribose hydroxyls of SAM to facilitate donor and acceptor substrate binding; this is important in maintaining the integrity of the complex, stabilizing METTL3, and enabling RNA substrate recognition [101]. Regarding m6A demethylases or "eraser" proteins, FTO and ALKBH5 are two well-known m6A demethylases located in nuclear speckles within the methyltransferase complex. They belong to the group of Fe(II)/2-oxoglutarate (2OG)-dependent dioxygenases [102] and act on both DNA and RNA. FTO demethylates m6A through oxidative reactions that generate two intermediate hydroxymethyl6A and formyl m6A [103]. ALKBH5 catalyzes the direct removal of m6A, oxidizing the N-methyl group of the m6A site to a hydroxymethyl group, as its m6A demethylation function affects total RNA synthesis and mRNA export [104]. The "reader" proteins belong to the YTH domain family proteins, and in humans include YTHDF1–3 and YTHDC1–2. YTHDF1 stimulates mRNA translation to interact with eIFs and ribosomes. YTHDF2 binds to m6A, located in the 3'UTR leading mRNA to become processing bodies for degradation in a methylation-dependent manner [105]. While YTHDC1 protein binds to m6A, as well as pre-mRNA splicing factor SRSF3 to its mRNA-binding elements, they are close to m6A sites but block SRSF10 mRNA binding, promoting exon exclusion, and modulating mRNA splicing by recruiting pre-mRNA splicing factors [106].

Damage to DNA is derived from replication stress, telomere shortening, UV light, chemical toxins, and ROS - but RNA can suffer alterations and respond to damage agents. This variety of DNA lesions is removed in cells by protein complexes in specific repair systems (**Figure 2**). Until recently, response to damage was unique to DNA, while analyzing the response of DNA to UV, with Xiang et al. [107] changing that view. They found an accumulation of m6A on poly(A) + RNA two minutes after UV irradiation in response to DNA damage. They observed that the methyltransferase complex (METTL3, METTL4, WTAP) and Poly(ADP-ribose) polymerase (PARP) is localized to sites of UV-induced damage, with FTO demethylase recruiting. DNA polymerase translesion (Pol κ) is necessary for METTL3 and METTL4 recruitment. The authors suggested that PARP, METTL3, m6A RNA, and Pol κ could be alternative repair pathways to respond to UV-induced damage, with m6A in the main role for rapid recruitment of Pol κ to damaged sites. Colocalization of Pol κ with m6A to sites with a high content of cyclobutene pyrimidine dimers (CPDs) corroborate that m6A RNAs have a regulatory role in the NER pathway. Svobodová et al. [108] found that m6A RNAs are diffuse to damaged DNA, but

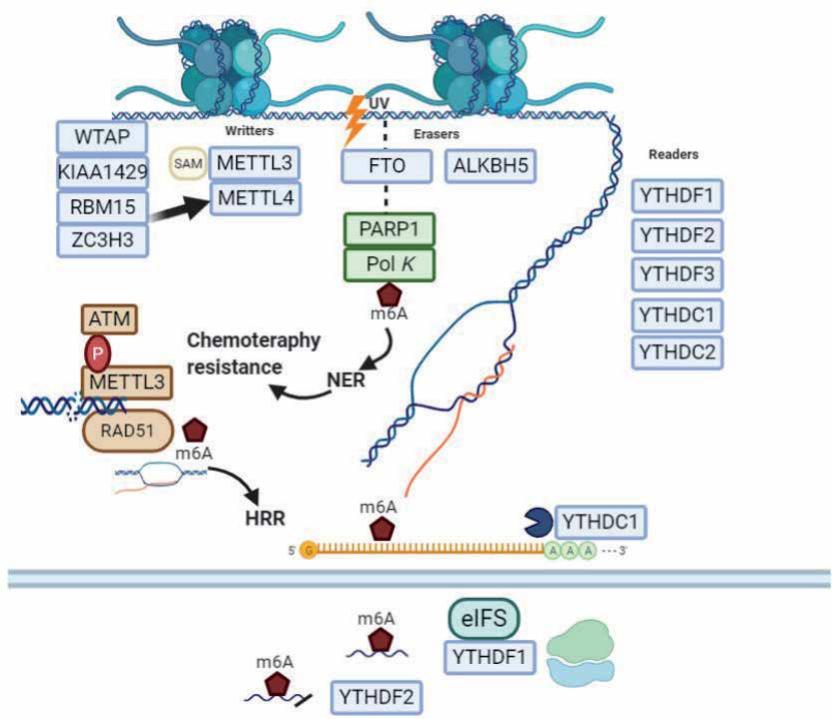

Figure 2.
Regulatory complexes of m6A in DNA damage repair. The m6A mark on mRNAs is involved in some repair pathways in response to DNA damage. It has been observed that the interaction of writing, eraser and reader proteins with other proteins that are part of canonical repair pathways is essential for the response to damage. SAM: S-adenosylmethionine; UV: ultraviolet light, NER: nucleotide excision repair, HRR: homologous recombination repair, eIFS: eukaryotic initiation factors, phosphate group: red circle.

a new participant, METTL16, accumulated 20–30 min after induced damage in a subset of irradiated cells. This response was specific to CPDs, as the authors observed that m6A RNAs' accumulation pattern was specific to repair of CPDs' sites, which do not accumulate in other lesions, such as NHEJ.

Apyrimidinic or apuric (AP) sites can arise by spontaneous hydrolysis, cleaving the N-glycosidic bond through elimination of an incorrect or damaged base by DNA glycosylases or ionizing radiation. Considered one of the most predominant lesions in the genome, it is repaired by the BER pathway, which has PARP-1 as the first sensor and responder, activated by poly(ADP-ribose) polymerase [1]. Recently, Xiang et al. [107] found it was associated with m6A in RNA. AP lyases cleave 5′ or 3′ to AP sites to further processing by DNA polymerase and ligase. ALKBH1 is another member of the AlkB family that participates in demethylation of histone H2A in mouse stem cells. It is capable of cleavage to DNA at AP sites, using a lyase mechanism to produce a DNA nick on the 3′ side of a basic site, leaving a product that is missing a 3′-phosphate and an adduct in the 5'-DNA product. ALKBH1 acts on both ss-DNA and ds-DNA and can produce DSBs related to AP lyase activity. Human ALKBH1 possesses m6A demethylation activity, although this is not its main function. It cannot be ruled out this has a role in epigenetic gene silencing. Due to its abundance in mitochondria, it could play a primary role in mitochondrial DNA repair and function [109–111]. Other AlkB, such as ALKBH3, function as RNA repair enzyme; it is equally distributed in cytosol, the nucleus, and is active on RNA and ssDNA [112]. Zhang et al. [113] showed how METTL3 is phosphorylated by ATM protein for its localization to DSBs, where it catalyzes m6A in RNAs. METTL3 stimulates the recruitment of RAD51 to DSBs in a DNA-RNA

hybrid-dependent manner, promoting efficient homologous recombination-mediated DSB repair. The upregulation of METTL3 may contribute to resistance to chemo- and radiotherapy.

6. Cancer drugs: resistance and epigenetics

Alterations in m6A and proteins responsible for its regulation on the RNA level were shown to interfere with the response to cancer treatment. Cyclophosphamide (CTX) is an alkylating chemotherapy drug used in cancer treatment; it forms adducts at the N7-guanine position, which are unstable and therefore undergo spontaneous dissociation. These features are exploited in rapidly dividing cells, which are likely to be disrupted before repair takes place [114]. Little is known about CTX effect on RNA epigenetic complexes, yet it was shown to increase m6A levels, inhibiting the gene and protein expression of FTO, YTHDF1, YTHDF2, YTHDC1, and YTHDF3 in a time- and concentration-dependent manner [115].

Cervical squamous cell carcinoma (CSCC) is an example of increased chemotherapy resistance; Zhou and colleagues [116] found that β-catenin is an FTO target: they observed FTO overexpression and reduced m6A β-catenin levels, with the effect of this change upregulation of β-catenin protein and the subsequent activation of ERCC1, a critical player in NER, which contributes to chemotherapy resistance and a poor prognosis. In case of BRCA-mutated epithelial ovarian cancers (EOC), the use of Olaparib has been clinically beneficial: it is a poly(ADP-ribose) polymerase inhibitor (PARPi), which detects and binds DNA SSBs and DSBs, using the N-terminal DNA binding domain [117, 118]. When DNA is damaged, PARP-1 can recognize damaged sites and their formation; the binding exposes the enzymatic site of PARP-1, resulting in its activation and the recruitment of XRCC1, the first protein for assembly and activation of DNA bases excision repair machinery [119]. The resistance of tumor cells to PARPi is not well-known, but in the study of Fukumoto and colleagues [120], FZD10 was observed as a receptor in Wnt/β-catenin signaling, increasing m6A modification of mRNA in resistant cells, thus stabilizing. This increase contributes to PARPi resistance by upregulating the Wnt/β-catenin pathway in BRCA-deficient EOC cells.

Xiang et al. [107] showed that FTO could be recruited to damaged γH2AX chromatin after irradiation, strengthening FTO participation in DNA damage repair. In melanoma, FTO is upregulated and promotes cell proliferation, cell migration, invasion, and cell viability. Yang et al. [121] demonstrated that FTO regulates PD-1 expression that also promotes mTOR signaling. FTO can promote resistance to anti PD-1 blockade in melanoma through m6A, mediating PD-1 (PD-1 or PDCD1 is a negative regulator of T-cell activity). This supports the anti PD-1 blockade with an anti-tumor response in advanced cancers and reduces immune-related adverse side effects, vs. with ipilimumab.

Glioblastoma is a common and aggressive primary brain tumor in adults, and is highly resistant to treatment such as surgery, irradiation, and adjuvant TMZ chemotherapy, which failed to improve the outcome. One cause of poor response to TMZ, as suggested by Visvanathan et al. [8], is that GSCs show high levels of m6A and METTL3, supporting the proposal that METTL3 is key in GSC maintenance, making those cells resistant to therapy and refractory to radiotherapy by efficient repair of DNA. Here, METTL3 alters the DNA repair efficiency and radiation sensitivity through m6A sites in SOX2–3'UTR, stabilizing it in GSC, as the recruitment of human antigen R (HuR) to m6A modified mRNA is crucial for SOX2 stabilization by METTL3. This supports a role for METTL3, shared with SOX2 in another repair pathway, mediated HR: SOX2 protects GSCs from radiation-induced cytotoxicity

by promoting HR repair, implying an oncogenic role for METTL3 and m6A. At the same time, FTO becomes a promising target to develop FTO inhibitors like Rhein, Meclofenamic acid (MA2), or its ethyl ester form, MA2 [122].

Hepatocarcinogenesis is correlated with abnormal m6A modifications, high METTL3 and YTHDF1 expression in hepatocellular carcinoma (HCC) is associated with poor prognosis, with its combination as a malignant marker, according to Zhou et al. [123]. The participation of the machinery regulating m6A was clarified by the work of Chen et al. [124]. They found a significant increase in mRNA m6A levels, supporting the role of m6A in liver cancers. When METTL3 is downregulated, it is unable to act on tumor suppressor SOCS2, being silenced by METTL3 through m6A-YTHDF2. SOCS2 transcripts are a direct target of YTHDF2 with mediated mRNA decay, promoting tumor progression. Recently, Lin et al. [125] found that METTL3 depletion leads to a resistant phenotype in HCC with sorafenib treatment through regulation of FOXO3 expression. FOXO3 m6A methylation maintains stability, but its absence accelerates degradation. The modification is read by YTHDF1, which stabilizes m6A-labeled RNA and promotes protein synthesis. These results contrast with those obtained by Taketo and colleagues [126] when they established a METTL3-KD using a pancreatic adenocarcinoma cell line. The authors concluded that these cells had higher sensitivity to gemcitabine, 5-fluorouracil, cisplatin, and irradiation. It is clear that METTL3 plays a key role in resistance to therapy, but the way this gene behaves in different types of cancer is not yet understood. In a study on colorectal cancer (CRC), it was found that c-Myc activates the YTHDF1 gene expression. YTHDF1 is overexpressed in CRC and has been associated with lymph node metastasis and poor prognosis, as the evidence in vitro with YTHDF1 knockdown indicates that cancer cells are sensitized to the exposure of 5-Fluorouracil and L-OHP (oxaliplatin) [127].

7. Conclusions

The SWI/SNF complex has been shown to be a central regulator in DNA repair, over other epigenetic complexes such as Polycomb repressive complex 1 (PRC1) and 2 (PRC2), which require the cooperation of their subunits and epigenetic markers to trigger the on–off signaling cascade, generated by acetylation, methylation, or ubiquitination of genes involved in response to DNA damage. We see that participation of epigenetics in the cellular responses goes far beyond DNA promoter methylation and histone modification. Methylation of RNA has a critical role in cell maintenance, changing our notion about RNA functions. We do not know whether deregulation of the m6A machinery could result in cancer development or progression by altering DNA damage response, but knowledge of molecular mechanisms of regulation of m6A cellular modification in tumor cells may develop a combined therapy for m6A regulator proteins as targets that facilitate a better cancer response.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

Genomic Instability and DNA Repair in Cancer

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Abstract

Mutations in genome are essential for evolution but if the frequency of mutation increases it can evince to be detrimental, for a steady maintenance there exist a detailed complex system of surveillance and repair of DNA defects. Therefore, fault in DNA repair processes raises the probability of genomic instability and cancer in organisms. Genome instability encompasses various aspects of mutations from indels to various somatic variants. The chapter tries to present an overview of how cancer puts up several ways to ensure suppression of the fidelity in our DNA repair system. Cancer cells assure failure of efficient DNA repair mechanisms by innumerous ways, by mutation and epigenetic modifications in repair genes themselves or genes controlling their expression and functions, other by some catastrophic events like kataegis, chromothripsis and chromoplexy. These are clustered mutations taking place at a particular genomic locus which deluge the repair process. Cancer generation and evolution is dependent largely on genome instability, so it applies many strategies to overcome one of its basic obstacles that is DNA repair, targeting these DNA repair genes has also demonstrated to be helpful in cancer therapy; but an intricate understanding of recalcitrant process and mechanisms of drug resistant in cancer will further enhance the potential in them.

Keywords: genome instability, DNA repair, cancer, epigenetic modifications, clustered mutation

1. Introduction

Genome is the basis of life of an organism and mutation in genome is essential for adaptation [1]. A mutation is a change in genomic sequence, they are the result of mistakes a cell makes while copying a piece of genome during replication or sometimes mutation is influenced by exogenous agents. Mutations have the capacity to influence gene expression depending on their location in the genome, gene structure and intergenic region. They possess this power to affect with such consequences because mutation in coding region of the genome might give rise to a truncated protein with no use or might compromise its fidelity. Alternatively, it can also endow the protein with some advantages with its function. Their presence in the regulatory region may increase or decrease its expression. This change in level of expression also affects cellular mechanisms since proteins are required by the cell in specific amounts. Therefore, these changes give either an advantage or a disadvantage depending on the effect it may produce but with a higher rate of mutation cell loses

its capacity to maintain genome integrity, that give rise to genome instability, it is a range of DNA alterations which irreversibly change information content of the genome. To keep a check on all such mutational process cell has an elaborated system of DNA repair and checkpoints [2, 3].

In unicellular organisms, a delicate balance exists between maintenance of genome stability and the tolerance to genome instability. They have harnessed this instability to mediate phase and antigenic variation that instead imparts them advantage for survival [4]. But any catastrophic changes in the genome are detrimental to them, in simple words genome instability for unicellular organisms is deleterious if it becomes impossible for them to take a control on it. For complex multicellular organisms like humans, we accumulate DNA damage over years that lead to genome instability and this genome instability is one such reason for aging [5]. A lot of studies are available that clearly state that the genome instability is a hallmark of aging and cancer. Cancer as we all know is a disease where the cell's own regulatory system goes wrong and there is uncontrolled division and to relief itself from cell cycle checkpoints and escape immune surveillance and apoptosis a cell must accumulate enough mutations. Cancer usually arises from benign tumor; these are localized abnormal growth of cells that cannot spread to other parts of the body. They undergo some more mutations and turn malignant now they have the capability to spread. Although there are certain types that do not form benign tumors such as leukemias, lymphomas and myelomas because of their nature [6].

There are several ways by which cell resist accumulation of DNA damage such as scavenging DNA damaging molecules, repairing erroneous DNA and at last if the cell is damaged beyond repair then apoptosis [7]. Despite all these measures the cell sometimes gathers enough mutation for the genome to be unstable and it to be cancerous [8]. Reasons behind DNA damage can be endogenous or exogenous. These damages are first perceived by the cell and a process of DNA repair is triggered on. Any discrepancies in the process of DNA repair predispose the cell to malignant transformation. Therefore, detection and repair of changes in the genome is prime to maintain cellular integrity. From this we can very well understand the importance of the role by DNA repair mechanism on maintaining genome integrity [9].

Its role can also be understood in hereditary cancers where most of the time one allele of a gene involved in one of the repair systems remains mutated at birth and the other turns mutated in course of time and the cancer arises. There are other example like the famous Breast Cancer genes; namely BRCA1 and BRCA2 where mutation in any one of them predisposes to breast and ovarian cancer. About 5–10% of people having mutation in BRCA genes encountered cancer once, mutation in these genes also predisposes the individual to cancer recurrence. We all know mutations owe their effectiveness to their space, therefore not all mutation in these genes is potentially effective in predisposing the person to cancer. Several mutations are identified till date and categorized by its influence. Both the genes BRCA1 and BRCA2 are involved in transcriptional regulation in response to DNA damage, most of these functions are mediated by the cellular proteins that interact with them [10]. BRCA1/2 is a tumor-suppressor and gets recruited in DNA damage loci, it has many other functions like damage induced cell cycle checkpoints activation, its association with homologous recombination as well as non-homologous recombination has also been established [11]. The exact mechanism behind BRCA controlling all these processes is still unclear but there is evidence of its direct association [10]. There is a specific type of drug available acting on these cells with mutation in BRCA genes; poly ADP-ribose polymerase (PARP) inhibitors. Their effect is specific to the cancer cells as they are usually deficit in homologous recombination, we will discuss this later in the chapter [11]. Current chapter gives a description on mutations and epigenetic modifications of the DNA repair genes which aid genomic instability in cancer.

2. An introduction to genome instability in cancer

Genomic instability refers to chromosomal changes, ploidy change or changes in nucleic acid sequence. In multicellular organisms, genome instability is fundamental to carcinogenesis [12]. Genome instability in cancer is associated with the ploidy change, chromosomal translocations, inversions, insertion, deletions, DNA breaks or any abnormal changes in DNA tertiary structure that can cause DNA damage, or the misexpression of genes (**Figure 1**). Ploidy change is accumulation of extra copies of chromosome(s) or parts of chromosome. Chromosomal translocations are phenomenon when a part of the chromosome breaks and get attached to some other chromosome. Inversions are breakage of a piece of DNA and getting attached to the same position in an inverted orientation. It happens when the DNA undergoes breakage and rearrangement. Insertion as the name suggests is the insertion of nucleotides on a locus in the genome. In deletions, there is deletion of nucleotide from specific loci in the genome. DNA breaks are either double stranded or single stranded depending, single stranded are easily repaired because here template is readily available but there are specific repair pathways dedicated to double stranded breaks since template is lost and the repair pathways has to bring it from the other pair and in some cases, there is error prone repair [8, 13]. Microsatellite instability is another such phenomenon influencing genomic instability. Genome instability fuels the cancer cells with changes that help it to evolve and escape death. Additionally, it also plays a critical role in cancer initiation and progression by overcoming immune surveillance, attaining uncontrolled cell division, more DNA damage, etc. [14]

In normal cells, genome is protected at every stage of the cell cycle, every step from DNA replication to chromosome packaging. Every step is very precisely monitored for faults. Several processes are involved in this such as cell cycle checkpoints, DNA damage checkpoints and DNA repair. When a normal cell turns cancerous, these fault monitoring systems are manipulated; presence of genomic instability itself indicates the failure of one or many of these safety nets (**Figure 1**) [13]. Interpreting the underlying mechanisms for imbalance in genome integrity would yield new avenues for precision therapies and clinical decision-making. Lot of research has been centered to genome instability to understand it and hold control over its initiation and progress in a hope to conquer cancer, which is the world

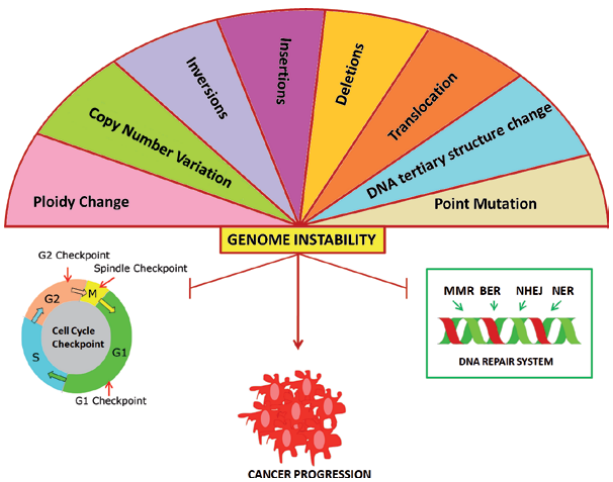

Figure 1.
Genomic instability influencing cancer.

leading cause of death. But still a clear picture of its origin or prevalence is far from our understanding [15].

All the mutations which are results of genomic instability are not hazardous, some of them just exist without much influential function. The ones with potent negative effects are termed as driver mutations and the one without are termed as passenger mutations. Even passenger mutations contribute to tumorigenesis but has less influence. Diver mutations are usually the mutations that are present in tumor suppressors, DNA repair genes, etc. A lot of studies are done to understand these driver mutations but again there is no clear evidence [15].

In hereditary cancers, genomic instability arises from mutations in DNA repair genes that drives cancer development, as predicted by the mutator hypothesis. On the other hand, sporadic cancer, the molecular basis of genomic instability remains unclear, but recent high-throughput sequencing studies suggest that mutations in DNA repair genes is one of the major mechanisms of inducing genomic instability. Still there remains a debate on either tumor suppressor genes or the DNA repair genes are the major source for genome instability in cancer, but it is established that DNA repair mechanisms is one of the prime targets [12].

3. Influence of cancer on DNA repair pathways

Source of mutation in DNA of normal cell are either from faults in DNA replication procedure or from carcinogens. The fidelity of eukaryotic DNA polymerase is very high with about 10^{-10} mutation per 1000 nucleotides, yet sometimes it miss, and mutation occur. Carcinogens encompasses all the factors that cause DNA damage and evoke carcinogenesis, they are such as ionizing radiations, UV radiations; some non -radiating ones are such as alcohol, tobacco, cigarette smoke and such other abusive products. These products include reactive oxygen species, deaminating agents, alkylating agents, polycyclic aromatic hydrocarbons, base analogs, intercalating agents, etc. [16]. Not only these, but there are also some microbes such as *Helicobacter pylori* and Human papilloma virus which induce inflammation leading to generation of various reactive oxygen species and generation of cancer. But still till date a specific reason for cancer cause has not been elucidated [17]. Several theories describe cancer cause but a proper picture of its causal and turning deadly is not yet established. It is very clear that cancer is not a one step process while it is complex, associated with several factors coming together to generate a system for freeing itself from crunches of checkpoints for regulating cell division. In normal cells these mutations are checked on by DNA damage checkpoints or cell cycle checkpoint, these are a series of biochemical pathways that are in constant surveillance to ensure integrity of the genome upon encountering any defects, they halt cell cycle progression and activate DNA damage repair mechanisms, then ensure repair of the faults and resume cell cycle progression [18] as shown in **Figure 2**.

There are several type of repair pathways namely: nucleotide excision repair (NER), base excision repair (BER), DNA mismatch repair (MMR) and double strand break repair which includes homologous recombination (HR) and non-homologous end joining (NHEJ). Every one of them are specialized in a specific type of repair pathway [4]. A detailed description of them with the mutation and modifications that are present in genes involved in these pathways in cancer cells can give us much understanding of exploitation of the process by cancer.

Nucleotide excision repair (NER) repairs bulky lesions and large adducts that distorts double stranded helix under conditions when only one of the two DNA strands is affected, UV radiation and chemical mutagens include such damage. These mutagens cross-link adjacent pyrimidine bases and purine bases and creates

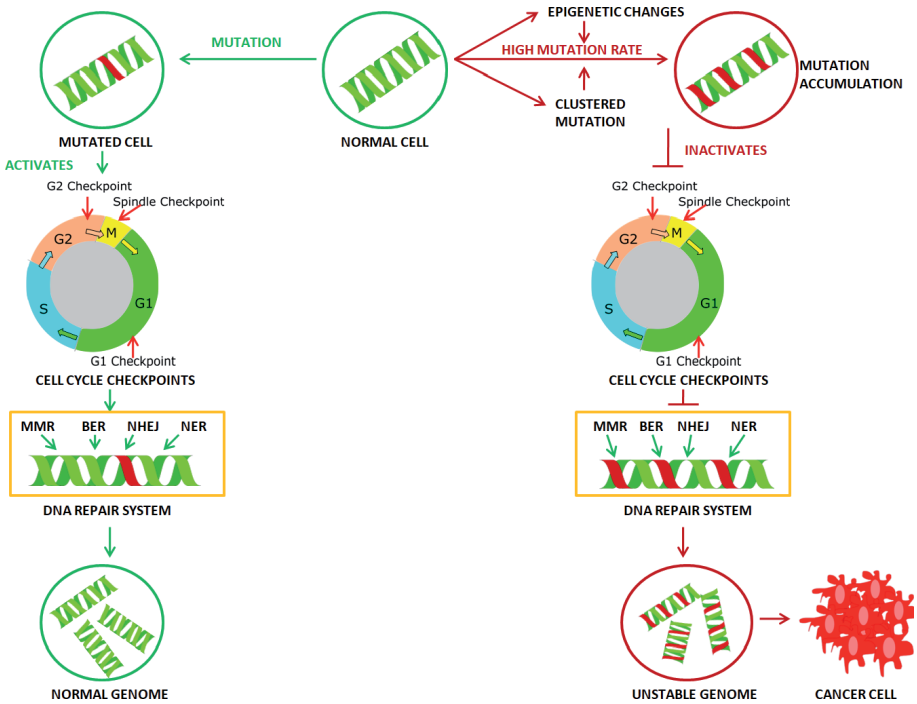

Figure 2.
Schematic illustration of relationship between mutations, DNA repair system and cancer.

intra-strand adducts, such damage blocks basic cellular systems like DNA replication and transcription [19]. NER can adapt to many structurally unrelated types of damage, to perform its diverse work, NER employs two sub pathways: global genome repair (GG-NER) and transcription coupled repair (TC-NER). The sub pathway names hint their distinctive roles, transcription coupled repair act on lesions that block progression of an active transcription site, a locus in the genome where an RNA polymerase is actively functioning and global genome repair is active in all phases of the cell cycle, repairing damages [20]. NER first recognize the damage, gather its associated complex and unwinds that specific region of DNA locus, makes incision on both points, remove the damage, synthesizes new nucleotides using other DNA strand as template strand and ligates the DNA [21]. Several different proteins are involved in the process; a nine-unit complex called transcription factor IIH (TFIIH) executes the first phase of repair, this complex includes two helicases, XPB and XPD and two other proteins, XPA and RPA that open the helix. It remains attached to the DNA while two different endonucleases, XPG and XPF, the latter acting in conjunction with ERCC1 perform precise cutting on one side of the damaged strand, several nucleotides away from the damage. After this initial step, RPA mediates the assembly of a second repair complex where Replication factor C (RFC) binds to excision region and facilitates PCNA that binds to DNA polymerases δ and ε, preventing them from falling off the strand before the reconstruction has been done and ultimately Ligase I attaches new repaired strand to the pre-existing ones [22].

The base excision repair (BER) pathway corrects damage occurring from oxidation, alkylation, deamination, and ionizing radiation, these lesions produce mild damage involving few bases the mutation usually cause base mispairings [23]. In this case mutagens are usually endogenous; here by the term mutagens we mean mutation causing agents, here only the damaged nucleoside is removed by cleaving

its *N*-glycosidic bond, leaving an abasic (AP) site [24]. One of BER's exclusivity depends on its 11 damage-sensing glycosylases, these glycosylases remain bound to the site to ensure that the fragile AP site never remains unattended, APE1/Ref1, or AP endonuclease/Redox Factor 1, process the loose ends creating special termini to accept the new base [25]. XRCC1 stabilizes the damaged area and coordinates sequential binding and release. XRCC1 also acts as a helicase, the process then includes a sliding clamp PCNA (proliferating cell nuclear antigen) and an additional stabilizer that also inserts the newly synthesized nucleotides, replication factor-C, or RFC [26].

During DNA replication, proofreading polymerases sometimes may fail to detect errors made by DNA polymerase. Mismatched repair (MMR) in a post-replicative repair mechanism steps in this scenario to clear the errors. MMR's damage sensors can differentiate between the parental DNA strand and the newly synthesized DNA strand and only remove the mismatch in newly synthesized strand, using parental strand as a template, MMR remove the mismatch and synthesizes new DNA strand [27]. MMR corrects single-base mismatches and small insertion/deletion loops by one of the two damage recognition complexes MSH2:MSH6 or MSH2:MSH3, MSH2:MSH6 recognizes single-base substitutions and the smallest insertion/deletion loop and MSH2:MSH3 insertion/deletion loop involving up to 10 nucleotides [28]. Then they recruits a complex, comprising MutL homolog 1 (MLH1) and its binding partners, post-meiotic segregation increased protein 1or 2 (PMS1 or PMS2), MSH and MLH complexes form sliding clamp that moves along the DNA sequence till it encounters a single-strand DNA gap, replication Protein A (RPA) functions as indication at the damage site. Then MutL complex encounters the cluster, it allows a DNA exonuclease (Exo1) to enter the DNA structure, guided by the MLH:MSH complex, Exo1 removes the damage portion plus some more nucleotides and a DNA polymerase, Pol δ synthesizes DNA in those loci of excision. Finally, Ligase I joins the new DNA to the existing daughter strand [29].

Double-strand breaks (DSBs) are the most serious, hazardous, very complex to repair, they are rare but there are instances of its occurrences; most common ones are due to breaks in replication forks when polymerases stall at the site of unrepaired base lesions and by exogenous agents like ionizing radiations, etc. Anticancer treatments comprising of chemotherapeutics and radiation therapy can also induce multiple kinds of double-stranded DNA damage [30].

DSB repair faces many challenges such as loss of physical integrity on both strands due to which there is loss of information, like when one stand is damaged one can retrieve information from the other strand but if there is a double strand break there is no template for synthesis of the new strand, to repair such damage, human cells employ two main pathways: nonhomologous end joining (NHEJ) and homologous recombination repair (HR). Cell cycle checkpoints evaluate end processing required, which partially dictates how DSBs would be repaired [31]. NHEJ operate during any cell cycle phase but is most active in G0 and G1 (before DNA replication), whereas HR is active during S and G2 phases (after replication), its prone to error whereas HR is template based therefore its fidelity is higher and this makes the process a little complicated. Damage sensor complex is common to both NHEJ and HRR is Mre11-Rad50-Nbs1 (MRN). MRN's functionality is only one of many mysteries of DSB repair, there are still a lot to be discovered regarding HR and NHEJ. The following paragraphs summarize the main HR and NHEJ pathways [22, 32].

Nonhomologous end joining (NHEJ) rejoins DSB ends without a template, it does not search for or use a large segment of DNA for determining which bases were present before the damage occurred and search which is the other end of the breakage. Therefore, repair proceed quickly with the potential for loss of nucleotides from either side of the DSB junction, alteration of base pair sequences at

the breakpoint or getting attached to some other end that does not belong to the sequence in case of multiple breaks. Thus, NHEJ can contribute to a large amount of mutation but it is preferred by the cell [33]. The main challenge of NHEJ is to collect the two free ends into immediate proximity and protect them from nucleolytic attack. The Ku heterodimer, a damage sensor and a lyase imparts protection and recruit other proteins for end processing. Then DNA-dependent protein kinase catalytic subunit (DNA-PKcs) binds to Ku and becomes DNA-PK, a docking port for various kinds of DNA end processing enzyme. Another complex XRCC4 + Ligase IV + XLF create a filament to bridge the ends. Ligase IV ligate across gaps and join processed DNA ends. Many aspects of NHEJ still remain a mystery, including whether its steps are sequential, iterative or flexible according to the complexity of the damage. The most studied form of NHEJ is V(D)J recombination, which occurs only in T and B cells and is essential for their development, maturation and generating lymphocyte diversity [34].

Homologous recombination repair (HR) is a complex template directed DSB repair mechanism, it gets activated after DNA is copied in S phase but before it divides in M phase, so that the two strands are still together held by cohesion complex and HR takes advantage of the other full copy of adjacent DNA. This enables HR to find a large area of homology on the sister chromatid and use it as a template to reconstruct the damaged DNA strand [35]. HR plays significant role in maintaining genomic stability, in the basic step, MRN forms single-stranded DNA at the DSB end, ssDNA extends beyond the original breakpoint enabling Rad51 to attach to 3′ end, RPA binds to the naked stretch of DNA so that Rad51 can sit on the ssDNA and find DNA sequences like the 3′ overhang. When Rad51 encounters the locus with homology it invades the double strand, creating a DNA heteroduplex. Rad51 facilitates exchange of homologous DNA sequences within the sister chromatid. The overhang progressively extends as new nucleotides are generated beyond the original breakpoint, Nbs1 recruits other repair proteins to the site, Rad50 serves as a tether; MRE11 possesses both exo- and endonuclease functions [35]. During synthesis, as the loop is pushed, an X-shaped structure develops, called a Holliday junction, at the end this Holliday junction is resolved and ends are ligated Many HR genes like BRCA1, BRCA2 are involved in genomic instability generation and cancer [36]. Above mentioned facts have lightened up the understanding that cell have a very elaborately designed DNA repair system for damages now let us see how cancer wangle it through various measures.

4. Mutations in DNA repair genes in cancer

DNA, the genetic component of a cell, often gets damaged when exposed to any endogenous or exogenous agent like radiation, smoke, macrophages, ROS, etc. Different DNA repair pathways like base repair, mismatch excision repair, homologous recombination etc. help in repairing these DNA damages. But the expression of genes involved in these repair pathways sometimes gets reduced due to germline mutation, epigenetic alterations, somatic mutation, etc. As a result, the unrepaired DNA damage accumulates in cells. This accumulation might lead to further increase in epigenetic or somatic alteration, which helps in multiplying the altered field defects as well as different driver mutations that ultimately helps in the progression of cancer [22]. Details of few alterations affecting DNA repair genes are mentioned below.

4.1 Germline mutation associated with DNA repair pathways

Germline mutations of DNA repair pathways usually results in predisposing ones to cancer or having it by birth itself if abnormal gene is inherited from one

of the parents and other gene copy gets inactivated in a somatic cell later in life or both copies of a gene can get deactivated. Ultimately, that results in loss of heterozygosity which leads to a deficient response of DNA repair genes. Such affect from mutations are also seen if it is present in tumor suppressor cells [37].

Genes involved in the mismatch repair like MLH1, MSH6, MHS1 are often associated with Lynch syndrome, hereditary non-polyposis colorectal cancer (HNPCC), is the most common cause of hereditary colorectal (colon) cancer when undergoes monoallelic mutation but are associated with Constitutional mismatch repair deficiency syndrome due to biallelic mutation across children and adolescent [38].

Genes involved in homologous recombination like BRCA1/2 and BRIP1 are associated with hereditary breast and ovarian cancer syndrome when undergoes biallelic mutation. Defect in these two genes often results in breast cancer [38].

4.2 Somatic mutational signatures are associated with DNA repair pathways in cancer

Somatic mutations are basic to cancer and finding their occurrence on DNA repair genes is expected, researchers have well-established effect and consequences from a few of them. The substitution mutation signatures with homologous recombination genes like BRCA leads to homologous recombination failure in breast cancer, other than that promoter methylation also contributes to BRCA gene defects in different tumors [38]. Apart from substitution, rearrangement signatures are also involved in BRCA of breast cancer, these signatures include tandem duplication, inversion, deletion, translocation, etc. For example, tandem duplication is associated with the BRCA1 mutation, whereas small deletions are associated with BRCA1/2 inactivation, indel rearrangement signature is associated with BRCA deficiency [39].

Unlike homologous recombination repair, substitution mutation signatures are also associated with the Mismatch excision repair system. For example, high rates of substitution and indels are seen in C.G to T.A transition at NpCpG sequences. Also, rearrangement signatures are associated with MMR genes, which are often found in breast cancer [39].

High expression of *XRCC-1,* interacts with DNA ligase III, polymerase beta and poly (ADP-ribose) polymerase is associated with early tumor stage in oral squamous cell carcinoma. Accumulation of single strand breaks downregulates protein APE1 responsible for DNA incision during BER helping the conversion of single strand breaks to double stranded ones [40].

Overexpression of *ERCC1* in prostate cancer has association with the formation of chromosome aberrations. It is shown to inhibit apoptosis in esophageal squamous cell carcinoma. Some of its polymorphisms also indicates prognostic markers [41].

5. Epigenetic alterations associated with DNA repair pathways

Likewise mentioned earlier cancer applies several methods to take control over repair mechanism, epigenetic alteration is another such technique, here the DNA sequence remains intact, but expression and activity of the gene is affected. It's the technique due to which we have different type of cells in our body despite having the same genetic makeup and most importantly it's hereditary. Epigenetics include covalent modifications like methylation, ubiquitylation, sumoylation, phosphorylation to histones or DNA, sRNA, miRNA [41]. The following information

is on promoter methylation, miRNAs and chromosome remodeling by histone modification.

5.1 Epigenetic alterations by promoter methylation

One of the most common ways of epigenetic alteration is by promoter methylation, this is often regulated by cytosine methyl transferases, genes get inactivated by methylation in 5-carbon of cytosine of 5′-CpG-3′ dinucleotide sequence at either promoter regions. Two of the genes involved in base excision repair namely Methyl-CpG Binding Domain 4, DNA (MBD4) and Thymine-DNA glycosylase (TDG), both are glycosylases with same function of removing mismatches by hydrolyzing carbon-nitrogen linkage between the sugar and the phosphate backbone of DNA and mis paired thymine. Due to promoter methylation in these two genes, it is found that BER pathway often gets suppressed in cancers like colorectal, myeloma, ovarian, etc. [41].

The *XPC* gene which encodes a protein that is a key component of the XPC complex involved in GG-NER, promoter methylation of this gene often leads to NER function loss in cancers like bladder cancer. Other genes of the nucleotide excision repair pathway, RAD23A and ERCC1 genes also get inactivated by promoter methylation in different cancers like RAD23A in multiple myeloma cancers and ERCC1 in glioma cancer. Genes of the mismatch repair pathway namely MLH1, MSH2, MSH3, and MSH6 also gets suppressed by promoter methylation in various cancers like ovarian, gastric, etc. [41, 42].

Two genes BRCA1 and BRCA2 of homologous recombination system also have compromised activity by promoter methylation in different cancers like breast, gastric, uterine, etc. One of the genes, involved in non-homologous end-joining, XRCC5 encodes the heterodimer Ku (composed of K70/K80), which facilitates binding to nascent DNA breaks often gets epigenetically inactivated by promoter methylation is seen to be associated with cancers like adenocarcinomas [43].

Direct reversal of DNA damage is the most energy efficient repair system, but its capabilities encompasses only certain damage categories such as pyrimidine dimers formed by UV radiation, O6 adducts like alkyl groups on nucleotides from chemotherapy. *O*6-methylguanine-DNA methyltransferase (MGMT), catalyzes transfer of methyl groups on DNA to its own molecule, methylation on its promoter inactivates it like the other DNA repair genes, this is often associated with cancers like glioblastomas, colon cancer, lung cancer, lymphoma etc. [44].

5.2 Epigenetic alterations due to chromosome remodeling and histone modification

The miRNA is synthesized as primary non-coding RNA these are then processed into mature effective ones which can alter expression of its target genes. On those target genes it influences the methylation status in the promoters and we know that the methylation status of promoters are related to their expression levels or they can directly target epigenetic factors, such as DNA methyltransferases or histone deacetylases, regulating chromatin structure for altered expression. Some genes of mismatch excision repair like MLH1, MSH2, and MSH6 are inactivated by such process, by the action of miR-155 (**Table 1**) [45].

Low expression of miRNA-15 suppresses promoter activity of BRCA1 by recruiting an enhanceosome mediated by HMGA1 [46]. miRNA-16 influence transcriptional activation of HMGA2 protein that again suppress ERCC1, is required for the repair of DNA lesions such as those induced by UV light or formed by electrophilic compounds including cisplatin. HMGA (High Mobility Group proteins with AT

DNA Repair Pathways	DNA mutation (Germline)	DNA mutation (Somatic)	Epigenetic Changes	References
BER		*APE1,XRCC-1*	MBD4, TDG	[40, 41]
NER		*ERCC1*	XPC, RAD23A, ERCC1	[41, 42, 47]
MMR	MLH1, MSH6, MHS1	MSH2, MSH6	MLH1, MSH2, MSH3, MSH6	[38, 39, 45]
Homologous Recombination	BRCA1/2, BRIP1	BRCA1/2, PALB2	BRCA1, BRCA2, HMGA1	[38, 39, 43]
NHEJ			XRCC5	[43]
Direct Reversal DNA Damage			MGMT	[44]

Table 1.
DNA repair pathway genes affected in various ways in cancer.

hook) code for a chromatin-associated protein that can modulate transcription by altering the chromatin architecture, HMGA1 and HMGA2 are two of its types [47].

As mentioned above that cancer cells put up several techniques to ensure faulty DNA repair system in the cell. The faulty DNA repair system now provides cancer cells with ability to produce more and more mutation. This higher rate of mutation gives the cancer cell advantage to manipulate cell machinery for uncontrolled growth. After choreographing the regulation of DNA repair system cancer then effects its fidelity, for the purpose it brings into the picture clustered mutation, which is a specific characteristic of cancer and cancer cells owe it to faulty DNA repair systems. An elaborate analysis of the clustered somatic mutations can identify error-prone DNA repair mechanism as a common source of mutations in active chromatin in human tumors [48].

6. DNA repair and clustered mutation in cancer

Clustered mutations, as the term suggest is localized hypermutation. There are three of its types namely- chromoplexy, chromothripsis, kataegis.

Chromoplexy refers to a class of complex DNA rearrangement observed in active regions of the genomes of cancer cell. The mechanism underlying complex rearrangements has not been established. But a proposed model says in the process DNA is brought together by the transcription factor working in a co regulated manner on different genes [49]. The process makes DNA in those places vulnerable to breakage and malfunctioning of DNA repair system make a jumbled-up repair of those broken pieces. Although this model has not been established but it's taken into account because chromoplexy is prevalent in only areas where there is active transcription and it can explain how DNA from multiple chromosomes may participate in a single chromoplexy event [50].

Chromothripsis is another such mutational process in which a number of chromosomal rearrangementsoccur in localized genomic regions in one or a few chromosomes together. The process takes place in a single event, where in the genomic space arise several double strand breaks. These breaks are then again joined by DNA repair system in a non-homologous manner. Once again, the crucial role of DNA repair system in the process of cancer survival and evolution is entrenched [51].

Kataegis mutational clusters are several hundred base pairs long, alternating between a long range of C → T and G → A substitutional pattern. This says it takes place in one of the two template strands during replication. It is more common than chromoplexy and chromothripsis. Kataegis hypothesis includes mismatch repair to activate and repair on locations of mismatch, making those regions single stranded and these single stranded regions are substrate to various modifying enzymes. These modifying enzymes then promote formation of mutation clusters along the entire track of breakage [52].

7. Evidence of clustered mutation influencing repair pathways

APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) enzymes and translesional DNA synthesizing enzyme are found to be associated with these events. There is literature available on this context. APOBEC enzymes are cytidine deaminase that is responsible for C → T transitions [53, 54]. H3K36me3 chromatin is normally protected from such somatic mutations, it is tri-methylation at the 36th lysine residue of the histone H3 protein, it relishes this protection from somatic mutation because of increased activity in canonical mismatch repair machinery at its locations. However, exposure to some carcinogens results in increased activity of a non-canonical, error-prone, mismatch repair pathway involving (POLH) DNA polymerase eta, which results in a relative increased mutation rate in H3K36me3-marked regions. This explains that some factors act as carcinogens not because they increase the mutation rate but because they relocate mutations to the more important regions of the genome. These environmental factors include alcohol, ionizing radiations, UV radiations etc. [6].

There are other evidence stating clustered mutations are driven by break induced replication (BIR) like mechanisms, which is associated with homologous recombination. Tremendous progress in whole genome analysis revealed that BIR is likely the mechanism of multiple genomic rearrangements in humans that give clustered mutation. To the date, there is no clear understanding of how BIR transforms from a beneficial pathway aimed at rescuing cells into a dangerous mechanism with high destabilizing potential [55].

These events are very common to cancer cells. They serve as source for catastrophically higher rate of mutational events giving rise to sustainable amount of genomic instability. And as mentioned several times before genomic instability is the prime mechanism for the cancer cell to hold control over cellular machinery for uncontrolled division these events are very specific to cancer cells and a proper process of these events has not yet been elucidated [51]. But we can clearly see the potential role of DNA repair systems in these clustered mutational events. Through clustered mutations the cancer cell tries to exhaust DNA repair pathways. Repair pathways are meant to repair the DNA at a specific rate, and they are designed to tackle a limited burden. When mutation rate become overwhelming for them, their fidelity exhaust and that is the opportunity cancer cells create to accumulate mutation [53].

Cancer cells first changes the expression and regulation of the DNA repair systems by either epigenetic modifications, mutating its coding sequence or regulatory sequence. This in turn gives error prone DNA repair system for clustered mutation. Again, the clustered mutation also exhausts the DNA repair systems leaving no chance for fixing the genomic instability taking place in the cell. There still lies a debate on how these catastrophic mutational processes occur. But there is proof that they are indebted to faulty repair systems for their birth [54].

8. Cancer therapy targeting DNA repair pathways

Presently there are a few chemotherapeutic drugs and some of them are even in phase 2 or phase 3 trials. This itself emphasize the crucial role DNA repair pathways and proves that it is an important chemotherapeutic target. Basically, DNA repair inhibitors are used as chemotherapeutic drug they make already fragile DNA repair system of cancer to collapse leading to destruction of cellular homeostasis ultimately leading to cancer death [13, 26].

8.1 MGMT inhibition

The *O*6-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein that removes alkyl group, was the target of the earliest attempt to develop a DNA repair inhibitor. MGMT is the most widely studied DNA repair mechanism [56].

In 1970s, nitrosoureas was introduced as a chemotherapeutic drug for glioblastoma and other malignant gliomas, it alkylates DNA at various positions on guanine, subsequently causing single- or double-strand damage which chemosensitiz cells to more damage by other drugs. Scientists quickly learned that something could reverse the DNA damage that they inflicted, that was MGMT. After some time, a potent MGMT inhibitor was used along with nitrosoureas but it did not work [26]. Although compromising MGMT fell short of expectations in chemo sensitizing tumors to alkylating agents, it was continued to be studied. There was evidence that in different cancer it is manipulated in different ways. But still there is not any effective drug involving this [57].

8.2 PARP inhibitors

The PARP is a nucleus specific enzyme that detects single strand breaks that are being formed spontaneously or during BER and binds to that position on the DNA strand. It then undergoes a structural change and begin synthesizing a polymeric adenosine diphosphate ribose (poly ADP-ribose) chain, which acts as signal for the other DNA-repairing enzymes. Three members of that family have roles in DNA repair, with PARP1 being the most important. It took a lot of time for PARP to be recognized as target for chemotherapeutic drug. First PARP inhibitor (PARPi) entered clinical trials, as a chemosensitizer like MGMT inhibitors. But its capacity as single agents to treat BRCA-deficient cell lines from germline breast cancers proved later. Olaparib was the first PARP inhibitor for ovarian cancer. Today, there are number of PARP inhibitors in clinical trials for not only breast cancer but also for other cancer types [58]. PARP's clinical efficacy on BRCA-deficient tumors is one of the most effective drug findings. PARPi function includes binding to PARP and inhibiting its function until next round of DNA replication, then accumulation of unrepaired SSBs will automatically get converted to DSB. Cells that are missing both alleles of BRCA 1, BRCA2 or PALB2 have no efficient HR functionality, which leaves repairs in the hands of NHEJ, its limited ability to repair extensive DSB damage leads to tumor cell death specifically because the cells with non-compromised HR can tackle these breaks very easily (**Figure 3**) [26]. That is why these are used as add on for effective cancer treatment. However, the effect of PARP inhibition is not as simple as it seems, there is lot more complexity to it like PARP's interactions with other proteins and PARP trapping [59].

PARP not only works with BER, but it also activates XRCC in HR pathway and is involved in a regulatory feedback loop with BRCA1. It also appears to inhibit the NHEJ pathway by inactivating DNA-PKcs and ATM's checkpoint activity. Moreover, it has a role in inflammation that proves its involvement in transcriptional regulation and many other biological functions associated to cancer. As mentioned earlier,

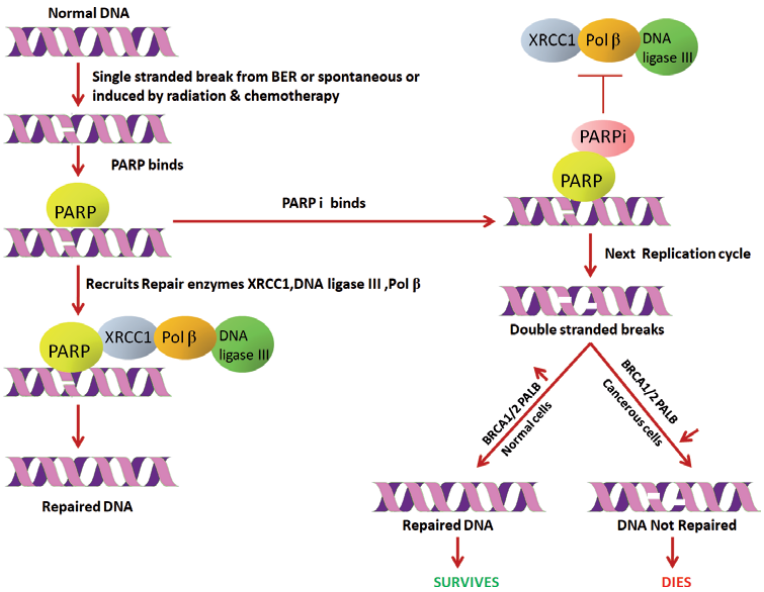

Figure 3.
Mechanism of action of PARP inhibitors.

cancers are notoriously clever when it comes to combat their survival, then they come up with new methods to the stress imparted to it by PARP inhibitors. Till date a lot of instances have been proved such as reverse mutation in BRCA, various ways of manipulating NHEJ, etc. [60].

9. Conclusion

Defects in the repair system assure genomic instability, this fuels disorderliness required for cancer to survive, sustain and evolve; that is why hereditary deficiencies in them makes the individual more susceptible to cancer. There are only some repair genes known to be exploited by cancer, a more extensive search of potential points might give a perspicuous picture. Researchers has been into understanding and finding cure to cancer since decades; but still till date we do not have a conclusion. This refers to its multiple techniques, different hierarchical steps and several process that it applies for its successful survival. DNA repair system is one of its basic targets, so cancer wangle it very well to establish its existence. It applies different mechanism from simple mutations to clustered mutations to various epigenetic changes just to assure a compromised repair system. A very elaborate venture of these changes can give us insight into generation of genomic instability by suppressing DNA repair in cancer. This information can help us get the much-sought effective treatment. Therapies targeting DNA repair genes already available are example to this.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 10

The Striatal DNA Damage and Neurodegenerations

Huifangjie Li and Jinbin Xu

Abstract

Reactive oxygen species (ROS) are produced during normal metabolic reactions in living cells. ROS causes oxidative damage to many types of biomolecules. An age-related increase in oxidative damage to DNA and RNA has been described in the human neurons, which play a vital role in the progression of age-associated neurodegeneration. As dopamine metabolism is believed to be the primary source of ROS, oxidative insults correlate with dopamine levels in the striatum during the progression of neurodegenerative diseases. Parallel changes in dopamine concentrations and vesicular monoamine transporter 2 (VMAT2) binding densities in the striatum were observed. Besides Fenton oxidation taking place, the packing of cytosolic dopamine into synaptic vesicles by VMAT2 inhibits its autoxidation and subsequent decay of dopaminergic neurons. The female bias in the DNA damage in the late-stage Parkinson disease (PD) patients suggests that the sex-determining region of the Y chromosome (SRY) genes are critically involved. ROS are involved in regulating the rate of the aging procession in healthy cohorts and an increased life span of patients with neurodegenerative diseases via stimulation of protective stress responses. Moreover, the DNA repair pathway's mechanism, as genetic modifiers determine the age at onset through a ROS-inducing mutation.

Keywords: DNA damage, striatum, neurodegenerative diseases, dopamine, sex, age

1. Introduction

Oxidative damage can come from harmful environments such as chemical agents and ionizing radiation, but the major oxidative damage is also caused by internally sourced reactive oxygen species (ROS) generated from the natural metabolic processes in living cells. As the brain has a relatively higher oxygen demand and lower levels of antioxidants than other organs, ROS generates mainly DNA damage in the brain [1]. Numerous studies show that the accumulation of neuronal DNA damage contributes to the progress of aging [2]. DNA bases frequently undergo lesions through modification by alkylation, oxidation, and deamination [3]. To protect against these destructive adducts, cells have developed an antioxidant defense system to be expressed by enzymes involved in base excision repair (BER). The imbalance between clearance and generation of ROS plays a critical role in disease pathogenesis. Except for healthy aging, insufficient DNA repair has been tightly associated with neurodegenerative disorders such as Alzheimer disease (AD), Parkinson disease (PD), and amyotrophic

lateral sclerosis (ALS) [4–9]. The elevated DNA strand breaks and the decreased DNA double-strand breaks (DSBs) repair proteins have been described in AD brains.

Additionally, the increase in β-amyloid (Aβ) and neurofibrillary tangles (NFTs) is closely linked to decreased oxidative damage—an early event in AD that decreases with disease progression [10]. What is more, the lesions to mitochondrial, a major source of ROS, have been reported in the PD cases, and mitochondrial dysfunctions have been associated with the disease pathophysiology. Historically, the first investigation involving mitochondria in PD relates to the observation that the presence of an impairment of complex I in the different forms of PD and Parkinsonism [11]. Dementia with Lewy bodies (DLB), Parkinson disease dementia (PDD), and PD have been aggregated conceptually as Lewy body disease (LBD) [12].

Striatal dopaminergic dysfunction probably is involved in both AD and LBD, while degeneration of nigrostriatal dopaminergic neurons is the classic pathology of PD; striatal dopaminergic dysfunction may also promote the motor manifestations of AD. The striatum consists of several subregions—caudate and putamen. The caudate nucleus is essential in many behaviors, including procedural learning and working memory; the dorsal posterior putamen receives its primary input from the motor and sensorimotor cortices and regulates the motor circuits [13–15]. Dopamine generates hydroxyl radical (•OH) through Fenton reactions in the presence of iron, which is believed to be responsible for the oxidative damage to lipids, proteins, and DNA in living cells and dopaminergic neurons [16]. Besides, as a chelator, dopamine can form different complexes with Fe(II) and Fe(III), decreasing catalytic productions of ROS [17]. Dopamine compartmentalization has been described by the vesicular monoamine transporter 2 (VMAT2)—correlates with dopaminergic neurons' vulnerability in Parkinsonism neurodegeneration [18]. There are close interactions among oxidative damage and dopamine concentration, and the antioxidant role of VMAT2 should be given more attention. Oxidative stress induced by genetics has been linked to the Y-chromosome gene products that modulate dopamine biosynthesis and motor function [19]. Further, DNA damage is associated with acceleration of the rate of aging, causing a variety of early symptoms such as gray hair, kidney disease, cataracts, osteoporosis, and neuronal atrophy [20]—factors which determine the health or disease people's life span and age at the onset of diseases.

Therefore, it is clear that there is an appreciable need for a better understanding of the correlations between oxidative damage and neurodegenerations. In this chapter, the striatal DNA damage was first focused, and its brain region concentrations in neurodegenerative diseases will be discussed with parallel changes of dopamine levels and VMAT2 densities. Moreover, original data on the association among striatal DNA damages, sex, life span, and the age of onset of diseases in neurodegenerative patients will be presented.

2. Oxidative damage of DNA in the striatum from patients with neurodegenerative diseases

It is widely recognized that 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) and 8-oxo-7,8-dihydroguanosine (8-oxo-G) may act as biomarkers of oxidative damage to DNA and RNA, respectively [1]. Studies by Li et al. have reported the levels of DNA adducts in the caudate and putamen of the disease groups and age-matched controls [21]. Compared to controls, remarkable reductions in DNA

oxidation adducts were observed in the caudate of PD and DLB brains, including males and females. However, in the caudate of AD brains, these levels were elevated. This finding was especially pronounced for male AD patients, as adduct levels were 36% elevated compared to controls (**Figure 1**). The concentrations of 8-oxo-dG in the putamen of the disease groups were similar to the controls. Comparing between caudate and putamen, there were impressive elevations in adduct levels in the caudate, especially for the AD brains. These data indicate that the caudate is more vulnerable to DNA damage than the putamen in advanced AD patients.

RNA bases are more exposed and vulnerable to oxidative damage than DNA as they are not protected by hydrogen bonding and specific proteins. RNA oxidation has been described as a "steady-state" marker of oxidative lesions [22]; however, DNA oxidation has been believed to be a historical marker of oxidative damage during disease pathogenesis and aging progression [23]. Increased 8-OHdG levels have been documented in PD patients [24–26], but as shown in **Figure 1**, a noticeable reduction of DNA oxidation adducts in the caudate was observed in the late-stage LBD brains. It was not unique; the urinary concentration of 8-OHdG in the MFB 6-hydroxydopamine lesion model started to elevate at day 3 with a significant increase to day 7 and gradually back to baseline at day 42 [27]. The increased 8-oxo-dG levels in the caudate of AD brains connected with the increase in dopamine levels of the same cases. These phenomena are most likely due to the Fenton reactions taking place—in response to dopamine release and dopamine compartmentalization by VMAT2 [28].

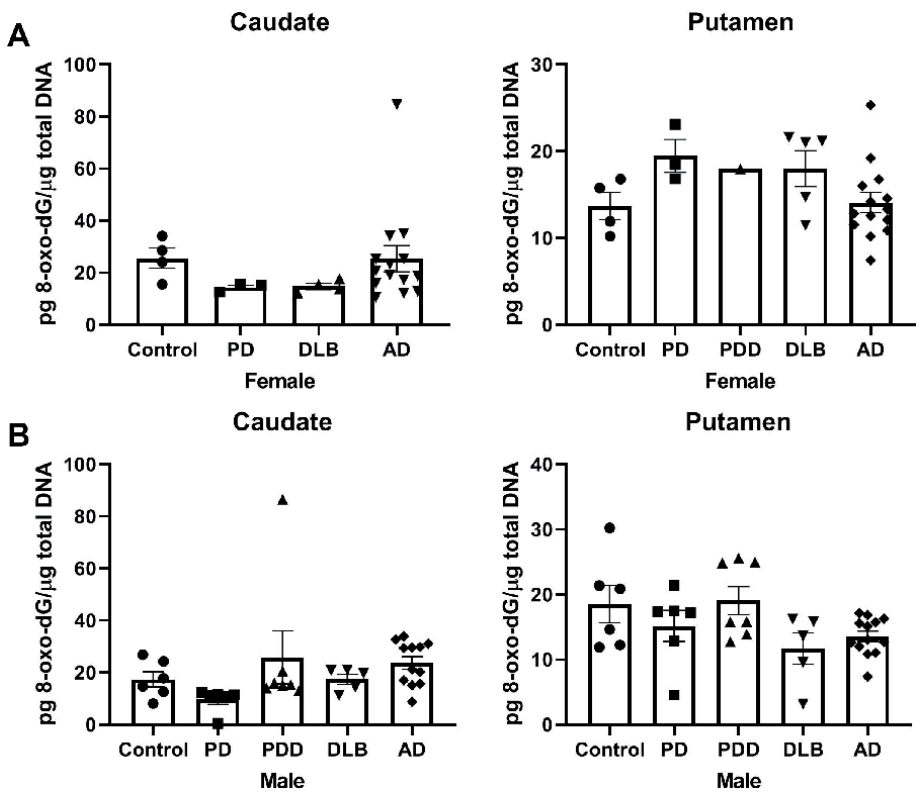


Figure 1.
8-oxo-dG levels in the caudate and putamen of patients with diseases (PD: n = 10, PDD: n = 7, DLB: n = 10, and AD: n = 26) and age-matched controls (n = 10). Values shown are means ± SEM as the concentration of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) (pg) per total DNA (µg). (a) Female and (b) male.

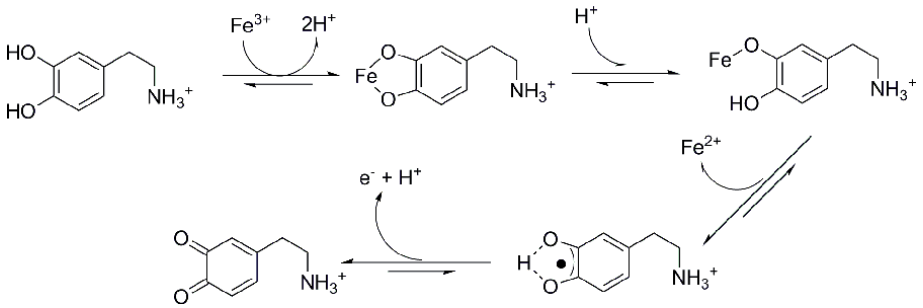
3. Interactions between oxidative damage and dopamine in the striatum of patients with neurodegenerative diseases

There are three biologically critical free radicals in our body, $O_2^{\bullet -}$, •OH, and $NO^{\bullet}$, mainly produced through Fenton oxidative reaction. Fenton reactions are catalytic oxidation reactions starting with transition metal ions, either iron or copper, and yielding both the hydroxyl radical (•OH) and higher oxidation states of the iron [29].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \quad (1)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\bullet}OOH + H^{+} \quad (2)$$

The relatively large amount of hydroxyl radical attacks adjacent to mitochondrial DNA strands and cytoplasmic RNA single-strands consequently produce an amount of oxidative adducts [29]. It is also critical to note that dopamine is metabolized enzymatically to produce a mass of H_2O_2 and, ultimately, dihydroxyphenylacetate, conversely promoting the dopaminergic exposure neurons to oxidative lesions. Put it another way, dopamine and related catechol are vulnerable molecules that can oxidize in the presence of transition metals to yield O_2^{-}, playing an essential role in nucleic oxidation as a major ROS source. O_2^{-}, a product of catechol autoxidation, can reversely oxidize catechol [30]. Either spontaneously or enzymatically, O_2^{-} can yield H_2O_2 and then •OH in the presence of transition metals [11]. The imbalance between clearance and generation of ROS promotes progressive dysfunction or increased death of dopaminergic neurons. Also, dopamine affects as a good metal chelator and electron donor and is capable of capturing iron and manganese [17]. Fe^{3+} has been reported as a catalyst for autoxidation of dopamine via the following mechanism [31]:



This mechanism can be proved further by a significant negative correlation between dopamine levels and 8-oxo-dG levels in the caudate of AD patients (**Figure 2**). Combined with a significant negative correlation between 8-oxo-dG levels and VMAT2 density in the same brain area from AD cases (**Figure 3**), these results are most likely owning to Fenton oxidation reactions taking place in the caudate from AD brains, which was believed to be a response to dopamine concentration and dopamine compartmentalization by VMAT2. As shown in **Figure 2**, dopamine concentrations in the caudate and putamen of disease patients and controls did not significantly differ. However, there were trends of decreasing and increasing dopamine levels in the LBD and AD patients, especially for the female cohorts.

PD has been described to be associated with both increased levels of nigral iron—a catalytic agent for yielding •OH—and enhanced Mn superoxide dismutase activity. As the midbrain levels of reduced glutathione were diminished, there was evidence of increased oxidative damage in the midbrain of PD patients, including not only lipid peroxidation, protein oxidation, oxidation of DNA, but also catechol oxidation in the same brain area of PD cases [11].

As shown in **Figure 3**, similar changes in VMAT2 density in the caudate and putamen are in line with the 8-oxo-dG and dopamine levels of the same cohorts. Compared to the controls, lower VMAT2 binding levels were found in the caudate from both female and male LBD cases. Diversely, a significant increase in VMAT2 density in female and male AD patients was observed. We can see significant negative correlations between 8-oxo-dG levels and VMAT2 density in the caudate (r_s = −0.451, p = 0.027) and putamen (r_s = −0.516, p = 0.024) of AD patients, as well as in the caudate (r_s = −0.683, p = 0.042) of PD patients. It might give

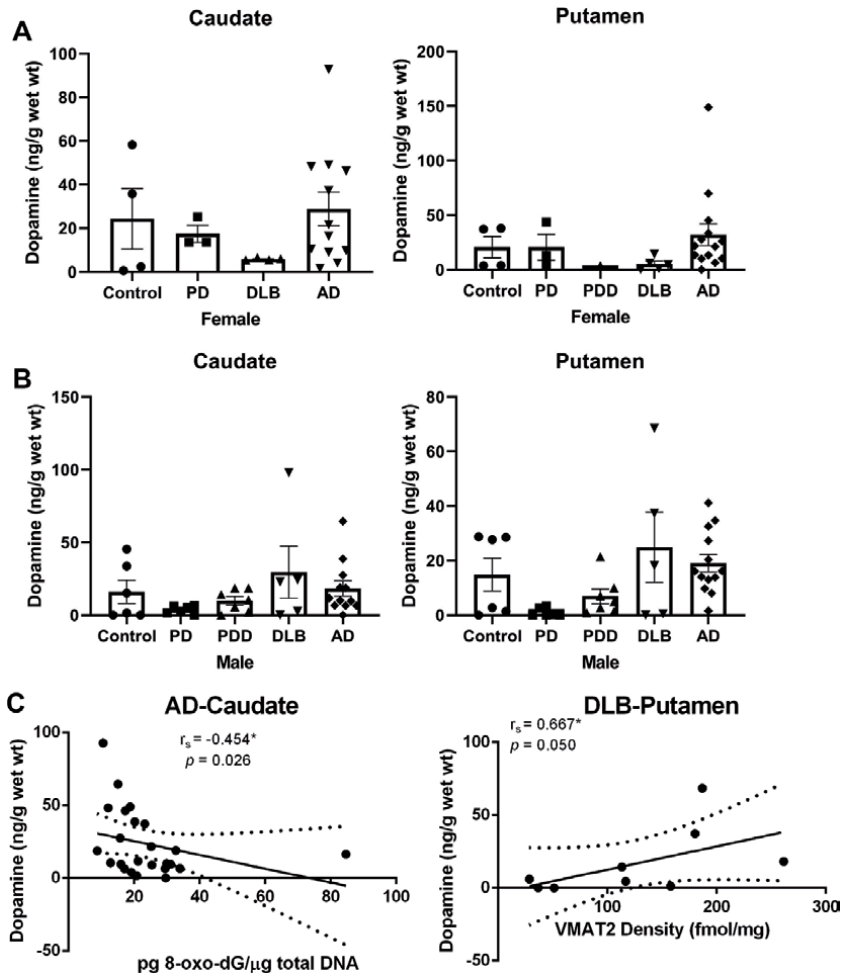


Figure 2.
Concentration of dopamine in the caudate and putamen from patients with diseases (PD: n = 10, PDD: n = 7, DLB: n = 10, and AD: n = 26) and age-matched controls (n = 10). (A) Female and (B) male. The values shown are means ± SEM. (C) Concentration of dopamine versus level of 8-oxo-dG in the caudate from diseases brains, significant association was observed only in the AD group (p = 0.026); concentration of dopamine versus VMAT2 expression in the putamen from diseases brains, significant association was observed only in DLB group (p = 0.050). r_s, the Spearman's rank correlation coefficient.

evidence that the reduction of vesicular storage increased dopamine release and then was conductive to produce hydrogen peroxide from MAO-catalyzed dopamine metabolism [32].

To better understand the correlations between oxidative damage and dopamine storage abilities, the spatial control of dopamine by VMAT2 and the antioxidation role of VMAT2 should be further elucidated. Bearing in mind the portrayal of oxidative lesions in the pathogenesis of PD, packing of cytosolic dopamine into synaptic vesicles by VMAT2 inhibits its autoxidation and the subsequent degeneration of dopaminergic neurons [33]. This theory conforms to the negative correlations observed between oxidative damage and VMAT2 density in striatum of both AD and PD patients. Reduced dopamine levels attenuated its uptake and transport functions by changing dopamine turnover. Thus,

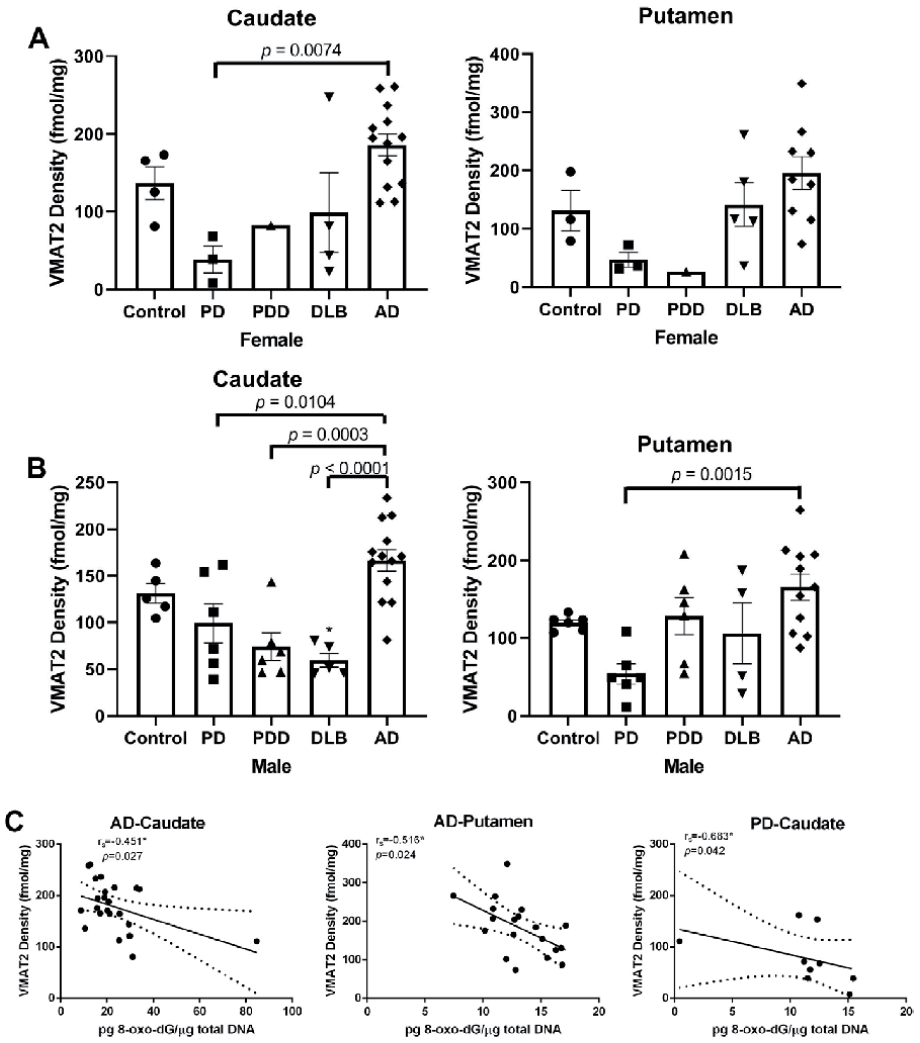


Figure 3.
*Quantitative autoradiographic analysis of VMAT2 density (fmol/mg) in the caudate and putamen from patients with diseases (PD: n = 10, PDD: n = 7, DLB: n = 10, and AD: n = 26) and age-matched controls (n = 10). (A) Female and (B) male. The values shown are means ± SEM. Statistical significance between two disease groups are indicated with brackets and corresponding p-values. A p value of <0.05 was considered significant: * indicates p < 0.05 versus the controls. (C) Density of VMAT2 as concentration of 8-oxo-dG in the caudate and putamen from AD brains (p = 0.027 and p = 0.024, respectively) as well as that in the caudate from PD brains (p = 0.042). Rs, the Spearman's rank correlation coefficient.*

VMAT2 expression correlates with the severity of Parkinsonism and cognitive impairment in DLB [18, 34]. The inhibition of dopamine metabolism by MAO-B attenuates hydrogen peroxide production, as a two-edged sword, it also increases the risk of dopamine autoxidation and subsequent augmentation of the cytosolic dopamine pool [32].

4. The interactions between oxidative damage in the striatum, sex, life span, and the age of onset of diseases in neurodegenerative patients

Many neurological diseases show significant sex differences in their susceptibility, severity, and progression [35, 36]. Specifically, a male bias has been found for disorders such as PD and attention-deficit hyperactivity disorder (ADHD), both of which are associated with abnormal levels of dopamine [37–39]. Considerable studies have supported the hypothesis that gonadal sex steroid hormones, especially estrogen, act as protectors in females by modulating dopamine release, metabolism, and dopamine receptors' activity. However, there is numerous evidence that genetic factors, especially sex-specific genes, influence either healthy or diseased dopamine systems [40–42].

As shown in **Figure 4**, Kendall's tau_b analysis revealed a significant positive correlation between sex and 8-oxo-dG levels in the caudate of PD cases. The result indicates that there is a sex difference concerning DNA damage in late-stage PD patients. Postmortem brain studies have revealed that the expression of PD-related genes in the substantia nigra pars compact (SNc), such as α-*synuclein* and *PINK-1*, is higher in men than women [43]. Sex-chromosome genes are critically involved, particularly the sex-determining region of the Y chromosome (*SRY*) gene [44]. The dopaminergic toxin, 6-hydroxydopamine (6-OHDA), has been described to significantly elevate *SRY* mRNA expression in human male dopamine cells, accompanied by an increase in the expression of GADD45γ, a DNA damage-inducible factor gene and a known *SRY* regulator. Interestingly, SRY upregulation initiated by dopamine cell damage is a protective response in males; however, the effect diminishes significantly with the gradual loss in dopamine cells [19].

DNA damage may be unique in its ability to promote multiple symptoms associated with old age. Exposure of rodents to ionizing radiation leads to the premature

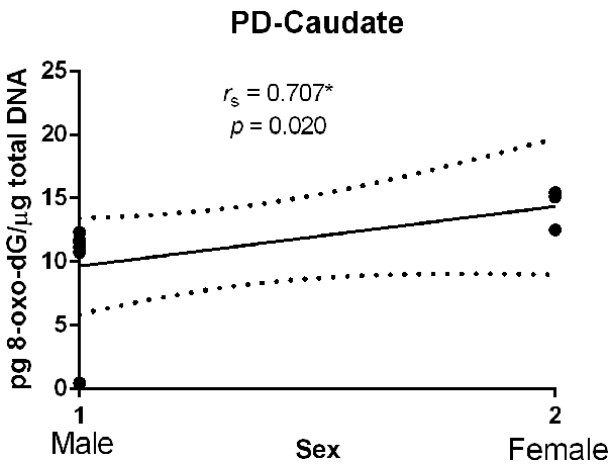


Figure 4.
Kendall's tau_b analysis of the correlation between sex and 8-oxo-dG levels in the caudate from PD brains (p = 0.020).

appearance of numerous histological features of healthy aging—gray hair, kidney disease, cataracts, osteoporosis, neuronal atrophy, and muscle atrophy. A classic mouse species survive a maximum life span of 2–4 years, whereas humans can live up to 122 years [45]. Body mass can account for approximately 60% of the mammalian life span variance, while another 40% is attributed to other factors. The mitochondrial electron transport chain yields superoxide, a reactive form of oxygen, which can damage proteins, lipids, and DNA. Superoxide generates immediately into hydrogen peroxide, promoting several forms of oxidative damage. Animals engineered to have reduced rates of oxidative lesions, make efforts to exhibit average life spans [46], which provides insights into the significant negative correlation between life span and 8-oxo-dG levels in the putamen of healthy aging groups (**Figure 5**). It seems conceivable that transcription-associated DNA damage is critically involved in the aging process of mammals.

The reverse is precisely the PDD cases, as shown in **Figure 6**, there is a significantly positive correlation between life span and 8-oxo-dG levels in the caudate of PDD patients. The result supporting a role for ROS in regulating the rate of aging was characterized as "at best equivocal" in a published comprehensive review of aging in the mouse [47], which can be explained as ROS increases life span by stimulating protective stress responses [48].

On the other side, quite a few repair enzymes recognize and remove many types of DNA damage from the genome, and failure of these mechanisms can lead to the accumulation of damage in the neurodegenerative diseases. Failure to repair DNA, in reverse, may cause the synthesis of defective proteins, which definitely will repair DNA less efficiently [49].

Genome-wide association studies (GWAS) of Huntington's disease (HD) have focused on genes associated with DNA damage repair mechanisms as modifiers of age at onset, defining an age-related mechanism shared in other hypotheses of neurodegeneration. Many ages at onset in neurodegenerative diseases are clarified to be caused by mutations in bona fide DNA repair factors—tyrosyl DNA-phosphodiesterase 1 (TDP1), aprataxin (APTX), and polynucleotide kinase/phosphatase (PNKP) [50]. Getting the picture of DNA repair defects in neurodegenerative diseases will shed light on why they affect the age at onset and the disease severity in HD.

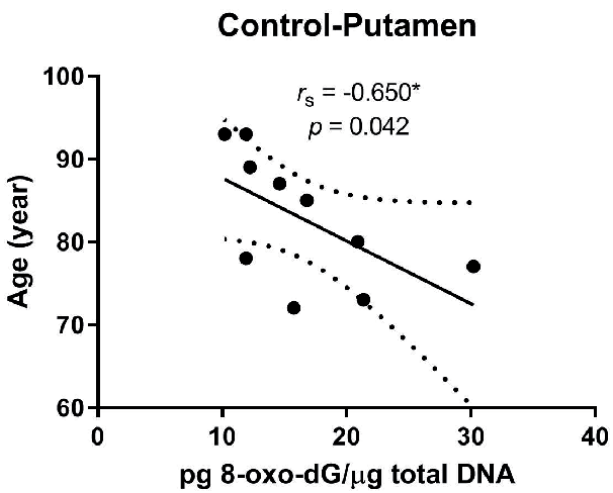


Figure 5.
Correlation between life span and 8-oxo-dG concentration in the putamen from the control brains (r_s = −0.650, p = 0.042). Rs, the Spearman's rank correlation coefficient.

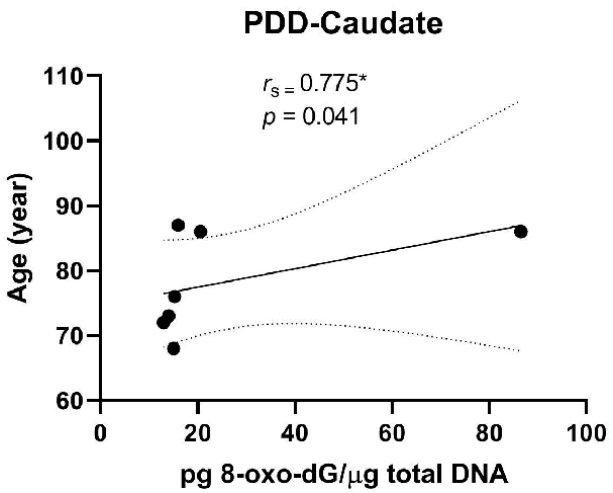


Figure 6.
Correlation between life span and 8-oxo-dG concentration in the caudate from the PDD brains (r_s = 0.775, p = 0.041). Rs, the Spearman's rank correlation coefficient.

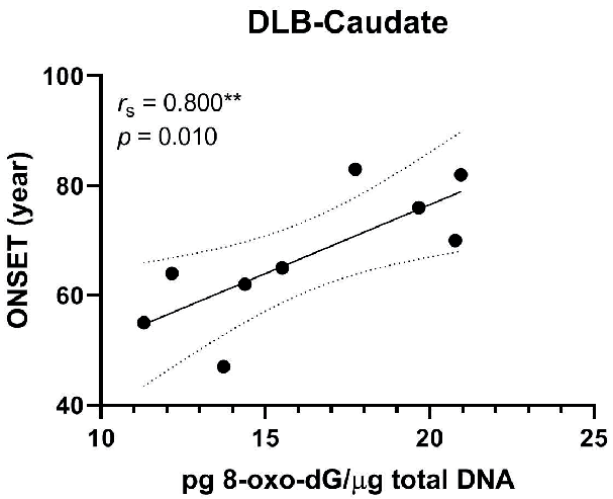


Figure 7.
Correlation between age at onset and 8-oxo-dG concentration in the caudate from the DLB brains (r_s = 0.800, p = 0.010). r_s, the Spearman's rank correlation coefficient.

An exomic sequencing study in a rare age-related ataxia oculomotor apraxia (AOA) identified mutations in the DNA repair scaffold gene *XRCC1*, the knock-out of *XRRCC1* resulted in hyper-PARlation, and genetic ablation of PARP1 prevent disease onset in an AOA-XRCC1 mouse model [51]. N6-furfuryladenine (N6FFA or kinetin)—a natural human metabolite of the DNA repairing ROS damaged adenosine—was protective against neurodegeneration in HD and PD models. The discovery of N6FFA efficacy in HD and PD models indicates a critical signaling pathway between DNA damage and mitochondria, where messed branches of this pathway may lead to different diseases in the brain, with similarities of late age onset [52]. As shown in **Figure 7**, the significantly positive correlation between age at onset and 8-oxo-dG levels in the caudate of the DLB patients, probably indicating that the DNA repair pathway, as genetic modifiers, determines the age at onset. 8-oxo-dG mainly promotes the transversion from GC to TA, GC to AT, or GC to CG, and this

is an important mechanism of ROS-induced mutation [53]. The study examined the DNA-repair capacities of basal cell carcinoma (BCC) skin cancer patients and revealed that the age at the first onset of BCC positively correlated with DNA repair, suggesting that the earlier the age of onset, the lower was their DNA repair [54].

5. Conclusion

DNA damage might progressively alter chromatin conformation and, thereby, gene expression types with age. Oxidative damage to nucleic acid is altered in midbrain structures of PD, DLB, and other neurodegenerative disease patients, consequently, inhibiting mitochondrial function. Mitochondrial dysfunction may play a vital role in the pathogenesis of neurodegeneration. What is more, there is a chicken and egg paradox in the studies trying to correlate neuronal degeneration with the signs of DNA damage.

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Conflict of interest

The authors declare no conflicts of interest.

Other declarations

The research in this chapter was approved by the Charles F. and Joanne Knight Alzheimer disease Research Center (Knight ADRC) and Movement Disorders Center (MDC) Leadership Committees (Ethics approval reference number: T1705).

Abbreviations

ROS	reactive oxygen species
8-oxo-dG	8-oxo-7,8-dihydro-2′-deoxyguanosine
8-oxo-G	8-oxo-7,8-dihydroguanosine
PD	Parkinson disease
DLB	dementia with Lewy bodies
AD	Alzheimer disease
VMAT2	vesicular monoamine transporter 2
SRY	sex-determining region of the Y chromosome
BER	base excision repair
ALS	amyotrophic lateral sclerosis
DSBs	double-strand breaks
Aβ	β-amyloid

NFTs	neurofibrillary tangles
PDD	Parkinson disease dementia
LBD	Lewy body disease
ADHD	attention-deficit hyperactivity disorder
SNc	substantia nigra pars compact
6-OHDA	6-hydroxydopamine
GWAS	genome-wide association studies
HD	Huntington's disease
TDP1	tyrosyl DNA-phosphodiesterase 1
APTX	aprataxin
PNKP	polynucleotide kinase/phosphatase
AOA	ataxia oculomotor apraxia
N6FFA or kinetin	N6-furfuryladenine

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Edited by Payam Behzadi

DNA is the most important biomolecule ever discovered. Indeed, this molecule bears genetic information from one generation to another. In this regard, DNA bases have a key role in transferring genetic information and data safely. However, there are cellular, genetic, and environmental factors that may damage the different parts of DNA molecules. These damages may result in mutations and cell death. As such, several DNA repair mechanisms have evolved. Over three sections, this book examines many of these mechanisms.

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