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Recent Advances in Noncoding RNAs

Edited by Lütfi Tutar

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Meet the editor

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Contents

Preface

Noncoding RNAs (ncRNAs), which play important roles in regulating gene expression in animals, plants, and various human diseases, have had a huge impact on RNA biology. This book provides an overview of current ncRNA research dealing with miRNA- and ncRNA-related human diseases and molecular diagnosis, plant ncRNAs, and the role of lncRNAs in cancer.

The introductory chapter provides an overview of ncRNAs, and the subsequent chapters constitute an essential resource for researchers in the field of ncRNAs, miRNAs and lncRNAs. Chapter 2 discusses the origin of lncRNAs, their biogenesis, their mechanism of action and their role in many biological and pathological processes such as epigenetics, genome imprinting, several cancers and autoimmune diseases. Chapter 3 examines the advantage of noncoding RNA in molecular diagnosis and looks in detail at the roles of miRNAs in various cancers. The processes leading to the identification of their target molecules are described, and the latest diagnostic strategies using miRNAs are discussed with specific examples. Chapter 4 focuses on the role of ncRNAs in lung cancer pathogenesis as well as their potential use as biomarkers or therapies against lung cancer. Chapters 5 and 6 describe the roles of miRNAs in pancreatic ß cell functional modulation and breast cancer. Chapter 5 discusses the multiple effects of different types of microRNAs on ß cell physiology in the context of maintenance and function in type 2 diabetes. Chapter 6 describes the SNP prediction in mature miRNAs dysregulated in breast cancer. Chapter 7 describes the roles and molecular mechanisms of lncRNAs in neural stem cell self-renewal, neurogenesis, gliogenesis and synaptogenesis over the course of neural development. Chapter 8 describes lncRNA and miRNA interactions in digestive system tumors and the vital role played by ncRNAs (both miRNA and lncRNA) in some digestive system tumors, either as oncogene promoting cancer viability, invasiveness, proliferation, and metastasis or as tumor suppressor inhibiting tumorigenicity or inducing apoptosis. Chapter 9 looks at the miRNA-mediated regulatory mechanism related to male infertility. The final chapter summarizes current knowledge on miRNA involvement in oil palm plant response to abiotic stress and suggests possible miRNA-based strategies for the genetic improvement of oil palm salinity and drought stress tolerance. Overall, this book provides a unique perspective for scientists in the field of ncRNA biology.

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Chapter 1

Introductory Chapter: Noncoding RNAs–A Brief Introduction

Lütfi Tutar

1. Introduction

The discovery of ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) in 1960s led to first steps of noncoding RNA (ncRNA) research [1–3]. In 1980, 90–220 nucleotides long small nuclear RNAs (snRNAs) were involved in splicing reported by Lerner *et al.* [4]. When microRNAs (miRNAs) were discovered in the late 1990s, the noncoding RNA research gained a momentum [5, 6]. Furthermore, development of the next-generation sequencing technologies and advanced bioinformatics opens a new era for noncoding RNAs (ncRNAs).

Ubiquitously, ncRNAs are present in all three domains of life and less than 2% of the human genome code for proteins. Generally, ncRNAs do not code for proteins, but there is some exceptions coding for small bioactive peptides [7, 8]. The two major groups of ncRNAs may be separated from each other by having less or more 200 nucleotides (nts): small noncoding RNAs (sncRNAs) are shorter than 200 nts, and long noncoding RNAs (lncRNAs) are longer than 200 nts.

Noncoding RNAs may be grouped into some categories; tRNA (transfer RNA), rRNA (ribosomal RNA), snRNA (small nuclear RNA), snoRNA (small nucleolar RNA), Telomerase RNA, tRF (transfer RNA-derived RNA fragments), and tiRNA (tRNA-derived stress-induced RNAs) are all "housekeeping" RNAs. "Regulatory" RNAs are miRNA (microRNA), siRNA (small interfering RNA), piRNA (P-element-induced wimpy testis (PIWI) interacting RNA), eRNA (enhancer RNA), lncRNA (long noncoding RNA), circRNA (circular RNA), YRNA, crasiRNA (centromere repeat associated short interacting RNA), and TelsRNA (telomere-specific small RNA). Based on structure, LincRNA (long intergenic noncoding RNA), TUCRNA (transcribed ultraconserved RNA), eRNA, and NAT (natural antisense transcript) are "linear" RNAs while ciRNA, ecircRNA (exonic circular RNA), and elciRNA (exon–intron circRNA) are "circular" RNAs. Based on the location, RNAs may be grouped as sense, antisense, intronic, intergenic, and bidirectional RNAs. Some of the ncRNA families are briefly reviewed below. Rfam is a public online database, which provides information for all RNA families, each represented by multiple sequence alignments, consensus secondary structures, and covariance models (https://rfam.xfam.org/) [9].

2. miRNAs (microRNAs)

miRNAs are ~20 nts long molecules that generally regulate gene expression by inducing mRNA degradation or translational repression. miRNAs originated from

pri-miRNA and mature miRNAs regulate protein coding genes. Understanding the regulation of gene expression is changed after the discovery of first miRNA called lin-4 on *Caenorhabditis elegans* in 1993 [5]. Furthermore, miRNAs are potential biomarkers for several human diseases since they are responsible for many biological processes in the cell such as cell differentiation, cell proliferation, cell death, and development by inducing mRNA degradation or translational repression. Especially, oncomirs such as mir-21 are generally upregulated in cancer patients, and miRNAs attract researchers because of their potential as diagnostic and prognostic biomarkers. miRNA gene sequences are publicly deposited on miRBase catalogs (https://www.mirbase.org/) [10].

3. siRNAs (small interfering RNAs)

siRNAs are about 20–22 nts in size and evolved from double-stranded RNAs. They perform silent transcription of genes via inducing mRNA degradation. siRNAs are very attractive for therapeutic applications in cancer and other diseases [11]. Especially, siRNAs target genes within the tumor cells to inhibit expansions of tumors by RNAi (RNA interference) technology [12]. Moreover, RNAi is a powerful tool in agriculture for crop improvement, including development against biotic or abiotic stress, seedless fruit development, improvement of nutritional quality, and induction of male sterility [13]. Furthermore, a database of siRNA sequences called siRNAdb located on http://sirna.cgb.ki.se/ provides information for siRNAs [14].

4. piRNAs (P-element-induced wimpy testis (PIWI) interacting RNAs)

piRNAs are small noncoding RNAs, which originated from long single chain precursor transcripts. These RNAs are about 21–35 nts long and interact with PIWI proteins to form the piRNA silencing complex (piRISC). piRNAs play key roles in transposon repression, DNA methylation, silencing transposable elements, regulating gene expression, and fighting with viral infections. Since their abnormal expression is reported for many cancer types, they have potential to be diagnostic tools, prognostic markers, and therapeutic targets for cancer [15]. There are two important databases for piRNAs on the web, piRNAdb and piRNABank. piRNAdb is a piwi-interacting RNA sequences storage and search system, providing some other relevant information such as alignments, clusters, datasets, and targets of these piRNAs on its URL (https://www.pirnadb.org/index). piRNABank provides comprehensive information on piRNAs in the three widely studied mammals, namely Human, Mouse, Rat and one fruit fly, Drosophila on the http://pirnabank.ibab.ac.in/ web address [16].

5. lncRNAs (long noncoding RNAs)

lncRNAs with longer than 200 nts are not translated into functional proteins and evolved from multiple ways and mainly transcribed by RNA polymerase II (and also by other RNA polymerases). lncRNAs regulate gene expressions in multiple ways including chromatin structure modulation, regulation of function and the transcription of neighboring and distant genes, and alteration of RNA splicing, stability and translation by interacting with DNA, RNA, and proteins. Moreover, lncRNAs can play roles in the formation and regulation of organelles [17]. Furthermore, they take roles in chromatin remodeling, cancer cell invasion and metastasis, and cell differentiation

by acting as cis- or trans-regulators in biological processes [18]. lncRNAs are linked to some human diseases including hepatocellular carcinoma, Alzheimer's disease, and diabetes [19]. Owing to their key roles in diseases, lncRNAs have a potential to be therapeutic targets. Recently, antisense oligonucleotides (ASOs) technology is used to perform therapeutic lncRNA targeting [20, 21]. However, since lncRNAs lack of functional open reading frames, targeting them with CRISPR–Cas system is more difficult compared with targeting protein-coding genes [22]. There are too many lncRNA databases on the internet, but most comprehensive lncRNA database is RNAcentral located at https://rnacentral.org/ on the World Wide Web [23]. RNAcentral offers integrated access to a comprehensive and up-to-date set of ncRNA sequences.

6. Future perspectives

Mounting data, evidence, and recent discoveries of novel short and long regulatory noncoding RNAs have changed the understanding of genome and transcriptome in the cell. Advanced bioinformatics, public databases, and sequencing technologies led to fast understanding of biogenesis and function of ncRNA types and families. ncRNAs are potential biomarkers to perform therapeutic applications for diseases and to improve crops for agriculture. Further illumination of function, biogenesis, and interactions of ncRNAs will empower our understanding of natural dogma with the aid of developing bioinformatics, sequencing, and biochemical techniques.

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Chapter 2

Long Non-Coding RNAs: Biogenesis, Mechanism of Action and Role in Different Biological and Pathological Processes

Ishteyaq Majeed Shah, Mashooq Ahmad Dar, Kaiser Ahmad Bhat, Tashook Ahmad Dar, Fayaz Ahmad and Syed Mudasir Ahmad

Abstract

RNA or ribonucleic acid constitutes of nucleotides, which are ribose sugars coupled to nitrogenous bases and phosphate groups. Nitrogenous bases include adenine, guanine, cytosine and uracil. Messenger RNA, ribosomal RNA and Transfer RNA are three main types of RNA that are involved in protein synthesis. Apart from its primary role in synthesis of protein, RNA comes in variety of forms like snRNA, miRNA, siRNA, antisense RNA, LncRNA etc., that are involved in DNA replication, post-transcriptional modification, and gene regulation etc. LncRNAs regulate gene expression by various ways including at, transcriptional, post-transcriptional, translational, post-translational and epigenetic levels by interacting principally with mRNA, DNA, protein, and miRNA. Among other biological functions, they are involved in chromatin remodelling, transcriptional interference, transcriptional activation, mRNA translation and RNA processing. In this chapter we shall be discussing the origin of lncRNAs, their biogenesis, their mechanism of action and their role in many biological and pathological processes like epigenetics, genome imprinting, several cancers and autoimmune diseases.

Keywords: long non-coding RNAs, epigenetics, chromatin remodelling, gene expression regulation, cancer, autoimmune diseases

1. Introduction

The genome of higher organisms contains less than 3% of protein coding genes while rest of genome is known as junk/or non-coding. With recent advances in science and technology we are learning more about the complexity of organisms, which has led to the discovery of the remarkable complexity of RNA. Large-scale projects such as ENCODE and FANTOM, for the systematic annotation and functional depiction of genes have pointed that 80% of genomic DNA of mammals

are effectively transcribed and intricately controlled with majority belonging to noncoding RNA genes [1]. The figure of ncRNA genes varies by species. Frequency of ncRNA genes but not protein-coding genes, is strongly linked with the complexity of an organism emphasising the growing relevance of ncRNAs [2]. On the basis of molecular size ncRNAs can be classified into two groups. One group includes short RNAs that are less than 200 nucleotides in length such as microRNAs (20–25 nucleotides Length), piwi-interacting RNAs etc. The other group includes Long-non coding RNAs of around 200 nucleotides or more in length. Among the ncRNAs, long non coding RNAs represent a greater portion. Regulatory non coding RNAs with length ≥ 200 nucleotides belong to long non coding RNAs (lncRNAs). Due to their low expression levels, lncRNAs in the beginning were considered to be transcription noise. According to HUMAN GENCODE statistics, there are around 16,000 lncRNA genes, although some estimates put the number at over 100,000 [3, 4]. RNA polymerase II is primarily responsible for lncRNA transcription, but other RNA polymerases are also involved. The resultant lncRNAs are frequently capped at their 5′ ends with 7-methyl guanosine (m7G), polyadenylated at their 3′ ends, and spliced similarly to mRNAs. lncRNAs, in contrast, have a median turnover of 3.5 h, while it is 5.1 h for mRNAs. After transcription, the widely held lncRNAs stay in the nucleus [5]. LncRNAs regulate gene expression at different domains including the ones at epigenetic, transcriptional, post-transcriptional, translational, and post-translational levels by interacting principally with DNA, mRNA, protein, and miRNA [5]. LncRNAs regulate gene transcription by promoting or inhibiting the formation of transcription loops and recruiting or blocking regulators. LncRNAs also operate as precursors to other ncRNAs, such as microRNAs (miRNAs), and influence mRNA splicing [6]. They are involved in RNA processing, transcriptional interference, chromatin remodelling, transcriptional activation, and mRNA translation, among other biological processes. Besides these roles they also act as oncogenes or tumour suppressors by regulating different signalling cascades [7]. Based on their functions lncRNAs are classified into three types: non-functional lncRNAs, these are products of transcriptional noise; lncRNAs for which the act of transcription is adequate for their activity but the transcript itself is not required and functional lncRNAs that act in both cis and trans ways [8]. In recent years, lncRNAs which account for the immense majority of non-coding RNAs (ncRNAs), have become a hot topic in disease diagnostics and target therapeutics in recent years.

2. Biogenesis of lncRNAs

Understanding the biosynthesis of these lncRNAs is not only crucial but also ineluctable in order to decipher their functional value, relevance, and differentiation from other forms of RNAs. LncRNA biogenesis is both cell type and stage specific, regulated by stage and cell specific stimuli [9]. Enhancers, promoters, and intergenic regions are among the DNA components in eukaryotic genomes that transcribe diverse types of lncRNAs [10]. LncRNA biogenesis involve ribonuclease P for cleavage to create mature ends, the production of protein (snoRNP) complex caps at their ends, small nucleolar RNA, and the development of circular structures [11, 12]. During specific lncRNAs biogenesis, “paraspeckles” (sub-nuclear structures) have been discovered [13]. The identity of 4 paraspeckle proteins (PSPs) required for paraspeckle formation was made possible by RNAi analyses of 40

paraspeckle proteins [14]. Overall, the biology dealing with regulation, synthesis of various lncRNAs is still unknown.

3. Origin of LncRNAs

Compared to protein-coding genes, very less information regarding origins and evolution of lncRNAs is available. They reveal that among mammals, sequence conservation is poor and evolution is fast [15]. For the origin of lncRNAs, various evolutionary assumptions have been considered. The first idea is that the protein-coding gene undergoes transformation as a result of a gene-duplication process [16, 17]. Throughout evolution, one copy of a protein-coding gene acquires mutations and loses its capacity to code for proteins. Then, among other coding fractions, a new functional lncRNA gene is generated with polyadenylation sequences, splicing signals, regulatory elements and exon sequences [16, 18].

X inactive-specific transcript (XIST), meant for dosage compensation in mammals is assumed to have arisen from the chicken protein-coding Lnx3 gene [16, 17, 19], was classified as a pseudo gene [18]. The 5-UTR of Lnx3 exons 1 and 2 were used to create the XIST promoter region. Lnx3 exons 4 and 11 were also used to create exons 4 and 5 of the XIST gene of humans [20]. Other lncRNA genes, such as short and long non-coding RNAs, can duplicate segments or whole genes to generate lncRNAs. According to genomic research large homologous protein-coding gene families, protein coding gene duplication is ubiquitous; yet apart from protein coding genes, there is minimal proof for the entire duplication of lncRNAs. This might be owing to the fast sequence divergence of lncRNAs. The duplicated lncRNA mouse nuclear-enriched abundant transcript 2 is paralogous to non exonic sequences in the mouse genome [18]. The generation of lncRNAs appears to be aided by segmental gene duplication within antisense non-coding RNAs (ancRNAs).

De novo creation is another option for lncRNA origins. Examples include genome changes viz. chromosomal rearrangement, creation of (proto-) splice sites and (proto-) promoters transformed non-functional genomic stretches into functional lncRNAs [16]. Genesis of lncRNAs via insertion of transposable elements is the most recent and final hypothesis [17, 18, 21]. The bulk of human lncRNAs have TE segments as related to other genes such as pseudo genes, tiny lncRNAs, and protein-coding genes. Internal exons, transcription start sites, polyadenylation (polyA) sites, or a mix of these components can all include TEs. According to this research at least 75% of human lncRNAs contain at least one exon containing partial TE origin.

4. Mechanism of action of LncRNAs

4.1 LncRNAs as chromatin regulators

The figure of lncRNAs with known roles is gradually increasing, and the majority of the studies focus on their controlling potential. Histone modifications, DNA methylation, and chromatin remodelling are all functional steps where lncRNAs regulate chromatin structure.

LncRNAs are frequently used as important regulators (modulators) of protein coding gene expression by acting in cis and trans ways [22]. Binding of lncRNAs to histone modification complexes like PRC1 and PRC2 [in-specific] causes methylation

of lysine 27 on histone 3, a histone signature connected to suppressed transcriptional state. Xist, a lncRNA abundantly generated from inactive X chromosomes in females (Xi), enhances PRC2 recruitment to the Xi to mute gene expression, according to studies on mammalian X chromosome inactivation [23, 24]. Other example includes HOTAIR lncRNA, synthesised from the HoxC gene cluster but targets histone H3K4me1/2 demethylase LSD1 and PRC2 complex to induce transcriptional gene suppression at the HoxD locus in trans [25]. Some well-studied lncRNAs, including ANRIL and KCNQ1OT1, bring epigenetic modifiers to particular loci, allowing for chromatin remodelling. For example, KCNQ1OT1, binds to PRC2 as well as the methyl-transferase G9A, whereas ANRIL binds to both polycomb repressive complexes [26]. Many different lncRNAs act as scaffolds and work by leading restrictive histone modifying complexes to particular loci [26]. By forming complexes with the trithorax group (TrxG) and the polycomb repressive complex 2, lncRNA steroid receptor RNA activator also plays a role in transcriptional control [27].

Examples of lncRNA that interact with PRC1 complex include FAL1 RNA (focally amplified lncRNA on chromosome 1). BMI1, a PRC1 subunit, interacts with FAL1. FAL1 controls not only the stability of BMI1, but also its connection with target promoter regions, altering target gene expression [28].

4.2 Transcriptional regulation

At gene promoters transcription is thought to be regulated by the interaction of transcription factors and chromatin modifying factors. LncRNAs regulate gene expression by interacting with other molecules such as Proteins, RNA and DNA near their target genes promoters or enhancers. LncRNAs have a number of ways for controlling transcription.

I. Enhancer RNAs: Enhancer RNAs (eRNAs) are a sort of long noncoding RNA (lncRNA) derived from gene enhancer regions that act along with DNA to upregulate gene transcription via two mechanisms: transcriptional machinery tracking and enhancer-promoter looping [29]. Kim et al. found a 2 kb eRNA transcribed bidirectional from active enhancers. The function of enhancer region was correlated with the expression of this eRNA, suggesting that eRNAs play a task in enhancer function and influence gene transcription [30, 31].

II. Activating ncRNAs: Class of lncRNAs that acts as a transcriptional activator and is produced from independent loci instead of enhancers. In an RNA-dependent way, activating ncRNAs need the activation of the coding gene promoter and exclusively trigger the transcription of neighbouring coding genes. The mediator complex has been connected to a variety of activating ncRNAs, and attenuation of such complex prevents looping between the activating ncRNAs locus and its target gene.

III. By recruitment of chromatin modifiers: LncRNAs can modulate target genes by triggering epigenetic alteration viz. DNA methylation, histone modification, and by bringing chromatin remodelling complexes to specific genomic loci, typically promoter regions. LncRNAs might have two purposes. Firstly, by attaching to a protein or protein complex, lncRNAs act as a bridge scaffold for chromatin conformational changes [32]. Second, lncRNAs lead chromatin modifying enzymes to particular DNA patterns by acting as a tethered scaffold. For instance, the lncRNA HOTAIR serves as an epigenetic protein scaffold with several binding domains for

various proteins. HOTAIR aids in the demethylation of H3K4 by interacting with LSD1 (lysine-specific histone demethylase 1A), REST (restriction element 1-silencing transcription) factor & REST corepressor1 at the 3′ end. HOTAIR (At the 5′ end) is a transcriptional gene silencing factor derived from the HOXC locus that leads to transcriptional gene silencing in trans across 40 kb of the HOXD locus by inducing a repressive chromatin state, recruiting PRC2, & reinforcing H3K27 methylation [33].

Long non-coding RNAs also function as cofactors, influencing the activity of transcription factors. For example, the ncRNA Evf2 is produced from a conserved distal enhancer and attracts DlX2 (TF) to the same enhancer, causing neighbouring protein-coding genes to be expressed.

4.3 Regulation after transcription

Because ncRNAs can recognise complementary sequences, they can affect how mRNAs are processed after transcription, including capping, splicing, editing, transport, translation, destruction, and stability. LncRNAs compete with microRNAs to impact mRNA levels by changing mRNA stability, degradation, and translation [34].

I. **Regulation of mRNA splicing by LncRNAs**: Alternative splicing can be aided by lncRNAs in a number of ways. LncRNAs interact with splicing factors most of the time to regulate gene splicing. MALAT1, a notable lncRNA is involved in pre-mRNA splicing. MALAT1 is needed for the precise localization of SRSF1 and numerous other splicing factors to nuclear speckles. Deletion of MALAT1 is associated with the change in the alternative splicing of a group of transcripts [35]. Other lncRNA like Gomafu/MIAT, restricted to a nuclear domain and expresses neuronally, may influence mRNA splicing and obstruct spliceosome formation by sequestering splicing factor1 [36]. Chromatin-mediated splicing control is the other way through these ncRNAs can modulate alternative splicing. For instance, nuclear antisense lncRNA generated from FGFR2 locus promotes FGFR2 epithelial-specific alternative splicing. The lncRNA establishes a chromatin environment that precludes the binding of a restrictive chromatin-splicing adaptor complex required for mesenchymal-specific splicing by attracting Polycomb-group proteins and KDM2a (Histone demethylase) [37].

II. **Regulation of mRNA stability**: Because of interactions between their 3′-UTR and RBPs (RNA Binding Proteins), many genes have a limited mRNA half-life. Because lncRNAs can interact with RBPs, this type of interaction is expected to alter not just the number of RBPs in the pool, but also the functioning of lncRNAs that share binding sites with other genes, both coding and noncoding. By this way, mRNA molecules are either stabilised or destabilised. RBPs of different kinds may have a function in mRNA stability and, as a result, mRNA level. HuR is destabilised when the lncRNA OCC-1 binds with it and recruits the ubiquitin E3 ligase-TrCP1 to it [38]. HuR works as a stabilising factor for a wide number of mRNAs, causing HuR-targeted mRNAs to be down regulated. Linc-RoR interacts with hnRNP I (stabilising factor) and AUF1 (destabilising factor) in the opposite way that it interacts with c-Myc mRNA, according to findings [39].

III. **Regulation of protein stability:** Many studies have discovered that lncRNAs can influence protein stability through ubiquitination or phosphorylation. DINO, a

damage-induced noncoding RNA (lncRNA) that is p53-dependent, affects p53 stability [40]. DINO participates in p53-mediated phenotypes such as Apoptosis and cell cycle arrest with respect to DNA damage. Eminently, DINO binds to and stabilises p53, allowing induced p53 to favourably regulate downstream targets like DINO. Because of its capacity to stabilise the p53 protein, DINO is an important component of the DNA damage-p53 regulation network. lncRNA GUARDIN is another paragon, which is likewise a p53-responsive lncRNA [41].

4.4 LncRNA-mediated regulation of protein activity

Several studies have discovered that lncRNAs can influence protein stability through ubiquitination or phosphorylation. DINO (lncRNA) has a function in phenotypes regulated by p53, such lncRNAs also regulate protein activity in mechanisms other than transcription. The *Caenorhabditis elegans* lncRNA rncs-1 found by Hellwig and Bass (2008) and functions in the processing of short RNAs by binding to and blocking the Dicer enzyme [42]. Marchese FP et al. established the involvement of lncRNA CONCR in sister chromatid cohesion control [43]. CONCR is a cell cycle-regulated lncRNA that is essential for DNA replication and cell cycle advancement and is activated by MYC. CONCR interacts with DDX11 (DNA-dependent transcription factor) and regulates its activity. ATPase and helicase have an impact on DNA replication and sister chromatid cohesion. Liu et al. showed how lncRNA regulates kinase signalling in the setting of metabolic stress response. The LKB1-AMPK pathway promotes the expression of lncRNA neighbour of BRCA1 gene 2 (NBR2), which has also been found to interact with AMPK in the context of energy stress [44]. The association

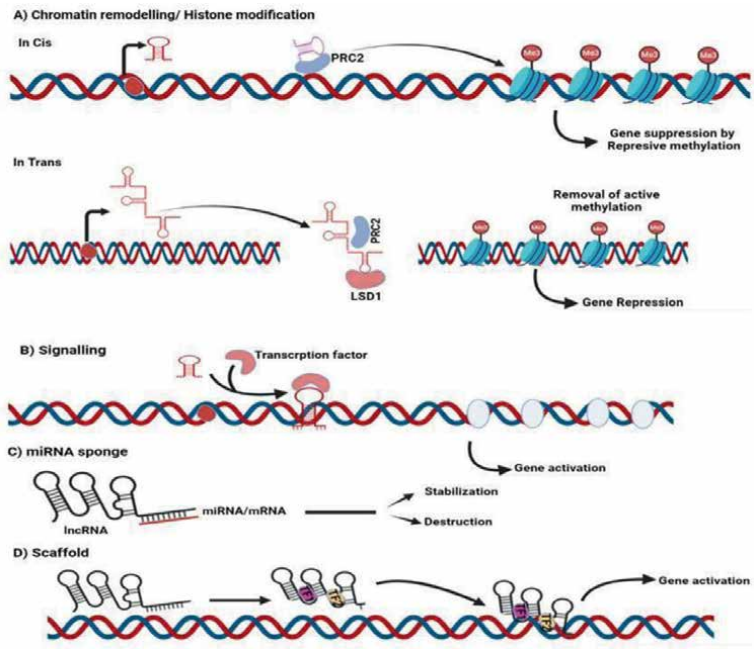

Figure 1.
Schematic representation of few mechanisms employed by lncRNAs in regulation gene expression. (A) Regulation by chromatin remodelling (B) Regulation through signalling (C) lncRNA sponging mRNA (D) By acting as scaffolds.

of lncRNA NBR2 with AMPK enhances its kinase activity under energy stress. AMPK activation is decreased in NBR2 loss, resulting in aberrant cell cycle, altered apoptosis/autophagy responses, and increase in tumour formation. The hypoxia-regulated lncRNA linc-p21 has been found to bind both HIF-1 and VHL, altering the VHL-HIF-1 interaction and increasing HIF-1 accumulation [45]. The importance of lincRNA-p21 in the control of the Warburg effect in tumour cells is highlighted by this positive feedback loop between HIF-1 and lincRNA-p21, which accelerates glycolysis under hypoxia. Certain lncRNAs control transcription factor activity in addition to impacting gene expression via mRNA interactions. As an alternatively spliced form of Evf-1 RNA, the lncRNA Evf2 is generated from the Dlx-5/6 ultra-conserved region. The lncRNA Evf2 forms a stable complex with the transcription factor Dlx2, boosting Dlx-5/6 enhancer transcriptional activation mediated by Dlx2 [46].

Despite the fact that lncRNAs are distinct from mRNAs exported to cytoplasm for protein synthesis [47, 48], few lncRNAs are confined in sub-nuclear compartments, suggesting possible activities in these compartments. The schematic figure that represents few mechanisms employed by lncRNAs in gene expression regulation (**Figure 1**).

5. Roles and functions of lncRNAs in different biological processes

An emerging view of lncRNAs is that they are key players in many biological processes. These lncRNAs have been discovered to be master regulators in different biological and pathological processes and their dysregulations leads to many life-threatening diseases including different cancers. Some of the pivotal roles and functions carried out by different lncRNAs are well documented.

5.1 Long non-coding RNAs in genomic imprinting: regulation of allelic expression

In layman's terms genomic imprinting represents a condition in which one of the alleles in an inherited paternal pair is active while the other one remains inactive. It is the parent of origin which will determines the differential expression of inherited parental alleles; in some cases, a genes allele is paternally imprinted, while in others, it is maternally imprinted.

Dna Methylation and Genomic imprinting: The process of adding a methyl (CH3) group to a cytosine known as DNA methylation is commonly located at CpG dinucleotides in mammals and is believed to regulate gene expression. CpG sites are very rare in the genome with the exceptions of CpG islands (which have high CpG amount/density). Generally found near or around promoter region these islands are usually unmethylated. Outside of CpG islands, CpG sites are often methylated, resulting in a bimodal methylation pattern across the genome. A family of DNA methyltransferases catalyses the acquisition of DNA methylation. DNMT1 is a DNA methyltransferase homologue that has an affinity for hemi-methylated DNA and is responsible for sustaining methylation following DNA replication. DNMT3A and DNMT3B catalyse de novo DNA methylation, while DNMT3L is a cofactor with no methyltransferase activity [49]. DNA methylation leads to epigenetic mechanisms and epigenetics leads to allow the transcriptional machinery of the cell to distinguish the two parental chromosomes at imprinted loci.

Imprinting is typically accomplished by altering the histone and/or DNA of a given locus, however lncRNAs have recently been identified to have a role in these

phenomena [50]. Imprinted genes must be regulated by cis-regulatory mechanisms since expressed and repressed alleles share the same nucleus. The breakthrough in genomic imprinting was established with the discovery of gametic differentially methylated regions (these are actually control elements) that contain the "imprinting mark" acquired in oocytes and sperm. These markers are then transmitted down through the generations, guiding parental-specific allelic expression in children and embryos.

Control of imprinted lncRNAs is achieved by shared regulatory mechanisms such as parent-of-origin-dependent differentially methylated regions (DMRs) & lncRNAs within a single imprinted cluster [51]. Allele-specific DNA methylation occurs in a separate ICR in the germline in well-studied imprinted clusters, termed as principal DMRs or germline-derived DMRs and persists beyond fertilisation. Epigenetic changes (parent-of-origin-specific) such as DNA methylation, influence parentally inherited allele expression patterns in ICRs in imprinted clusters [52]. There are approximately 35 imprinted gDMRs in the human & 24 in Mouse genome (Monk et al., 2018). For embryonic development to govern imprinted gene expression, gDMRs must be established on paternal or maternal alleles [53]. In early primordial germ cells epigenetic markers including DNA methylation and histone modifications are substantially erased genome-wide. Depending on the parent-of-origin, DNA methylation of ICRs is reinstated in germline cells in gametes. After fertilisation, gDMRs are impervious to subsequent global epigenetic reprogramming. The data on DNA methylation at the ICRs of imprinted areas is retained. Imprinted loci gDMRs establish themselves strongly throughout germline development and are hence resistant to genomic reprogramming after fertilisation. Imprinting markings, on the other hand, are passed down from one generation to the next [54]. The allele-specific methylation statuses of gDMRs are generally identified by transcription regulators in maintaining parent-of-origin specific regulation of imprinted genes for example ZFP57 protein. The development of monoallelic gene expression requires differential methylation statuses of gDMRs on parental alleles. In early embryonic and adult lineages, imprinting control regions regulate DNA methylation and chromatin organisation, resulting in the survival of imprinting patterns through generations and their perpetuation in adult tissues [55].

To explain the control of gene regulation within an imprinted cluster, two fundamental methods have been proposed [56]. The lncRNA model is the earliest and arguably most frequent model. According to this concept, imprinted lncRNAs control imprinted gene expression. In this model, imprinted lncRNAs are closely linked to ICRs. Imprinted lncRNAs are defined by their ability to suppress imprinted genes in the same cluster [57]. As shown by the imprinted cluster Kcnq1/Kcnq1ot1. On paternal allele, the constantly expressed imprinted lncRNA Kcnq1ot1 can repress multiple imprinted genes bidirectionally throughout their gene area. The insulator paradigm, wherein parental allele-specific epigenetic changes at ICRs contribute to topological variations of imprinted gene areas, resulting in gene silence or activation of certain alleles, has been observed in additional imprinted regions. The insulin-like growth factor 2/H19 locus controls imprinted genes mechanistically, according to this concept. The following are some well-known lncRNAs and their roles in genomic imprinting:

I. **Airn lncRNA:** 108-kb nuclear-localised transcript that is synthesised in the opposite direction from 3.7-kb ICE of Igf type-2 receptor gene [58]. This lncRNA have been shown to influence the regulation of three parent of origin-specific genes viz. Igf2r, Slc22a2 & Slc22a3. Mice with an ICE deletion show biallelic expression of 3 genes (including Igf2r) and are smaller at birth [59].

II. **Kcnq1ot1:** Another lncRNA with a well-known involvement in imprinting is Kcnq1ot1. It is a 91-kilobyte RNA with many protein-coding genes that comes from the 1-megabyte Kcnq1/Cdkn1c locus on chromosome 7 [36, 60]. Because of CpG methylation, this lncRNA is suppressed on maternal chromosome and assumes paternal specific expression. The paternal chromosome specific expression is associated with inhibition of some 8–10 protein-coding genes that span many megabases. Kcnq1ot1, like Airn works in cis on the paternal chromosome to mute Phlda2, Kcnq1, Slc22a18, & Cdkn1c genes in all tissues (ubiquitously imprinted loci), Imprinting is a phenomenon that happens in a 600-kb region of mouse chromosome 7 called the imprinting cluster. H19 & Igf2, two genes in this cluster, are expressed maternally and paternally, respectively, and correlate to the human locus 11p15.5 [61, 62]. The H19 gene regulates expression of imprinted loci, forming the imprinted gene network, which is important for embryo development. H19 is a 2.3 kb lncRNA that has been demonstrated to generate an overgrowth phenotype and alleviate imprinting on Igf2 and other IGN genes when knocked out [63].

III. **DLK1-DIO3:** Human and mouse chromosome 14 & 12 respectvely both have a big imprinted cluster [imprinting status, in prototherians, metatherians and eutherians have also been determined] [64]. The paternally expressed genes DLK1 and DIO3 flank a 1 Mb area bordered by maternally expressed noncoding RNAs including the lncRNA MEG3 [65].

IV. **SNURF-SNRPN:** The SNURF-SNRPN area on chromosome 15q11-13, which spans over 2 Mb and has been related to the neurodevelopment diseases like Angel man Syndrome (AS) and Prader-Willi Syndrome (PWS) [66], is the biggest known imprinted cluster.

5.2 X-chromosome inactivation

Sex in most animals including humans is generally determined by X and Y chromosome system with men carrying XY and females XX chromosomes. In order to have the balance of products of sex-linked and autosomal genes, dosage compensation is required. X-chromosome inactivation (XCI) is a special kind of dosage compensation present in mammalian females involving random selection and transcriptional repression in one among the two X -chromosomes during the early stages of embryonic development [67, 68]. However, humans lack imprinted XCI and instead have XCD (X chromosome dampening) [69–71]. Most of the research with respect to this has been carried on mouse model and regulated by interactions of many lncRNAs [72]. At the heart of XCI/XCR (X-chromosome reactivation) regulation is a region of X chromosome namely Xic X-inactivation centre (containing many noncoding RNA genes whose expression is regulated by pluripotency factors) [73]. Developmental phase of mice includes two forms of XCI; one being imprinted and another random. Imprinted XCI, in which the paternally inherited X (Xp) is always inactivated, occurs during preimplantation development in the early embryo, when Xist becomes expressed on the Xp from the 2-cell stage onwards and is maintained in the placenta's extraembryonic tissues [74].

Xist lncRNA regulates all three stages (initiation, establishment, and maintenance) of XCI [75]. The X-linked minimum genetic region (XIC) contains many elements and genes like Tsix & Xist which are meant for XCI initiation [76]. Among the lncRNA loci reported in a 100–500 kb region of the mouse X chromosome and a 2.3 Mb syntenic

region of the human X chromosome are RepA Jpx, Xist, Ftx, Tsix, & Xite [75, 76]. XIC contains the lncRNA Xist, and is located 15 kb downstream of Tsix antisense [77].

In first phase, complex factors Xite, OCT4, Tsix & CTCF among others bind Xi and Xa independently to promote X chromosome pairing and counting in the embryo following fertilisation [78]. After counting and pairing, Tsix, Xist, and other genes are elevated, which is regulated by a network of genetic interactions including OCT4, Jpx, Rnf12, and RepA Tsix, Sox2 & PRDM14 [79]. They use alternative transcription fates when full initiation of XCI occurs, with one becoming the Xa and the other Xi chromosome [72]. RNF12, Tsix, and RepA, as well as pluripotency factors (NANOG, OCT4, SOX2) impact lncRNA Xist activation and expression in Xi. Xist binds to Polycomb repressive complex 2 via Repeat A, producing the Xist-PRC2 complex, while YY1 tethers the PRC2-Xist complex to the Xi nucleation centre via Repeat C, where RNA polymerase II gets the lncRNA Xist-PRC2 complex [80]. Upon completion of initiation phase lncRNA Xist recruits different protein complex factors (including heterogeneous nuclear protein U, meant for lncRNA Xist localization, heterogeneous nuclear ribonucleoprotein K for Xist-mediated chromatin modifications) and gene-silencing factor Spen which binds to C, B, F, and A repeats at the 5′ end of the lncRNA Xist and causes the lncRNA Xist topologically associated domains meant for (epigenetic modification and chromatin compaction) to spread along the Xi chromosome at the established phase [76, 81]. ATRX directs PRC1 and PRC2 that cause epigenetic silencing by acetylation of histone H3 and H4 and methylation of CpG islands [56]. The protein complexes SHARP, HDAC3, LBR, Airn and Kcnq1ot1, U1 snRNP, Rsx, and CdK8 are all implicated in the lncRNA Xist spreading process. LncRNA Xist attracts restrictive complexes (like H3K27me3, H2AK119Ub, & the CpG island), which cause immediate histone modifications and DNA methylation, and coats the Xi to generate Xi [82]. Continuous synthesis of lncRNA Xist RNA in an inactive state has been used to establish and maintain the Xi.

6. Role of LncRNAs in cancer

The deadliest disease on the planet is linked to abnormal gene expression. Non-coding areas of the genome have been related to the majority of malignancies. Cancer genomic mutations are found in areas that do not code for proteins [83]. These areas, however, are frequently translated into long non-coding RNAs (lncRNAs). Anomalies in these lncRNAs is thought to have the tumour suppressor or carcinogenic effects and thus play a vital role in tumour establishment. Since lncRNAs have expression that are unique to a tissue, expressed in regulated manner and in association with other gene sets impact cell cycle regulations, immunological responses, survival etc. all of which affect cancer cell transformation [84]. The role of lncRNA is associated with their specific subcellular location. LncRNAs regulate gene expression by different interactions both in the nucleus as well as in the cytoplasm. In nucleus regulate expression at both epigenetic and transcriptional levels including histone modifications [24, 85], DNA methylation regulation [86], chromatin remodelling [87, 88], chromatin modification complexes [89, 90], transcription regulators [91] and proteins present in nucleus [92] while in the cytoplasm these lncRNAs regulate gene expression at both transcriptional and translational levels including interplay with proteins present in the cytoplasm [93], Control of mRNA metabolism [94], as endogenous competitive RNA (ceRNA) interacts with microRNA [95]. As a result, lncRNA play a significant role in cancer cell proliferation, transition, invasion, and treatment resistance [96, 97].

LncRNAs like LUCAT1, KCNQ1OT, HOTAIR, ANRIL, MALAT1(metastasis-associated lung adenocarcinoma transcript 1), Taurine-upregulated gene 1, LINC00152, RP11-385 J1.2 and TUBA4B bring different epigenetic modifiers at their respective loci and modify the chromatin shape and their dysregulation is strongly associated with establishment of different tumours including LUCAT1: digestive system tumours, ANRIL: prostate cancer, MALAT1: breast, liver, and colon cancer [98], HOTAIR: breast cancer KCNQ1OT1: colorectal cancer etc.

Some of known lncRNAs with links to cancer is represented below in a table:

Symbol/emblem	Cancer phenotype	Cancer association	Mechanism	Reference
HOTAIR	Promote metastasis	Upregulated in many cancers including those of liver, breast, lung and pancreas	By acting a platform for the PRC2 and LSD1 (chromatin repressors). By turning off *HOXD* and many other gene loci	[99]
MALAT1	Promotes both cell metastasis and proliferation	Overexpressed in non-small cell lung cancer, pancreatic, Colon, prostate, breast and hepatocellular carcinomas	Similar to alternative splicing and active transcription. Unique tRNA-like sequence at the 3′ end cleaved off and transformed to produce a short tRNA-like ncRNA (mascRNA)	[35, 98, 100]
PTENP1	Impede cell amplification, relocation and carcinoma establishment	Locus preferentially deleted in sporadic colon cancer, prostate and other different carcinomas	Inveigle for microRNAs that attack *PTEN*	[101]
PR-lncRNA-1	Impede cell amplification and stimulates Apoptosis	Suppressed in colorectal cancer	Upregulates the transcription of p53	[102]
LUCAT1(SCAL1)	Controls cellular events, programmed cell death and resistance to cisplatin in NSCLC cells by attacking IGF-2	Overexpressed in clear cell renal cell carcinoma ccRCC tissues	Engage in control of amplification, relocation, invasion and impedance to drugs in multiple tumours	[103]

One of the first lncRNAs to be discovered as having a functional role in cancer formation was the HOTAIR. HOTAIR lncRNAs enhance cancer spread by triggering epigenetic alterations in the chromatin status of tumour cells [104]. Interestingly, various lncRNAs have been reported to originate from the HOX locus, implying that it plays a global regulatory role [21]. In tumours, lncRNAs are also implicated in the control of the epithelial-mesenchymal transition (EMT) [99]. TGF-activated lncRNA (lncRNA-ATB) stimulated the EMT cascade by upregulating the levels of zinc finger

E-box-binding homeobox (ZEB1 and ZEB2) [105]. Recent research has discovered that the transcription factor PNUTS has a corresponding lncRNA-PNUTS, which plays a role in breast cancer metastasis by influencing the EMT process [106]. lncRNA human ortholog RNA of Dreh (hDREH), inhibitor of EMT is seen downregulated in many types of cancers. Some tumour suppressor lncRNAs, on the other hand, are expressed at low levels in tumours [107]. It is now possible to increase the expression of these lncRNAs in order to treat cancer. lncRNAs have been implicated in stemness-related signalling pathways in a number of studies. The tumour suppressor long noncoding RNA (TSLNC8), for example, inhibit the STAT3 signalling pathway and thus acted as a tumour suppressor [108]. lncRNA MST1/2-antagonising for YAP activation (MAYA) participate in the Hippo-YAP signalling pathway. These lncRNAs might be directly regulating cell stemness [109]. Thus, these lncRNAs act as potential targets for cancer treatment.

7. Role of LncRNAs in immunology

The study of lncRNA biology is still in its early stages, but it has already revealed key roles in the formation and functions of immune cells. However, because the functions of most lncRNAs are unknown, the field must fill a significant gap in order to comprehend the breadth of their roles in immunity. The function of non-long coding RNAs in the immunology of humans even though in its infancy stage has become hot topic in recent research. In immune system, lncRNAs have a role in immune cell formation, survival, cell fate determination, differentiation, amplification, and activation. Although the functions of most lncRNAs are unknown, novel protein-protein interactions or partnering with RNA or DNA have been discovered to influence innate and adaptive immune responses transcriptionally or post-transcriptionally. According to the recent research lncRNAs have been found in many immune cells including T cells and B cells. Regulation of these lncRNAs including expression levels is considered to be linked to immune cell development, differentiation, and activation [110].

To carry out their functions these lncRNAs can associate with transcription regulators and signalling molecules (NF-B, STAT3) [111, 112], RNA binding proteins including (hnRNP, HuR) [113, 114], and chromatin remodelling components (PRC2, WDR5).

7.1 Long non-coding RNA in T cells

LncRNAs, such as TMEVPG1 (also known as LincR-Ifng-3′AS), have been identified in both mouse and human CD8+ T cells and demonstrated to be positioned inside a cluster of cytokines genes, and have been shown to control Theiler's virus load in CNS infection [115]. T-bet/Stat, a transcription factor exclusive to Th1 cells that collaborates with TMEVPG1, promotes interferon gamma expression [116]. Interaction of lncRNA called NeST with WDR5, significant component of MLL H3K4 methyltransferases, leads to increase in methylation state of histones at Ifng gene in CD8+/Th1 cells [117]. Hundreds of lncRNAs were found in both CD8+ T cells of animal and human spleens after a genome-wide expression examination utilising a proprietary array, indicating that lncRNAs are important for lymphocyte differentiation and stimulation [118]. To get clearer picture of the role of lncRNAs in T cell growth and differentiation, Hu et al. used RNA-Seq to recognise 1524 genomic regions that produce lincRNAs in 42 subsets of mature peripheral T cells and thymocytes;

specific transcription regulators including T-bet, STAT4 for CD8+ descendants, & GATA-3 and STAT6 for CD4+ lineages seemed to be substantially responsible for lineage-specific expression of T cell lincRNAs (LincR-Ccr2-5′AS) [119].

7.2 Long non-coding RNA in B cells

B lymphocytes after being activated by antigen interaction, the major roles are to make antigen-specific antibodies, operate as APCs, and develop into memory cells. In compared to T cells, less is known regarding lncRNA roles in B cells. Bolland et al. [120] have documented how lncRNAs play their part in the chromatin remodelling that occurs during V/D/J gene recombination, which is required for the production of epitopes (Ig or TCR). Further study discovered that the synthesis of such antisense and sense lncRNAs is related to VH portions looping next to DJH areas during pro-B cell recombination, and as a result, it has been dubbed the Igh locus whole transcriptome of sense and antisense transcripts [121]. SAS-ZFAT gene expression which is restricted to CD19+ B cells of peripheral blood lymphocytes may be important for B cell function and AITD development [122].

7.3 Long non-coding RNA in macrophages

Macrophages are white blood cells that surround and destroy pathogens, destroy dead cells and stimulate other immune system cells. Macrophages as APCs also contribute to the optimal operation of both intrinsic and adaptive immune responses. LncRNA acknowledgement and purposes in monocytes/macrophages have received little attention. Macrophages have capital baseline level of ptprj/CD148 (a tyrosine phosphatase with tumour inhibitor-like role), as expected; its level is modulated by LPS, TLR, and CSF-1 treatments in various animals. Dave et al. investigated ptprj-as1, a 1006-nt lncRNA species that is produced antisense to ptprj. Transcripted ptprj-as1 is highly regulated in tissues augmented with macrophages that has been temporarily activated by TLR ligands, similar to ptprj [123]. Thus, the ptprj coding transcript may guide to inflammatory alteration that is promptly connected to macrophages. When myeloid and CD11c + dendritic cells are stimulated by lipopolysaccharide, a TLR4 signalling activator via NFkB, they express lncRNAs-COX2 (Ptgs2) [84]. Li et al. investigated how lincRNA expression changed when innate immune signalling was activated in THP1 macrophages and discovered that an unannotated LincRNA called THRIL was a critical factor in TNF- control and that its activity was clearly lower during the acute period of Kawasaki disease [114]. LncRNA has been observed to regulate healthy and pathological inflammatory immune responses through an RNA-protein complex with hnRNPL [124]. Many lncRNAs discovered as unique binding ally for lincRNA-cox2 in the nucleus and cytoplasm of macrophages, are key switch of immune genes [113].

7.4 Long non-coding RNA in natural-killer cells

Wright et al. uncovered an intron 2 promoter in many KIR genes that generate spliced antisense transcripts. KIR antisense lncRNA is found in progenitor cell lines, and its abundance in NK cells results in the under expression of KIR protein coding genes. KIR's antisense lncRNA corresponds to exons 1 and 2 of the KIR gene, and also the proximal promoter upstream of KIR. Myeloid zinc finger one (MZF-1) appears to impact KIR antisense lncRNA transcription, resulting in KIR silence via an unknown mechanism [125].

7.5 Functions of immune related lncRNAs

The following table represents the functions of some immune related lncRNAs:

S. no.	lncRNA	Functions	Reference
1	NEAT1	In response to viral infection, activates IL-8 transcription	[126]
2	Lnc-DC	Plays a role in transformation of monocytes of humans into dendritic cells	[112]
3	LincRNA-COX-2	increases the production of proinflammatory genes (IL-6) & suppresses the synthesis of anti-inflammation genes in non-stimulated cells	[113]
4	NeST	controls IFN-encoding chromatin epigenetic tagging, IFN-expression, and susceptibility to viral and bacterial pathogens	[117]
5	Hotair	Through epigenetic changes in the chromatin state, promotes cancer spread and progression	[127]
6	LincRCcr2–5′AS	In mouse CD4+ TH2 cells, it controls the synthesis of many chemokine receptor genes via STAT-6 pathway	[119]
7	LincRNA (THRIL)	Promotes TNF Transcription	[114]
8	IL1β	Modulation of chromatin	[128]
9	IL1β-RBT46	In monocytes regulates IL-1 homeostasis	[128]
10	IL1b-eRNA	Expression of CXCL8 and IL-1β (proinflammatory mediators)	[128]
11	Lethe	Activated during inflammation	[111]
12	NRAV	Transcriptional regulation of numerous interferon-stimulated genes (ISGs), including MxA and IFITM3	[129]
13	Morrbid	Control the longevity of myeloid cells with brief lives (neutrophils, eosinophils, and monocytes)	[130]
14	FAS AS1	Increase programmed cell death (Fas-driven) in B cell Carcinomas	[131]
15	lincRNA-EPS	Restriction of inflammation	[132]
16	TH2-LCR	Th2 cytokine gene transcription regulation	[133]
17	linc-MAF-4	Th1 cell differentiation	[134]
18	lnc-EGFR	Treg differentiation stimulation	[135]

8. LncRNAs in auto-immune diseases

Auto-immune diseases are conditions in which our immune system wrongly assaults our bodies, assumed to be the result of a complex interplay of genetic, immunological and environmental variables.

8.1 Systemic lupus erythematosus (SLE)

TLR4 signalling dysregulation has been connected to the progression of SLE. A lncRNA has been associated to SLE and is controlled by TLR4 signalling. Nuclear enriched abundant transcript 1 (NEAT1), a lncRNA, has been connected to the

pathogenesis of SLE [136]. In a study NEAT1 levels were found to be elevated in PBMCs of SLE patients in comparison with healthy individual. In LPS-stimulated human monocytic cell lines, silencing NEAT1 with siRNA results in lower production of Interleukin-6, CCL2, and CXCL10, all of which have been related to SLE pathogenesis. NEAT1 regulates the stimulation of late mitogen-activated protein kinase signalling pathways, which play a role in the TLR4-driven inflammatory response. SLE patients have lower Gas5 expression in plasma, and in both CD4+ T cells and B cells than in healthy people [137].

8.2 Rheumatoid arthritis (RA)

Is an autoimmune illness characterised by inflammation and proliferation of synovial joint resulting in significant joint damage. Several recent investigations have found that dysregulated lncRNAs play a significant part in aetiology of RA. In 2015, Song et al. discovered that PBMCs and serum exosomes of RA patients have higher expression of HOTAIR than in the healthy controls. Furthermore, increasing HOTAIR increases active macrophage migration, whereas decreased HOTAIR suppresses the synthesis of matrix metalloproteinase (MMP)-2 and MMP-13 in developed osteoclasts and rheumatoid synoviocytes. These results suggest that abnormal HOTAIR expression could perform a part in the development of RA [138]. Low levels of lincRNA-p21 have been linked to increased NF-B activity in persons with Arthritis. Spurlock et al. observed that amount of phosphorylated p65, a hallmark of NF-B activation, was greater in the whole blood of patients suffering from RA however the expression of lincRNA-p21 was found to be downregulated. In comparison with MTX-treated RA patients, untreated counter parts had reduced levels of lincRNA-p21 activity and upregulated levels of p65 [139]. Lu et al. observed that T cells of patients suffering from RA have upregulated lncRNAs LOC100652951 and LOC100506036 in comparison with healthy individuals [140].

8.3 Sjogren's Syndrome (SS)

Chronic systemic auto-immune illness characterised by symptoms of driest mouth and eyes caused by gland inflammation (exocrine). The lncRNA Tmevpg1, which modulates Th1 responses, may have a role in the aetiology of SS, according to one research. The researchers discovered a connection between SS and the lncRNA Tmevpg1 [141].

8.4 Polymyositis and dermatomyositis (PM/DM)

Muscle inflammatory illness that is persistent. PM/DM has been connected to the expression of the RNA component of signal recognition particles, lncRNA 7SL [142]. Using microarray research, Peng et al. discovered 1198 differently transcribed lncRNAs in diabetes patients muscles compared to normal ones. uc011ihb.2, ENST00000583156.1, ENST00000551761.1, ENST00000541196.1 & linc-DGCR6-1, were among the five lncRNAs confirmed. As per their computational predictions, the linc-DGCR6-1 gene controls USP18, a kind of type 1 interferon-inducible gene found predominantly in the perifascicular portions of muscle fibres of diabetes patients [143].

9. Conclusion

Although there has been immense research going on to understand the role of lncRNAs, still there are many questions that need to be addressed. Efforts are being

carried out to elucidate the role of lncRNAs as potential regulators in different biological and pathological processes. The advancement in technologies will further help to pave way in clarifying the mechanisms underlying of lncRNA influence in different diseases. Studying lncRNA role in regulation of host-pathogen interactions can be helpful in identifying different lncRNAs that can serve as potential drug targets and can also serve as novel biomarkers.

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Chapter 3

Advantages of Noncoding RNAs in Molecular Diagnosis

Tomomi Fujii, Tomoko Uchiyama and Maiko Takeda

Abstract

Noncoding RNAs contribute to physiological processes by regulating many intracellular molecules participating in the life-supporting mechanisms of development, differentiation, and regeneration as well as by disrupting various signaling mechanisms such as disease development and progression and tumor growth. Because microRNAs (miRNAs) target and regulate the functions of key proteins, it is very useful to identify specific miRNAs that contribute to cellular functions and to clarify the roles of their target molecules as diagnostic and therapeutic strategies for cancer prognosis and treatment. In this section, the roles of miRNAs in various cancers and the processes leading to the identification of their target molecules are described, and the latest diagnostic strategies using miRNAs are discussed with specific examples.

Keywords: microRNA, cancer, molecular diagnosis

1. Introduction

Noncoding RNAs (ncRNAs) comprise the majority of gene transcripts in eukaryotes. ncRNAs play little biological role, but microRNAs (miRNAs), first reported in 1993, have important roles in gene expression [1, 2]. One of the most well-known miRNAs, let-7, a heterochronic gene in C. elegans, is considered important for developmental differentiation and regulates the larva-to-adult transition during larval development [3]. As a summary of the biosynthetic process of miRNAs, double-stranded RNA, the source of RNA interference, is sequentially processed into single-stranded RNA fragments of 21–23 nucleotides (nt) by the action of various enzymes [4, 5]. Let-7 encodes a small 21-nt ncRNA that binds to the 3′-untranslated region (UTR) of lin genes, resulting in RNA–RNA interactions and negative regulation of mRNA [6]. More than 30,000 miRNAs have been identified from more than 250 species, ranging from prokaryotes to eukaryotes, invertebrates to vertebrates, and even viruses [7, 8]. More than 5000 of these miRNAs have been identified in humans [miRBase: The microRNA database; cited 2018 Aug; Available from: http://www.mirbase.org].

miRNAs function as posttranscriptional regulators (negative regulators) of various gene expressions by binding to complementary sequences, primarily in the 3′-UTR of their target genes. miRNAs can target a large number of genes, and the mRNAs targeted by miRNAs mRNAs targeted by miRNAs account for more than 60% of all genes. Most miRNAs are encoded within the introns of coding mRNAs, but

some can also be found within the 3′-UTR sequences of noncoding or coding mRNAs but can also be found in the 3′-UTR sequence of noncoding or coding mRNAs [9]. When miRNAs bind to target mRNAs, these are destabilized by deadenylation factors becoming more susceptible to degradation. As a result, mRNA is degraded; however, sometimes the mRNA is not degraded and translation is regulated, a mechanism that is not yet unclear. Alterations in the genetic structure of miRNA-associated regions are known to occur in many pathologies, especially cancer. Such chromosomal aberrations include amplifications (e.g. miR-26a in gliomas), deletions (e.g. miR-15a/16–1 in chronic lymphocytic leukemia), mutations (e.g. miR-125a in breast cancer), translocations [e.g. miR-125b in acute myeloid leukemia with t(2;11)(p21;q23)], single-nucleotide polymorphisms (e.g. miR-608 in colorectal adenocarcinoma), and heterozygous deletions (such as the 14q32 cluster in acute lymphoblastic leukemia) [10–15]. These changes can occur not only in the sequence of the mature miRNA itself but also in the promoter region/primary transcript (pri-miRNA) sequence or in the miRNA binding site of the target gene, and as somatic or germline mutations [16–18]. A number of transcription factors, as well as vital protein-coding genes, influence the expression levels of miRNAs, and this regulatory action is assumed to be particularly important for tissue specificity and developmental stage control. Conversely, loss of Dicer, DGCR8, Drosha, and Argonaute2(Ago2), components of pathways involved in the miRNA biosynthesis mechanism, is known to result in the inability to sustain life and early pregnancy death due to severe developmental defects. [19–22]. Loss of reproductive function due to the deletion of components involved in miRNA biosynthesis, and developmental defects in the heart, lungs, and neuromuscular tissue have been reported [23–30]. Dicer mutations have also been reported to cause tumor development and progression [31, 32]. Dysfunction of the miRNA biogenesis pathway causes the disruption of important life-support mechanisms associated with various diseases, among which cancer development and progression are strongly influenced. In particular, abnormal expression of Dicer and Drosha is known to lead to poor prognosis in various cancers [33–39]. Although there is a vast amount of evidence that miRNAs play fundamental roles in the mechanisms of many diseases, including cancer, unfortunately, they have not yet been fully exploited in diagnostic and therapeutic settings. It is therefore necessary to determine how miRNAs can potentially be utilized in clinical practice in the future. miRNAs likely have the greatest and most direct potential as novel biomarkers of diagnosis and prognosis, as well as predictors of therapeutic efficacy. It is also clear that miRNAs regulate or disrupt the expression of numerous target mRNAs, resulting in maintenance of vital activity or disease, which cannot be resolved by a single miRNA. Therefore, gene expression analysis by miRNA expression profiling in disease must accurately discriminate between disease diagnosis and tumor stage of development [40].

A more accurate diagnosis of tumors can be obtained by morphological histopathology using formalin-fixed paraffin-embedded (FFPE) tissues and profiling of expressed proteins via immunohistochemical staining. Genetic analysis using FFPE is also emerging as being important for therapeutic selection. However, the degradation of nucleic acids is a problem for FFPE, and nucleic acids extracted from aged tissues are degraded [41]. A particularly attractive property of miRNAs is their stability against chemical and enzymatic degradation. This implies that they can be purified from routinely prepared FFPE biopsy materials and measured robustly [42]. As a result, retrospective miRNA expression studies are underway, using FFPE as a conserved and valuable resource. The stability and clinical utility of miRNAs are due to their presence in extracellular biological fluids, including blood, as well as in

tissues and cells. Tumor-associated miRNAs are found at higher levels in the serum and plasma of cancer patients than in healthy controls. Therefore, the use of miRNAs as potential noninvasive biomarkers of disease has attracted much attention, and miRNAs have been detected in many biological fluids, including plasma, serum, tears, urine, cerebrospinal fluid, breast milk, and saliva [43, 44]. The widespread use of biomarker testing, which is noninvasive and more reliable than the conventional histopathological diagnosis of cancer that invasive to the patient, could pave the way for screening programs, improve the early detection rate of disease, and enhance cancer prevention. Despite the growing functional importance of miRNAs as diagnostic and therapeutic strategies for cancer, there are still many knowledge gaps despite the potential to develop miRNA markers in the future.

2. Role of miRNAs as oncogenes and tumor suppressor genes

The role of miRNAs in regulating the genes involved in cancer growth and progression may be to suppress cancer progression primarily through suppressing target gene expression or to inhibit the function of genes that suppress cancer progression, therefore positively affecting cancer progression. The results obtained by detailed profiling of miRNA expression in cancer according to organ or cancer differentiation level revealed that the expression pattern of miRNAs varies depending on the organ, histological type, and differentiation level of cancer, indicating that the role of miRNAs is highly segmented [40]. Many miRNAs function in a suppressive manner in cancer. The expression of the aforementioned let-7 miRNA is downregulated in lung cancer, targeting and suppressing the expression of Ras, an important oncogene. This indicates that let-7 may function as a cancer suppressor gene [45]. The expression of miR-15 and miR-16 is absent or decreased in chronic lymphocytic leukemia, suggesting they act in a tumor-suppressive manner by targeting B cell lymphoma-2, an antiapoptotic factor [46]. Human miR-373 binds to the E-cadherin (CDH1) promoter and induces gene expression [47]. In addition to gene silencing through base pairing with DNA and mRNA target sequences, miRNAs are known to inhibit the function of regulatory proteins (decoy activity). miR-328 binds to poly C-binding protein 2 (PCBP2), also known as heterogeneous ribonucleoprotein E2. This binding does not involve the seed region of the miRNA, but it inhibits its interaction with the target mRNA. In chronic myeloid leukemia, downregulation of miR-328 inhibits myeloid differentiation via PCBP2, leading to tumor progression [48]. It is also important to understand the molecular mechanisms of miRNAs to define the mechanism of their suppressive properties against cancer. miRNAs primarily target the 3′-UTR of mRNAs and downregulate the cytoplasmic expression of protein-coding genes. As evidence for this, miRNAs are usually localized in the nucleus [49]. In addition to the 3′-UTR, other genetic regions at the DNA and RNA levels, that is, the 5′-UTR, promoter, and coding regions, as well as proteins can be targeted (**Figure 1**) [50–52]. By binding to various functional regions of genes, miRNAs can upregulate or downregulate gene expression, interact with other ncRNAs and RNA transcripts, and modulate biological networks [53, 54]. It has become clear that the actions of these miRNAs on their target molecules lead to the regulation of gene expression [55]. It has been shown that miRNAs not only bind to key sites in other RNAs but also to promoter regions at the DNA level, thereby affecting transcriptional activity. As a specific example, in terms of localization sites, miRNAs have been shown to function both in the cytoplasm and nucleus [56]. Investigation of the subcellular localization of miR-29b revealed

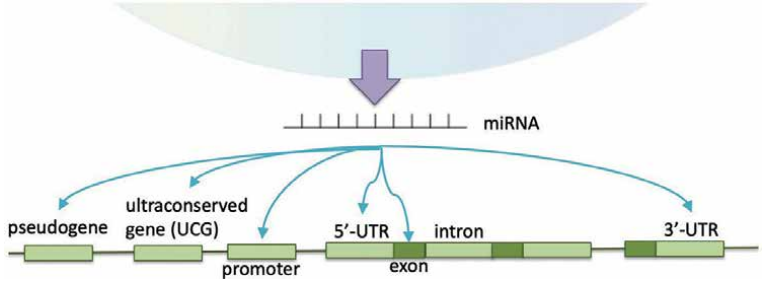

Figure 1.
Predicted binding sites for miRNAs in genes. miRNAs have binding sites not only in the 3′UTR but also in every site of the gene.

it was mostly localized to the nucleus. The characteristic hexanucleotide terminal motif of miR-29b functions as a metastable nuclear localization element that induces the nuclear localization of the miRNA or small-interference RNA to which it binds [49]. It has been shown that miRNAs bind to adenylate-uridylate-rich elements and upregulate translation in cells that have undergone cell cycle arrest. This suggests that miRNAs can affect not only cell differentiation and proliferation stages in a broad sense but also the cell cycle [57].

3. Expression dynamics of miRNAs in cancer and expectations for clinical application

Cancer is a malignant disease in which a population of cells with genetically abnormal protein expression proliferate autonomously, destroying host tissues as they grow, metastasize, and settle in other organs via vascular flow. As a result, cancer causes the dysfunction of normal tissues and ultimately leads to organ failure. There is evidence that carcinogenesis is a multistep process in which malignant cells accumulate epigenetic and genetic changes that gradually transform normal cells into malignant cells. Triggers for carcinogenesis include tobacco, chemical exogenous stimuli such as chemicals and dust from air pollution, and accumulation of genetic damage due to the aging of individual cells [58]. The proliferation of cancer cells progresses by causing structural changes in genes that promote cancer cell proliferation (oncogenes), genes that prevent their proliferation (tumor suppressor genes), and abnormal gene expression [59–62]. Malignant cells lose cell discipline in morphology and differentiation and acquire autonomous proliferation, antiapoptosis, and invasiveness properties. miRNAs have been analyzed in various human malignant tumors and can be involved in the pathophysiology of benign and malignant tumors in various ways. Although there are differences in miRNA expression profiles between normal and tumor tissues, many of these miRNAs are only indirectly altered by genetic changes that occur during carcinogenesis, epigenomic dynamics, and physiological changes in cell biology and do not directly trigger tumor development. Differentially expressed miRNAs between tumor and normal tissues have been identified in lymphomas, breast cancer, lung cancer, papillary thyroid cancer, glioblastoma, hepatocellular carcinoma, pancreatic tumor, pituitary adenoma, cervical cancer, brain tumors, prostate cancer (PCa), kidney and bladder cancer, and colon cancer [63–76]. The involvement of miRNAs in the carcinogenic process may involve changes in the components of

miRNA biosynthesis pathway, and overall suppression of miRNA maturation by mutations in DROSHA, DGCR8, or DICER has been shown to affect the transcription and translation of multiple mRNAs, ultimately promoting cell transformation and tumorigenesis [77–79]. The fact that conditional loss of DICER or DGCR8 inhibits bone and cartilage growth and differentiation in mice and in vitro has been demonstrated, suggesting that components involved in the miRNA biosynthesis mechanism are also key molecules in carcinogenesis and cancer progression [80, 81]. The expression of Dicer, Exportin-5, TRKRA, TARBP2, DGCR8, and Argonaut-2 changes dynamically with changes in miRNA expression during the oncogenic phase in pancreatic cancer, suggesting that these miRNA biosynthesis-related molecules can cause cellular changes, including carcinogenesis [82]. Decreased expression of some components that play an important role in miRNA biogenesis, such as DICER, is associated with cancer prognosis and sensitivity to molecularly targeted drugs and has been shown to correlate with short survival in non-small cell lung cancer while increasing sensitivity to gefitinib [36, 83]. The methylation of DICER and DROSHA is also a potential biomarker for lung cancer [84]. Some miRNA precursors, known as mirtrons, have the unique feature of entering the miRNA biosynthesis pathway without undergoing Drosha-mediated cleavage while mimicking the structural features of pre-miRNAs. They are susceptible to epigenetic regulation and have been shown to exert epigenetic effects through interactions between various miRNAs and host genes in urothelial cell carcinoma [85–87]. Breast cancer susceptibility gene 1 (BRCA1) promotes the processing of primary miRNA transcripts and increases the expression of both precursors and mature forms of let-7a-1, miR-16-1, miR-145, and miR-34a [88]. BRCA1 interacts with Smad3, p53, and DHX9 RNA helicases by directly binding to DROSHA and DDX5 in the DROSHA microprocessor complex that regulates miRNA maturation and recognizes RNA secondary structures through its DNA-binding domain, which interacts with pri-miRNA via the DNA-binding domain.

Thus, from their discovery to the present, miRNAs have brought great promise and success in cancer diagnosis, prognosis, and treatment both in clinical practice and experimental medicine. Powerful technologies, such as miRNA arrays, short RNA deep sequencing, specific quantitative polymerase chain reaction (PCR) of miRNAs, and antisense technologies, have revealed the functions of numerous miRNAs. It is expected that they will continue to have a major impact on clinical oncology in terms of cancer diagnosis and prognostic treatment. Because miRNAs are important factors that define cellular characteristics, direct detection of miRNAs and miRNA target molecules from algorithms based on the vast amount of data obtained by profiling analysis could be used as a valuable tool for cancer diagnosis. miRNA expression profiling has been considered more efficient and informative than conventional mRNA profiling in classifying tumors with respect to their tissue of origin and differentiation [89–93]. These findings indicate that in clinical practice, miRNAs are useful biomarkers for tracking the tissue of origin of tumors [94–97]. miRNA expression profiles can be an important source of information for cancer progression prediction and prognosis. It has been shown that high expression of miR-21 and miR-155 in lung cancer and colorectal cancer (CRC) predicts efficient recurrence, poor prognosis, and reduced survival [98–101]. Functional analysis of miRNAs through experimental processes has been found to be valuable for therapeutic strategies based on the regulation of miRNA activity. As a therapeutic strategy against miRNAs acting as oncogenes (oncomirs), anti-miRNAs are expected to exert therapeutic effects by blocking oncomirs through the action of oligonucleotides that are complementary to miRNAs. For them to function stably, they must be chemically modified to enhance their

stability and deliverability to the target organs. As is the case with many drugs used in cancer therapy, issues related to the delivery of miRNA-containing compounds, their stability in vivo, and biological defense measures against toxicity are important issues that must be resolved as miRNAs progress from experimental to clinical trials. However, because miRNAs have multiple targets, inhibition of target molecules by miRNAs may cause serious side effects. These issues are expected to be resolved and applied clinically in the future.

4. Functions of miRNAs in various organs

Given their properties, miRNAs have many target molecules and each performs different functions during cell differentiation and proliferation. Therefore, miRNAs are expected to perform characteristic functions in various organs. Here, the functions of miRNAs in typical cancers are introduced.

4.1 Breast cancer

miRNA profiling studies in breast cancer have been conducted from various perspectives. The first miRNA shown to be highly expressed in metastatic breast cancer was miR-10b (using mouse and human cells), with clinical correlation in primary breast cancer [102]. Subsequent studies confirmed the elevated levels of miR-10b, miR-34a, and miR-155 in patients with metastatic breast cancer [103]. Furthermore, miR-10b and miR-373 were recently shown to be upregulated in lymph node-positive breast cancer [104]. In addition, miRNAs from previous miRNA profiling studies have been shown to play potential roles as tumor suppressor genes or oncogenes in breast cancer [65, 105, 106]. The decreased expression of many genes involved in cancer development and progression has become evident, and miRNAs and their target molecules that are downregulated in breast cancer tissues and cell lines have become the focus of research. Breast cancer is a cancerous tissue with high histopathological heterogeneity. Identifying miRNAs and their target molecules that are specifically expressed and function in this context and have diagnostic value is therefore difficult. miRNA profiling should facilitate the accurate classification of tumor subtypes and provide useful additional information to complement current classification methods and guide therapeutic decisions. Studies have nested to predict miRNAs in accordance with previous subtype classifications. Recent analyses of miRNAs have identified predicted miRNAs corresponding to hormone receptor and HER2 subtypes, including ER (miR-342, miR-299, miR-217, miR-190, miR-135b, and miR-218), PR (miR-520g, miR-377, miR- 527-518a, miR-520f-520c), and HER2 (miR-520d, miR-181c, miR-302c, miR-376b, and miR-30e). These expression kinetics classified cases with 100% accuracy compared to immunohistochemistry results [107]. Further classification is expected to increase miRNA profiling accuracy and the development of new diagnostic markers and therapeutic strategies based on miRNA target molecules.

4.2 Prostate cancer

Prostate cancer (PCa) is one of the most common causes of cancer-related death in men. Although most PCa progress very slowly and remain within the primary tumor,

some have an aggressive behavior that can spread to other organs via the vascular system. Prostate-specific antigen (PSA) and prostate acid phosphatase (PAP) are useful tumor markers for prostate cancer. However, PAP has low specificity and PSA, despite its superior specificity, is inadequate for predicting the tumor types in which it is not elevated and histological differentiation and progression. Many studies have indicated that miRNAs are involved in the development of PCa [108–112]. Aberrant expression of miRNAs has been detected in PCa cell lines, in experimental carcinogenesis using cancer xenografts, and in clinical samples obtained from patients [113–118]. These changes suggest that miRNAs play an important role in the pathogenesis of PCa.

Many miRNAs are expressed as oncogenes. For example, miR-375, miR-106a, and miR-194 have been reported to play important roles in tumor formation, proliferation, and progression [119–121]. When the expression of tumor suppressor miRNAs decreases, the expression of oncogenes increases, promoting cancer growth and progression. Experimental introduction of precursors of these miRNAs into cells and overexpression of these miRNAs to reproduce normal expression patterns suppressed the growth, invasion, clonogenesis, and metastatic potential of PCa cells. The most well-known miRNAs are miR-149-5p, miR-7-5p, miR-122, miR-124-3p, miR-188, and miR-129 [122–127]. Downregulation of these miRNAs is associated with increased cell proliferation, migration, and chemotherapy resistance, primarily through the regulation of cell growth and proliferation via the mitogen-activated protein kinase pathway.

Clinically practical markers in PCa include serum PSA measurement and digital rectal examination; however, their diagnostic value is limited and does not extend to prognostic prediction based on cancer progression or assessment of differentiation related to tumor growth potential. Human glandular kallikrein 2, urokinase plasminogen activator and its receptor, transforming growth factor-β1, and interleukin 6 and its receptor are biomarkers with high sensitivity and specificity for the early diagnosis of PCa. miRNAs are also clinically promising diagnostic markers for this cancer. miRNAs can be used as diagnostic and disease-monitoring markers for the presence of minimal residual disease and early detection of recurrence. Furthermore, the establishment of a diagnostic algorithm combining multiple oncomir and tumor suppressor miRNAs is expected to enable a more stepwise diagnosis of PCa. In this cancer, histopathological diagnosis by biopsy is highly invasive and often fails to detect microscopic cancers owing to the extremely small amount of visible tissue. To compensate for the shortcomings of local biopsy pathology diagnosis, the availability of profiling tests with circulating miRNAs has the potential to predict the onset of cancer and the degree of differentiation through its combination algorithm. From the time of diagnosis and initiation of treatment, the detection of circulating miRNAs could contribute to the early detection of hormone refractory PCa. The role of cancer stem cells in PCa has been elucidated in previous studies. The CD44-positive PCa stem cell population has tumorigenic and metastatic potential, which has been mentioned in conjunction with other useful markers for cancer stem cells [128]. Functional analysis of miRNAs has shown that miR-9-5p, miR-34a, miR-373, miR-708, and miR-520c directly target CD44 suppressing its expression [129–133]. The fact that the expression levels of these miRNAs are decreased in PCa suggests an increased potential for tumor invasion and metastasis. Profiling of miRNAs in PCa holds promise as a more precise diagnostic tool before performing prostate biopsy.

4.3 CRC

CRC is a common cancer worldwide and is the second most common cause of cancer-related deaths in the Western world. Although the 5-year survival rate for early-stage cancer exceeds 90%, it decreases to approximately 70% for advanced cancer and to approximately 10% when distant metastases are present. Although it is certainly a carcinoma for which early diagnosis is very useful, in reality early detection is less than 40% due to insufficient noninvasive screening. Although direct diagnosis by endoscopy is most effective in CRC, it often relies on a fecal occult blood test because of its invasive nature. However, the fecal occult blood reaction can be positive even for inflammatory or nonneoplastic polyps and lacks specificity in terms of cancer diagnosis. If CRC can be found to have miRNA expression characteristics, analysis of miRNAs using stool and the detection of circulating miRNAs may be useful in detecting marginally present tumor cells. Therefore, we searched for miRNAs that were significantly elevated in CRC using stool and plasma and found a number of miRNAs, including miR-17, miR-18a, miR-19a, miR-21, miR-92a, miR-200c, miR-221, and miR-106a as diagnostic markers [134–138]. miR-141 is a miRNA that functions in an inhibitory manner against colon cancer cells in terms of cell proliferation, invasiveness, and migration [139–142]. As a circulating miRNA, miR-141, together with miR-200 and miR-143, could be an independent prognostic marker for advanced CRC [143, 144].

CRC progresses from adenoma to colorectal adenocarcinoma as precancerous lesions. Investigation of miRNA expression in staged histological types has revealed that the expression of several miRNAs is altered in precancerous lesions, adenomas, and CRC tissues compared to that in normal colorectal tissue. This suggests that some miRNAs are involved in the process of CRC and may be important biomarkers in the transition of tumor tissue from benign to malignant. In addition, miRNAs are now being extracted from stored FFPE tissue specimens and retrospectively profiled for miRNA expression using next-generation sequencing (NGS) to understand the origin of CRC development and metastatic disease in cases with multiple lesions. The most studied miRNAs with respect to clinical prognosis are miR-21, miR-143, and miR-145. Although miR-21 suppresses the expression of various tumor suppressor genes, it also affects cell proliferation, apoptosis, invasion, and tumor progression. Increased miR-21 expression in CRC tumor tissue correlates with increased metastatic potential of the primary tumor and is associated with shorter disease-free and overall survival. Owing to the antiapoptotic effect of miR-21, its overexpression was also found to affect therapeutic efficacy by reducing the efficacy of 5-fluorouracil (5-FU) chemotherapy [145–150]. Decreased expression of miR-143 and miR-145 in CRC tumor tissue has been observed in several studies, suggesting that they behave as tumor suppressors [132, 151–154]. Their decreased expression has been associated with increased tumor growth and angiogenesis, and it is clinically associated with shorter disease-free survival. The expression of miR-143 directly correlates with the sensitivity of the colon cancer cell line HCT116 to 5-FU treatment. miR-215 is involved in the cell proliferative capacity of CRC and is sensitive to chemotherapy and molecular-targeted therapy. miR-215 is also involved in the prognostic prediction of CRC [155–160].

Although biomarker analysis using stool as a method to directly capture tumor cells in CRC is open for consideration, the establishment of a methodology for good-quality nucleic acid extraction that enables good analysis and sample quality maintenance is required to obtain reliable data due to the heterogeneity of sample processing.

In contrast, for the prediction of treatment potential and prognosis for advanced cancer, miRNA profiling can be performed by nucleic acid extraction from FFPE of excised tumor tissue, and sufficient technology is being established for accuracy and reliability.

5. Analysis of circulating miRNAs for their usefulness as biomarkers

In addition to serum and plasma, various body fluids, such as saliva, cerebrospinal fluid, and body cavity fluid, are candidates as analyzable materials for the detection of circulating miRNAs. However, in samples of these fluids from cancer patients, not only tumor cells but also blood cells, macrophages, exosomes, and microparticles are present; where the miRNAs are present, there is also the issue of how many of the targeted tumor-derived miRNAs are included or how reliable the analysis is based on which miRNAs are included. Nevertheless, regardless of the members, each of whom may possess miRNAs, if the changes caused by the coexistence of cancer can be captured for cancer patients, the usefulness of profiling will be well maintained. It is well known that analysis of exosome-derived miRNAs can detect more cancer-relevant miRNAs than analysis using untreated serum. An interesting aspect of the quantitative analysis of miRNAs in exosomes and microparticles is that they may be involved in cell–cell interactions, stimulate intracellular signaling, and modulate metabolic functions and homeostasis in a small number of stem cells. Quantitative analysis of circulating miRNAs is most useful for miRNAs quantitative real-time PCR (qRT-PCR), because it detects miRNAs from a very small amount of nucleic acids. Although NGS is superior for expression profiling, it requires a sufficient amount of nucleic acids and is likely to lack reliability due to detection errors or low sensitivity. Therefore, a single-miRNA assay using the TaqMan method should be performed on miRNAs with presumed abnormal expression at the transcriptional level, as detected from microarray analysis or NGS analysis based on FFPE, followed by qRT-PCR validation. Capturing abnormal miRNA expression in cancer is a highly useful diagnostic strategy but, because capturing circulating miRNAs involves sampling a physiological diverse environment, we may be quantifying miRNAs derived not only from cancer but also from blood or other organs. In addition, miRNAs derived not only from cancer but also from coexisting diseases may be captured; therefore, it is not always the case that miRNAs of cancer cells are captured. In other words, the possibility of observing the dynamics of miRNAs in a broad sense, including in the cancer microenvironment, must be fully understood. Nevertheless, the analysis of circulating miRNAs is highly useful as a potential candidate for blood-based biomarkers that can be tested repeatedly. Circulating miRNAs are promising diagnostic biomarkers owing to their high information content and disease-specific regulation. miRNAs inhibit the translation of various proteins involved in disease-related signaling pathways, including malignant and benign diseases, and alterations in physiological states, thereby inhibiting specific signaling pathways. Therefore, the analysis of the complex profiling of multiple miRNAs involved in complex pathways is expected to provide more specific diagnostic algorithms.

Biomarker development involves several important issues. The use of various analytical technology platforms, serum or plasma samples, extraction of miRNAs from whole serum/plasma or exosomes/microvesicles separated by specific disease-related markers, and sample collection, storage, and processing, all need to be addressed. Depending on the time point of blood sample collection (presurgery, during surgery,

and posttreatment) and patient treatment (surgery, chemotherapy, immunotherapy, and/or drug therapy), the expression profile of circulating miRNAs may change. It is also important to establish endogenous miRNA control and assess its consistency with the patient's clinicopathological data to assess prognosis.

6. Conclusions

miRNA expression profiling for cancer diagnosis can be subdivided into miRNA expression patterns based on signaling common to cancer, such as cell proliferation, invasion, migration, and antiapoptotic effects, and miRNA expression patterns according to various organ or tumor differentiation levels and tissue types. In addition, the expression patterns of miRNAs in cancers of various organs are different according to cancer differentiation levels and tissue types. In addition, the degree of differentiation and tissue classification are often heterogeneous among cancers of various organs, and the estimation of miRNAs based on the differentiation of each organ or developmental stage and the miRNAs that may be targeted may be important determinants for organ identification in so-called primary unknown cancers. A vast number of miRNA expression analyses in various cancers are expected to be reported in the near future. As more cancer-specific miRNAs become known, they will be systematically classified and miRNA expression profiling consistent with conventional immunohistochemical staining of cancer tissues is expected to have practical application.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 4

Role of Non-Coding RNAs in Lung Cancer

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Abstract

Lung cancer is the most common cancer worldwide, and the leading cancer killer in both men and women. Globally, it accounts for 11.6% of all cancer cases and is responsible for 18.4% of cancer-related deaths. The mechanisms underlying lung cancer development and progression have been widely studied, and roles for non-coding RNAs (ncRNAs) have been identified. Non-coding RNAs are a type of RNA molecules that are not translated into proteins. The main types of ncRNAs include transfer RNAs (tRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), small nucleolar/nuclear RNAs (snoRNAs, snRNAs), extracellular RNAs (exRNAs), tRNA fragments, and long non-coding RNAs (lncRNAs). In the past few years, there has been an increased interest in the role of ncRNAs in oncology, and lung cancer tumorigenesis specifically. Multiple ncRNAs were identified as tumor suppressors: tRNA fragments, snoRNAs, and piRNAs while others were reported to have tumor-promoting functions: circular RNAs (circRNAs), snoRNAs, piRNAs, YRNAs, natural antisense transcripts (NATs) and pseudogene transcripts. In this chapter, we discuss the latest body of knowledge regarding the role of ncRNAs in lung cancer pathogenesis as well as their potential use as biomarkers or therapies against lung cancer.

Keywords: lung cancer, miRNAs, adenocarcinoma, squamous cell carcinoma, NSCLC, SCLC, small RNAs, non-coding RNAs

1. Introduction

Lung cancer is responsible for 19% of cancer-related deaths, making it the number one cause of cancer-related mortality worldwide. A recent global cancer statistics report has estimated that approximately 2.2 million new cases and 1.8 million deaths were reported in 2020 [1, 2]. Similarly, in the United States, 230,000 new cases of lung cancer and 132,000 deaths were reported in the same year [1].

While smoking is a well-investigated major risk factor, responsible for approximately 80% of total lung cancer fatalities, not all lung cancers are associated with smoking, particularly in women [3]. Other environmental and occupational factors have been linked to lung cancer development, including second hand smoke exposure, ionizing radiation, arsenic in drinking water, and radon, silica, asbestos, heavy metals, and air pollution exposures. Moreover, genetic factors and other comorbidities such as

chronic obstructive pulmonary disease (COPD) have been identified as risk factors for lung cancer [4].

Lung cancer is defined as a cancer that starts in lung tissue. The condition is divided into two main types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The NSCLC type represents around 85% of all lung cancer cases, whereas the SCLC represents the remaining 15% [5, 6]. The NSCLC is further divided into several subtypes which include lung adenocarcinoma (LAC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). The World Health Organization (WHO) has classified lung tumors into multiple subtypes. This classification relies on the use of immunohistochemical characterization and microscopy and provides standardized criteria and terminology for diagnosis on small biopsies and cytology [7]. It also provides guidance for molecular testing, recognizing the therapeutic importance of targetable genetic alterations.

The molecular basis of lung cancer development is complex and involves a combination of genetic and epigenetic alterations that regulate tumor development. Multiple pro-oncogenic and tumor suppressor genes, as well as growth factors, transcription factors and other regulatory molecules are involved. Recent studies describing the pathways involved in lung cancer tumorigenesis have identified non-coding RNAs (ncRNAs) as important players in its pathogenesis regulation. Non-coding RNAs are a subtype of RNA molecules that regulate gene expression at the transcriptional and post-transcriptional level but are not translated into proteins. These ncRNAs are divided into two categories, short non-coding RNAs (less than 200 bp) an long non-coding RNAs (lncRNAs, more than 200 bp) [8]. The category of short ncRNAs consists of microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), small nucleolar RNA (snoRNA), tRNA-derived stress-induced RNA (tiRNA), YRNA and transfer RNA-derived RNA fragment (tRF). Further, the lncRNA makes up a heterogeneous category, which includes long intergenic non-coding RNAs (lincRNAs), bidirectional lncRNAs, intronic lncRNAs, overlapping sense lncRNAs, circular intronic RNAs (ciRNAs), T-UCR lncRNAs (transcribed from ultra-conserved regions), antisense transcripts, and enhancer RNAs [9, 10]. The lncRNAs display additional subtypes, and can also act as precursors to smaller ncRNAs such as snoRNAs, miRNAs, piRNAs, or siRNAs [10]. A summary of known non-coding RNA subtypes is provided in **Table 1**.

2. Role of ncRNAs in non-small cell lung cancer

2.1 Role of ncRNAs in NSCLC development

Lung adenocarcinoma is the most common lung cancer subtype, representing 40% of all lung NSCLC cases [11]. It is also the most common subtype diagnosed in never smokers. This subtype of lung cancer usually occurs in the lung periphery, evolving from mucosal glands, but can also be found in scars or areas of chronic inflammation [12].

In early 2000, Hanahan and Weinberg proposed a set of molecular and biochemical principles, that the most of human cancers share based on the knowledge accumulated at the time. The initial list of six traits included apoptosis evasion, self-sufficiency in growth signaling, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential with tissue invasion and metastasis [13]. Two decades later, new traits and principles were introduced in the list of hallmarks with

ncRNA	Full name
miRNA	microRNA
piRNA	PIWI interacting RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
lncNRA	long non-coding RNA
circRNA	circular RNA
siRNA	small interfering RNA
tRNA	transfer RNA
tRF	tRNA fragments
tiRNA	tRNA-derived stress-induced RNA
TERC	Telomerase RNA Component
NAT	Natural Antisense Transcript
T-UCR	Transcribed ultra-conserved noncoding RNA

Table 1.
Common ncRNAs and standard nomenclature.

the latest set containing emerging hallmarks and cancer enabling characteristics (**Figure 1**). The enabling characteristics involve unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, senescent cells, and polymorphic microbiomes.

The last few decades have seen a steep increase in interest and consequent number of studies related to ncRNAs role in lung cancer development. Significant attention is given to main ncRNA groups such as miRNAs, lncRNAs, snoRNAs, piRNAs and as well as tRNAs with its derivatives. In the next few sections, we will be discussing the roles that some groups within ncRNAs families play in lung cancer tumorigenesis, diagnosis, prognosis, and therapy.

2.1.1 MicroRNAs

MiRNAs are the most studied group of ncRNAs involved in lung cancer. MiRNAs make up a family of small noncoding RNAs with length of 21–25 nucleotides that can promote mRNA degradation by inhibiting their translation [15].

Several miRNAs can act on the same target mRNA, but a single miRNA can also regulate several different target mRNAs. Studies estimate that 30% of genes are regulated by miRNAs, and they participate various processes, such as gene regulation, apoptosis, cell differentiation and hematopoietic development [15, 16]. One of the hallmarks of cancer is sustained cell proliferation and unsuppressed cell growth [14]. Proteins such as kinases and kinase receptors play an important role in cell proliferation processes. For example, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) can attach to EGF receptor (EGFR), activating it, and causing further signaling pathway activation downstream. This leads to activation of the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways, two major signaling pathways, that play role in cell cycle progression and proliferation [17]. The EGFR is a direct target of numerous miRNAs, thus they play an important role in cell proliferation processes.

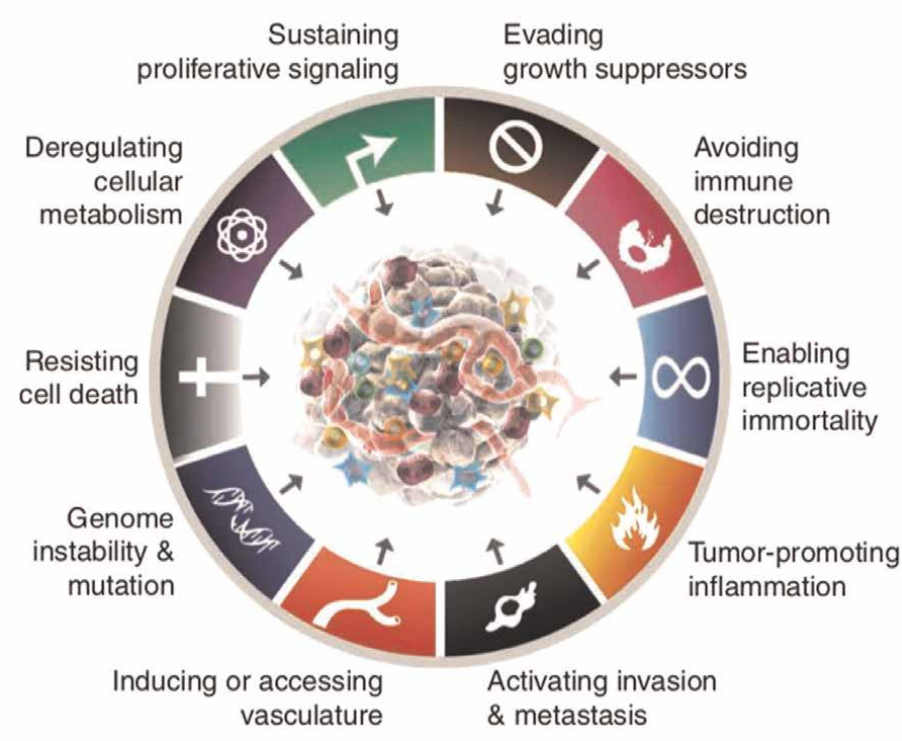

Figure 1.
The schematic representation of hallmarks of cancer (from Hanahan and Weinberg) [14].

These include, miR-146, miR-149, miR-7, miR-133, miR-27a-3p, miR-30, miR-34, miR-134, miR-145, miR-218, and miR-542-5p [15, 18–21].

The fusion proteins EML4 and ALK are also able to induce PI3K/Akt/mTOR and Ras/Raf/MEK/ERK pathways. The EML4 and ALK getting more interest as potential targets in NSCLC therapy, and miR-96 was shown to suppress ALK post-transcriptionally in a cell model [22]. The miR-760 had negative impact on cell proliferation in NSCLC cell lines by downregulating ROS1 expression, another potential therapeutic target. Same as previous kinase proteins, ROS1 promotes cell proliferation and survival via SHP-1/SHP-2, JAK/STAT, PI3K/Akt/mTOR, and MEK/ERK pathways [23, 24].

The Kirsten rat sarcoma 2 virus (KRAS) gene, encodes for K-Ras, which also acts through EGFR, ALK and ROS to activate Ras/Raf/MEK/ERK pathway [25, 26]. In a recent study, miR-193a-3p was reported to inhibit KRAS-mutated lung tumor growth by targeting KRAS directly [27]. Similarly, miR-181a-5p was reported to suppress A549 lung epithelial cells proliferation and migration by downregulating KRAS [28]. In addition, miR-148a-3p was found to suppress cell proliferation in NSCLC by downregulating SOS1, a protein involved in the Ras signaling pathway [29]. The miR-1258 was reported to suppress tumor progression by targeting GRB2/Ras/Erk pathway, while miR-520-3p was found to be involved in PI3K/Akt/mTOR pathway in vitro [30, 31].

The highly expressed miR-15a and miR-16 suppress cyclin D1, an important regulator of cell cycle progression. Cyclin D1 acts upstream of retinoblastoma (RB) pathway, and its suppression leads to RB upregulation and cell cycle arrest, which is a

necessary step leading to cell senescence or apoptosis [32]. The E2F3 gene, which encodes for transcription factor E2F3, is a target of miR-449a and has low expression levels in tumor tissues. The miR-449a upregulation suppresses E2F3 with consequent cell cycle blockage and cell senescence [33]. Finally, miR-641 and miR-660 were reported to promote lung cancer cells apoptosis through suppressing the p53 pathway by targeting MDM2 [34, 35]. **Figure 2** summarizes the list of miRNAs involved in pathways associated with cancer hallmarks.

In addition to regulating genes involved in cancer-related pathways, miRNAs are also involved in enabling cancer cells with unlimited replicative immortality. The miRNAs from the miR-29 family demonstrated tumor-suppressing effect in lung cancer cells in vitro by targeting DNMT3A and DNMT3B [36]. These DNA methyltransferases (DNMT) play a role in controlling telomere length similar to telomerase, being involved in cell replicative potential.

Another critical cancer feature that involves miRNAs is metastasis. An important factor in cancer metastasis is epithelial-to-mesenchymal transition (EMT), that normally takes place in the embryonic development stage. The EMT involves the loss of

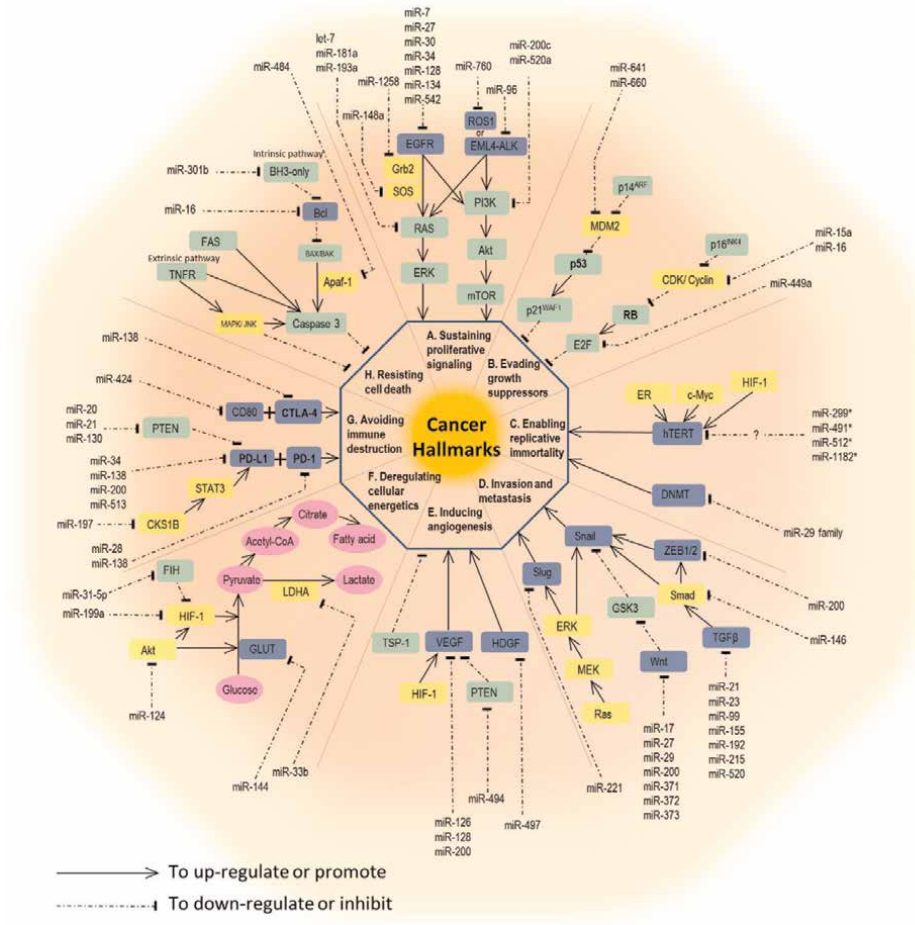

Figure 2.
A schematic diagram of miRNAs and their involvement in cancer hallmarks pathways (from Wu [15]).

E-cadherin-mediated cell adhesion and increased cell motility, which in turn promotes tumor invasion and metastasis. The miRNA 15a was reported to inhibit metastasis in NSCLC by targeting BCL2L2, and its overexpression significantly inhibited cell viability, invasion and migration [37]. There are several main transcription factors (TFs) related to epithelial-to-mesenchymal (EMT) transition that are directly targeted by miRNAs. These TFs are Snail, Slug, ZEB1, ZEB2 and Twist [38]. For example, miR-126 was reported to regulate NSCLC cell invasion and migration suppressing EMT by directly targeting PI3K/AKT/Snail pathway [39]. MiR-346 is upregulated in NSCLC and it promotes cell proliferation, metastasis and hinders apoptosis by targeting XPC/ERK/SNAIL/E-cadherin pathway [40]. Interestingly, miR-22 and miR-30a are downregulated in NSCLC, and they regulate EMT by directly targeting Snail [41–43].

In order to grow and spread, tumor cells require nutrients and oxygen, and that requires angiogenesis. Vascular endothelial growth factors (VEGFs) are the most studied group involved in angiogenesis process. MiRNAs from the miR-200 family were reported to suppress neovascularization in lung cancer cell lines by targeting the VEGF [44, 45]. Additionally, miR-126 and miR-128 were observed to target VEGF-A and VEGF-C, respectively, and inhibit angiogenesis in vitro when upregulated [46, 47]. When overexpressed, miR-497 was found to suppress angiogenesis in lung cancer by targeting an HDGF, and inducing a independent pathway [48]. However, there are miRNAs that promote angiogenesis as well. The miR-494 enhances angiogenesis by targeting a VEGF suppressor PTEN [49, 50]. Besides that, miR-23a activates VEGF pathway by targeting PHD1 and PHD2, inducing HIF-1α, and consequently promoting neovascularization [50].

Apoptosis, a programmed cell death process, is another cancer hallmark that miRNAs are involved in. The dysregulation of miRNAs can lead to downregulation of genes involved in apoptosis, as well as other tumor suppressor genes. Apoptosis can take place via two major pathways, extrinsic and intrinsic; the intrinsic pathway is triggered by genotoxic agents, metabolic aberrations, and transcriptional signals, while the extrinsic pathway is triggered by extracellular apoptotic signals [15]. These mentioned cues are picked up by BH3-only proteins, which results in deactivation of BCL-2 regulator proteins, with consequent activation of caspases, and eventual cell death [51]. In this context, there are miRNAs that can have antiapoptotic or proapoptotic effects. As central regulators of apoptosis, members of the BCL-2 family can be targets of several miRNAs in lung cancer cells. For example, BCL-2 is a direct target of miR-7 in NSCLC cells [52]. Another member of BCL-2 family, BCL-W was identified as a direct target of miR-335 in A549 and NCI-H1299 cells [53]. The miRNA-15a, that was mentioned earlier, besides inhibiting metastasis, also induces cell apoptosis by targeting BC2L2 [37]. In addition to BCL-2 related miRNAs, miR-608 has been identified as BCL-XL-induced miRNA and was found to be involved in regulating apoptosis in A549 and SK-LU-1 cells [54]. Another miRNA that induces apoptosis in lung cancer cells (A549, H460, and 95D cell lines) is miR-192. This miRNA acts by targeting the retinoblastoma 1 (RB1) gene [55].

When it comes to miRNAs with antiapoptotic effect, the activation of PPAR-γ/VEGF pathway by miR-130b leads to indirect Bcl-2 targeting and consequent suppression of NSCLC cell apoptosis [56]. A study showed that miR-214 modulates NSCLC cells radiotherapy response through regulation of p38 MAPK, apoptosis, and senescence [57]. Similarly, miR-197 is upregulated in cancer tissue, and miR-197 knockdown in NIH-H460 and A549 cells promotes apoptosis induction. It was also shown that miR-197 can act on the p53 pathway on various levels to hinder apoptosis, and to promote cell proliferation [58]. Other miRNAs known to have antiapoptotic

properties in lung cancer are miR-21, miR-212, miR-17-5p and miR-20a. The miR-21 targets negative regulators of the RAS pathway and targets proapoptotic genes in NSCLC [59].

MiRNAs also participate in cellular energetics deregulation processes in cancer cells. For example, a decreased expression of miR-144 in vitro can increase glucose uptake by upregulating the glucose transporter (GLUT1) expression [60]. Besides, miR-33b was found to suppress cell growth in NSCLC by downregulating an enzyme participating in glucose metabolism lactate dehydrogenase A (LDHA) [61]. MiR-199a impacts glycolytic pathways by suppressing HIF-1α and consequently inhibits cancer growth [62]. MiR-31-5p also supports glycolysis through downregulation of the inhibitor of HIF-1α (FIH) gene, thus promoting cancer development [63].

2.1.2 Long noncoding RNAs

Long noncoding RNAs include transcripts with sizes that exceed 200 nt and they make up the majority of ncRNAs [64]. There are several subgroups of lncRNAs which are involved in lung cancer. Among the lncRNAs that have gained more attention among investigators, natural antisense transcripts (NATs), transcribed ultra-conserved region RNAs (T-UCR), telomerase RNA components (TERC), circular RNAs (circRNAs), and pseudogenes transcripts are the ones that stand out.

LncRNAs cover a wide range of functions such as cell and tissue differentiation, gene imprinting, interaction with transcription mechanisms, regulation of mRNA splicing and degradation, protein translation modulation and sponging of miRNAs to name a few [65]. Given a wide range of biological functions, lncRNAs are also involved in lung cancer mechanisms as well, with members having tumorigenic or tumor suppressor properties.

One of the most studied lncRNAs in lung cancer is MALAT1 and it is known to have carcinogenic properties through modulation of miR-124/STAT3. It is reported to be overexpressed in lung cancer and to promote carcinogenesis via miR-124 sponging which is a STAT3 suppressor [66]. The MALAT1 was also reported to promote epithelial-mesenchymal transition and metastasis through miR-204/SLUG axis [67]. Another closely studied lncRNA is XIST, which was reported to inhibit NSCLC development when downregulated by activating miR-335/SOD2/ROS signal pathway [68]. The XIST was also reported to control NSCLC proliferation and invasion by modulating miR-186-5p [69]. Its silencing suppressed cell proliferation, migration, and invasion, and promoted apoptosis through regulation of miR449a and Bcl-2 [70]. Another reported lncRNA with pro-tumor effect is HOTAIR, which is overexpressed in a various cancer types, including NSCLC [71].

Natural antisense transcripts (NATs), are a group of lncRNAs that have transcript complementarity with other RNA transcripts [72]. The NATs NKX2–1-AS1, WRAP53, FAM83A and AFAP1-AS1 were found to be upregulated in lung cancer, with first two increasing lung malignant cell proliferation rates [73, 74], FAM83A promoting lung cancer cell progression [75], and AFAP1-AS1 promoting cell migration in NSCLC [76]. On top of that, pseudogene transcripts such as DUXAP8 and DUXAP10 were reported to be upregulated while SFTA1P was found to be downregulated in NSCLC [77–79]. Other lncRNAs that have been reported as involved in lung cancer are T-UCRs. For example, Uc.338 and Uc.339 increase malignant cell cycle progression and migration in cells, and they are upregulated in NSCLC, while Uc.454, having the same biological role is downregulated [80–82].

CircRNAs make up a large group of ncRNAs and are produced through an unconventional splicing event called back-splicing. CircRNAs are part of the lncRNAs with coding potential, they possess important biological functions such as acting as miRNA and protein inhibitors and regulating protein function [83]. Circular RNAs can have several action mechanisms: interact with chromatin histones, act as miRNA sponges, entrap transcription factors to prevent gene transcription, attach themselves to RNA polymerase, and encircle protein-coding exons [84].

The role of the interaction between circRNA, miRNA and mRNA in lung cancer development is gaining more attention. A study reported that circRAD23B is overexpressed in lung tumors compared to healthy adjacent tissue. In these tumors, the circRAD23B sponged miR-593-3p and miR-653-5p, and normalized the expression levels mRNAs CCND2 and TIAM1, respectively [85]. Similarly, the circRNA100146 sponged miR-361-3p and miR-615-5p as a result of its overexpression. The sponging of miRNAs leads to upregulation of their respective targets, miR-361-3p targeting COL1A1, NFAT5, and TRAF3, and miR-615-5p targeting MEF2C in H460 NSCLC cells [86]. Another finding shows how an apoptotic resistance takes place in NSCLC due to sponging of miR-497, a BCL-2 (anti-apoptotic gene) suppressor by circPVT1 [87]. On the other hand, some circular RNAs such as circHIPK3 can have tumor-promoting effect by binding miR-193, miR-124, miR-654, and miR-379. The non-coding RNA hsa_circ_0007385 behaves as a competing endogenous RNA to miR-181 and is an oncogenic circRNA. One of the contributors to the cancer proliferation and improved invasion capacity in lung adenocarcinoma is the upregulation of hsa_circ_001358 [88]. Another circRNA that acts as an oncogene is circ_0000735. It is upregulated in lung cancer tissue compared to normal tissue, and it sponges tumor suppressor miRNAs miR-11,179 and miR-1182. The association of circ_0000735 with the lung cancer cells capacity to self-renew was also reported [89].

Table 2 provides a list of studies that investigated the role of circRNA-miRNA-mRNA axis in the context of lung cancer. There are circular RNAs that can convey tumor-suppressing capabilities utilizing their ability to sponge pro-carcinogenic microRNAs. One example is circ-ITCH, a circRNA with tumor suppressing ability that stems from its interaction with miR-7 and miR-214 [97].

2.1.3 Transfer RNAs

Transfer RNAs are a type of small ncRNA that plays a major role in protein synthesis by serving as a link between mRNA and the growing chain of amino acids [98]. Besides its known conventional function, tRNA dysregulated expression has been found in some malignancies. Transfer RNAs can be cleaved by ANG (angiogenin) and form tRNA-derived stress-induced RNAs (tiRNAs), which in turn can be further changed into tRNA-derived fragments (tRFs) [99]. As an example of tRNA dysregulation, the tiRNAs ts-3676 and ts-4521 were found to be downregulated in a lung tumor tissue when compared with healthy tissue [100]. Also, ts-46 and ts-47, tiRNAs with tumor suppressing properties were found to be downregulated in lung cancer. The introduction of mentioned tiRNAs in two lung cancer cell lines demonstrated their ability to negatively impact cell proliferation rate and self-renewal capacity in tumor cells.

An association between the tiRNAs ts-101 and ts-53 and PiwiL2, a protein that plays an important role in silencing transposons, was also found in lung cancer [101]. The tRNAs-Leu and tRNAs-Val were found to be overexpressed in lung tumor tissue,

circRNA	Targeted miRNA(s)	Indirect target(s)	Biological effect(s)	Reference
circ-RAD23B	miR-593-3p	CCND2	↑ Cell invasion	[85]
	miR-653-5p	TIAM1		
circRNA 100,146	miR-361-3p	NFAT5, COL1A1, TRAF3	↑ Cell invasion ↑ proliferation	[86]
	miR-615-5p	MEF2C		
circPVT1	miR-497	BCL-2	↑ apoptosis ↓ cell proliferation	[87]
	miR-125b	E2F1	↑ tumorigenesis	[90]
circFGFR3	miR-22-3p	Gal-1, p-AKT, and p-ERK1/2	↑ cell invasion	[91]
circ_0004015	miR-1183	PDPK1	↓ survival ↑ cell viability ↑ proliferation ↑ cell invasion ↓ drug (gefitinib) resistance	[92]
circPUM1	miR-326	CCND1 and BCL-2	↑ cell proliferation ↑ cell invasion ↑ cell migration	[93]
circFLI1	miR-584-3p	ROCK1	↑ Metastasis	[94]
circABCB10	miR-1252	FOXR2	↑ cell proliferation ↑ cell migration	[95]
circHIPK3	miR-124	SphK1, STAT3 and CDK4	↑ Cell proliferation ↓ apoptosis	[96]

Table 2.
Oncogenic circRNAs acting as miRNA sponges in NSCLC, and their biological effects (adapted from Braicu et al. [84]).

in 37% and 26% of samples, respectively. On the other hand, tRF-Leu-CAG was found to be overexpressed in not only lung tumor tissue, but also in serum and cell lines. The involvement of tRF-Leu-CAG in cell cycle progression and cell proliferation was reported, and its overexpression was shown to be associated with NSCLC progression. This tRF seems to interact with protein AURKA and its role in lung cancer needs further investigation [102].

2.1.4 Small nucleolar RNAs

Small nucleolar RNAs (snoRNAs) are a class of noncoding RNAs that guide the chemical modifications of ribosomal RNAs, transfer RNAs and small nuclear RNAs. SnoRNAs are located in the region of introns of genes that code for proteins or lncRNAs [84]. The snoRNAs also known to be a source of piRNAs (piR30840) [103]. The analysis of The Cancer Genome Atlas (TCGA) data has revealed that snoRNAs U60, U51, U28, U63, U104, HBI-100, U59B, HBII-419, HBII-142, and U30 are overexpressed, and HBII-420 is under-expressed, in lung cancer. Interestingly, SNORD15A was found to be significantly downregulated in non-smoker lung tissue with an overall difference pattern better distinguished in non-smokers compared to smokers [104]. The fact that SNORD78 is overexpressed in vitro, leads to cell

proliferation, promotes EMT and consequently leads to enhanced invasion capacity, adds to the critical role snoRNAs play in NSCLC development [105]. Additionally, SNORD116–26 was found to be downregulated in tumor-initiating cells, while SNRA42 and SNORA3 were upregulated in the same cell type. Silencing of SNORA42 in cancer stem cells (CSCs) results in lower levels of tumorigenesis in lung cancer cells, while decreased expression of SNORA3 and SNORA42 in patients with lung tumors is associated with improved overall survival rate [106].

2.1.5 PIWI-interacting RNAs

The P-Element induced wimpy testis (PIWI) are a type of proteins highly conserved in plants and animals which are responsible for stem cell and germ cell differentiation [107]. The PIWI-interacting RNAs (piRNAs) make up the largest group of noncoding RNAs in animal cells and they originate from transposable elements (TE), mRNA and lncRNA (**Table 3**) [84, 113–115].

Type of ncRNA	Transcript	Expression in tumor vs. control	Biological function	Reference
piRNA	piR-34,871, piR-52,200	↑	↑ apoptosis ↑ cell proliferation	[108]
	piR-35,127, piR-46,545	↓	None	[108]
	piR-L-163	↓	↓ cell migration ↓ cell cycle progression	[109]
tRNA fragments	ts-46, ts-47, ts-101 and ts-53	↓	↓ cell proliferation	[101]
	tRF-Leu-CAG1	↑	↑ cell cycle progression ↑ cell proliferation	[102]
	tRF-Leu-CAG2			
YRNA	hY4 RNA	↑	↑ cell proliferation	[110]
SNORD	SNORA42	↑	Preserve tumor initiating make-up of cancer cells.	[106]
	U60, U63, U28, U51, U104, HBII-419, U59B, HBII-142, HBI-100, U30	↑	None	[104]
	HBII-420	↓		
	SNORD78	↑	↑ in vivo tumorigenesis↑ lung tumor cell proliferation	[105]
	SNORA47, SNORA68, SNORA78, SNORA21, SNORD28 SNORD66	↑	None	[111]
	SNORD33, SNORD66 SNORD76	↑	None	[112]

Type of ncRNA	Transcript	Expression in tumor vs. control	Biological function	Reference
NAT	NKX2–1-AS1	↑	↑ lung malignant cell proliferation.	[73]
	WRAP53		↑ lung malignant cell proliferation	[74]
	FAM83A		↑ tumor progression	[75]
	AFAP1-AS1		↑ cell invasion and metastasis	[76]
Pseudogene transcripts	SFTA1P	↓	↓ cell migration and invasion	[79]
	DUXAP8	↑	↑ malignant cell survival, proliferation, tumorigenesis	[77]
	DUXAP10		↑ malignant cell survival, proliferation, migration, tumorigenesis	[78]
T-UCR	Uc.338	↑	↑ malignant cell cycle progression, invasion, and migration.	[80]
	Uc.339	↑	↑ malignant cell cycle progression, and migration.	[81]
	Uc.454	↓	↓ malignant cell cycle progression, invasion, and migration	[82]

Table 3.
Examples of ncRNA involved in lung cancer and their biological effects (adapted from Braicu et al. [84]).

2.2 Role of ncRNAs in NSCLC diagnosis and prognosis

2.2.1 MicroRNAs

When diagnosing a lung cancer, several miRNAs can be used to determine the cancer subtype. As an example, miR-205 can be utilized to distinguish SCC from other lung cancer NSCLC subtypes, whereas miR-124a is known for its specificity to LAC [116, 117]. In addition, four upregulated miRNAs (miR-93, miR-221 miR-30e, and miR-205,) show specificity to SCC, while another five upregulated miRNAs (miR-100, let-7e, miR-125a-5p, miR-29b and miR-29c) are specific to LAC [118]. Being able to distinguish primary lung tumor from metastatic tumor plays an important role for diagnosis and prognosis. The high levels of miR-182 were reported to be associated with primary lung tumors, while overexpression of miR-126 points to tumors of metastatic nature [119]. In a similar fashion, overexpression of miR-552 and miR-592 helps to distinguish primary LAC from metastatic colorectal adenocarcinoma [120].

The possibility of performing diagnosis based on miRNA expression in bodily fluids (blood, plasma, sputum) remains a topic of high interest for researchers due to feasibility and high potential as a diagnostic tool. One of the suggestions is utilizing a set of miRNAs, a miR-test kit comprising of 13 miRNAs which include miR-140-5p miR30b-5p, miR148a-3p, let-7d-5p, miR-191-5p, miR-30c-5p, miR-328-3p, miR-331-3p,

miR-374a-5p, miR-29a-3p, miR-484, miR-223-3p, and miR-92a-3p [121]. A commercially available kit named microRNA signature classifier (MSC) uses the expression ratio among 24 miRNAs in lung cancer as a diagnostic tool [122].

MiRNAs play an important role as prognosis tools as well. For example, underexpression [123] of let-7 miRNA is associated with shortened postoperative survival. Another study reported that miR-21 and miR-155 were associated with poor recurrence-free survival for NSCLC patients [124].

2.2.2 Long noncoding RNAs

Regarding lncRNAs in the context of lung cancer diagnosis and prognosis, a number of lncRNAs were found to be of use. For example, the overexpression of HOTAIR promotes metastasis and indicates a poor prognosis for NSLC patients [71]. Similarly, MALAT1 in conjunction with protein thymosin beta 4 can be used to predict metastasis and survival in early-stage NSCLC determined using early stage and metastasized tumor tissue removed with surgery [125]. The lncRNA CDKN2B-AS1 with a more common name as antisense noncoding RNA in the INK4 locus (ANRIL) is associated with advanced lymph node metastasis and poor overall survival. A high expression of large intergenic RNA (lincRNA) PVT1 was shown to be associated with tumor advanced stage and advanced stage of tumor-node-metastasis (TNM) in NSCLC tissues, with consequent poor prognosis [126]. Other lncRNAs associated with poor NSCLC prognosis are listed in the **Table 4**.

LncRNA	Expression in NSCLC (vs. normal tissue)	Clinical association	Reference
MALAT1	↑	Poor prognosis	[125]
HOTAIR	↑	Poor survival and prognosis	[71]
SPRY4-IT1	↓	Poor prognosis	[128]
CDKN2B-AS1	↑	Poor survival	[129]
CCAT2	↑	Poor prognosis	[130]
PVT1	↑	Poor prognosis	[131]
IRAIN	↓	Poor prognosis	[132]
LCAL1	↑	Poor prognosis	[133]
SOX2OT	↑	Poor survival and prognosis	[134]
AFAP1-AS1	↑	Poor prognosis	[135]
TATDN1	↑	Poor prognosis	[136]
FOXD2-AS1	↑	Poor prognosis	[137]
SNHG1	↑	Poor survival and prognosis	[138]
ARHGAP27P1	↑	Aggressive tumorigenesis and poor prognosis	[139]
UCA1	↑	Poor prognosis	[140, 141]
HOXA11-AS	↑	Poor prognosis	[142]
LINC00473	↑	Poor prognosis	[143]

LncRNA	Expression in NSCLC (vs. normal tissue)	Clinical association	Reference
LINC00673	↑	Poor prognosis	[144]
BC087858	↓	Poor prognosis	[145]

Table 4.
LncRNAs associated with poor NSCLC prognosis (adapted from Osielska et al. [127]).

2.2.3 YRNAs

YRNAs and YRNA-derived small RNAs (YsRNAs) are also found in NSCLC patients, and they are gaining traction as potential lung cancer biomarkers. When assessed in A549 NSCLC cell lines, Ys4RNA was significantly downregulated. On the other hand, a deep sequencing expression analysis of small RNAs in plasma extracellular vesicles from LAC patients, SCC patients and healthy individuals showed upregulation of Ys4RNA [110]. The finding shows it may be used as a potential circulating biomarker for NSCLC diagnosis.

2.3 Role of ncRNAs in NSCLC therapy

The last two decades have seen a significant change in lung cancer management and treatment. Achievements in tumor genotyping and other molecular approaches helped to develop tools for personalized medicine adding targeted therapy and immunotherapy to traditional chemotherapy and radiation therapy. Investigation of PD-L1 status and genetic mutations within tumor helps to predict the most appropriate targeted therapy or immune checkpoint blockers (ICBs). The combination of platinum-based doublet therapy has served as a standard for advanced stage NSCLC patients [146]. Surgical resection remains as the most effective therapy for NSCLC in stages I and II, however, there is a high percentage of tumor recurrence, with varying 5-year overall survival rate depending on the NSCLC stage [147, 148]. In this section, we will discuss the roles of noncoding RNAs in NSCLC therapy.

2.3.1 MicroRNAs

Owing to their role that they play in gene regulation, miRNAs do also impact the response to cancer radiotherapy, chemotherapy, and targeted therapy [149]. MiRNAs have been shown to modify the sensitivity and resistance to the most common platinum-based therapy. For example, cisplatin sensitivity in NSCLC in vitro was reported to increase as a result of miR-106b overexpression, its further suppression of polycystin 2 (PKD2) levels, and consequent downregulation of P-glycoprotein [150]. Another study reported how miR-503 blocked drug efflux mechanism and suppressed a number of proteins associated with drug resistance (MRP1, MDR1, survivin, Bcl-2 and ERCC1), consequently improving cisplatin sensitivity [151]. Interestingly, the upregulation of miR-196 had an opposite effect on the same proteins, thus promoting drug efflux and cisplatin resistance [152]. When it comes to radiation therapy, miR-200c was shown to have a positive impact on radiotherapy by suppressing oxidative response genes and inhibiting DNA repair [153]. The upregulation of miRNA-138 was reported to induce radio-sensitization in NSCLC cells [154]. Similarly, miR-25 modulated the radiosensitivity in lung cancer cells by directly inhibiting BTG2 expression [155].

In the context of molecular targeted therapy, miR-21 were reported to induce resistance to gefitinib through activation of ALK and ERK and inhibiting PTEN in lung cancer [156, 157]. The ALK resistance is another aspect where miRNAs play a role, and it was reported that histone H3 lysine 27 acetylation (H3K27ac) loss and inhibition of miR-34a is one the ways how ALK-positive lung cancer acquire ALK inhibitor resistance [158]. Another study observed a fingerprint of 7 circulating miRNAs (miR-493-5p, miR-411-3p, miR-494-3p, miR-215-5p, miR-495-3p, miR-93-3p and miR-548j-5p) to have a strong association with improved survival in lung cancer patients following the nivolumab treatment.

Currently, the most studied therapeutic miRNAs are let-7, miR-150, miR-29b, miR-200c, miR-34, and miR-145. The miR-34a alters the p53/mir-34/PD-L1 pathway by targeting PD-L1 and liposomal mimic MRX34 is a first miRNA that was used in phase I clinical trials initially showing promising antitumor activity [159]. However, the trials had to be terminated due to adverse effects [18]. **Table 5** summarizes the list of miRNAs associated with drug therapy and radiation sensitivity and resistance.

miRNA	Target/Pathway	Reference	miRNA	Target/Pathway	Reference
Chemo-sensitive			**Chemo-resistant**		
let-7	LIN28	[160]	miR-15b	PEBP4	[161]
miR-7	EGFR	[162]	miR-21	PTEN, SMAD7	[163, 164]
miR-17, 20a, 20b	TGFβR2	[165]			
miR-17 and miR-92 family	CDKN1A, RAD21	[166]	miR-27a	RKIP	[167]
miR-34a	PEBP4	[168]	miR-92a	PTEN	[169]
miR-101	ROCK2	[170]	miR-106a	Unknown	[171]
miR-106b	PKD2	[150]			
miR-135b	FZD1	[172]	miR-181c	WIF1, Wnt/β-catenin pathway	[173]
miR-137	NUCKS1	[174]			
miR-138	ZEB2	[175]	miR-196	MDR1, MRP1, ERCC1,	[152]
miR-146a	CCNJ	[176]		Survivin and Bcl-2	
miR-181b	TGFβR1/Smad signaling pathway	[177]	miR-205	PTEN, Mcl-1 and Survivin	[178, 179]
miR-184	Bcl-2	[180]	miR-488	eIF3a-mediated NER signaling pathway	[181]
miR-218	RUNX2	[182]			
mir-296	CX3CR1	[183]			
miR-379	EIF4G2	[184]			
miR-451	c-Myc-survivin/ rad-51 signaling	[185]			
miR-503	Down-regulation of MDR1, MRP1, ERCC1,	[151]			
	Survivin and Bcl-2				

miRNA	Target/Pathway	Reference	miRNA	Target/Pathway	Reference
miR-9600	STAT3	[186]			
Radio-sensitive			**Radio-resistant**		
miR-29c	Bcl-2, Mcl-1	[187]	miR-21	PTEN, HIF1α, PDCD4	[163, 188]
miR-138	SENP1	[154]	miR-25	BTG2	[155]
miR-200c	PRDX2, GAPB/ Nrf2, and SESN1	[153]	miR-210	HIF1	[189]
miR-328	γ-H2AX	[190]	miR-1323	PRKDC	[191]
miR-449a	Unknown	[192]			
miR-451	c-Myc-survivin/ rad-51 signaling	[185]			
EGFR TKI-sensitive			**EGFR TKI-resistant**		
miR-126	Akt and ERK pathways	[193]	miR-21	PTEN, PDCD4	[194]
miR-134/miR-487b/miR-655 cluster	MAGI2	[195]	miR-23a	PTEN/PI3K/Akt pathway	[196]
miR-200c	PI3K/Akt pathway	[197]	miR-30 family	PI3K-SIAH2	[198]
miR-223	IGF1R/PI3K/Akt pathway	[199]	miR-214	PTEN/AKT pathway	[200]
miR-483-3p	integrin β3/ FAK/Erk pathway	[201]			
ALK TKI-sensitive					
miR-200c	ZEB1	[202]			

Table 5.
miRNAs associated with lung cancer therapy and radiation sensitivity or resistance (adapted from Wu et al.) [15].

2.3.2 Long noncoding RNAs

The MALAT1 is the first lncRNA used in targeted therapy studies with lncRNAs molecules. The targeted therapy decreased the amount of MALAT1 using antisense oligonucleotides (ASOs), consequently reducing lung cancer metastasis in a murine model [203]. The protein STAT3 can impact the MRP1 transcription by binding upstream, thus STAT3 activation is associated with MRP1 and MDR1 upregulation, which in turn enhances lung tumor cells resistance to cisplatin [204, 205]. In similar manner, the lncRNA KCNQ1OT1 expression was shown to be positively correlated with MDR1 expression, and KCNQ1OT1 knockdown can improve paclitaxel sensitivity in LAC cells [206]. A different study has demonstrated the ability of the lncRNA XIST to act as a competing endogenous RNA by sponging miR-144-3p, and thus regulating the expression of MRP1 [207]. In another study, the lncRNA SNHG14 was found to regulate the DDP-resistance of NSCLC cell through miR-133a/HOXB12 pathway [208].

The EGFR-tyrosine kinase inhibitors (EGFR-TKIs) prevent the lung cancer cells growth by inhibiting EGFR activity. The lncRNA LINC00460 eliminates miR-769-5p, which in turn promotes EGFR expression and therefore enhances NSCLC cells resistance to Gefitinib. The association between expression of LINC00460 and expression

of proteins with multidrug-resistance, such as BCRP, P-gp, and MRP1 was reported [209]. The lncRNA colon cancer-associated transcript-1 (CCAT1), targets the miR-130a-3p/SOX4 axis and promotes cisplatin resistance in NSCLC in vitro [210]. Another lncRNA that induces cisplatin resistance in NSCLC is SNHG7, and it does it by upregulating MRD1 and BCRP via PI3K/AKT pathway [211]. A larger list of lncRNAs involved in drug resistance in NSCLC can be found in **Table 6**.

Drug	lncRNA	Effect on NSCLC	miRNA Target	Expression in tumor vs. control	Reference
Gefitinib	LINC00460	Promote	miR-769-5p	↑	[205]
	LINC00665		EZH2		[213]
	RHN1-AS1	Inhibit	miR-299-3p	↓	[214]
Cisplatin	MALAT1	Promote	STAT3	↑	[215]
	CCAT1		miR-130a-3p		[207]
	SNHG7		PI3K/AKT		[211]
	ROR		Akt/mTOR		[216]
	LINC00485		miR-195		[217]
	BLACAT		miR-17		[218]
	XIST		miR-17		[219]
	HOXA-AS3		HOXA3		[220]
	PAX6		PI3K/AKT		[221]
	PVT1		miR-216b		[222]
	NBAT1	Inhibit	PSMD10	↓	[223]
	TUG1		miR-221		[224]
	MEG3		p53, Bcl-xl		[225]
Vincristine	MEG3	Inhibit	miR-650	↓	[226]
Paclitaxel	KCNQ1OT1	Promote	Not known	↑	[227]
	NEAT1		Akt/mTOR		[228]
Multiple	DGCr5	Promote	miR-330-5p	↑	[229]
	ITGB1		Snail		[230]
	SNHG12		miR-299-3p		[231]
	SOX21-AS1		p57		[232]
	TUSC-7	Inhibit	miR-146	↓	[233]
	FENDRR		HuR		[234]
	MBNL1-AS1		miR-301b-3p		[235]
Crizotinib	HOTAIR	Promote	ULK1	↑	[236]
	ROR		ALK		[237]
EGFR-TKIs	UCA1	Promote	Akt/mTOR	↑	[238]
	LINC00460		miR-149-5p		[239]

Table 6.
LncRNAs related to drug resistance in NSCLC (adapted from Zhou et al. [212]).

3. The role of ncRNAs in small cell lung cancer

Small-cell lung cancer makes up 15% of all lung cancer incidences with 250,000 new cases and at least 200,000 annual death rates globally [240]. Carcinogens present in tobacco are responsible for the initiation of SCLC with accompanying inactivation of tumor suppressors p53 and RB in the majority of SCLC patients [241]. Amplification of MYC family members and frequent mutations of chromatin modeling proteins (EP300, CREBBP and MLL2) and Notch family members were also observed in SCLC patients [241, 242]. The ability of ncRNAs to modulate gene expression at transcriptional, post-transcriptional and epigenetic levels makes them significant factors, with clinical implications and functional roles in SCLC.

3.1 Role of miRNAs in SCLC

The miR-335 is downregulated in multi-drug resistant human SCLC cell lines. When overexpressed, miR-335 leads to the sensitization of the human SCLC cell lines to chemotherapy and radiotherapy by targeting PARP-1 (Poly [ADP-ribose] polymerase 1) gene. On top of that, overexpression of miR-335 promoted cell apoptosis, inhibited cell migration ability in vitro, and inhibited tumor growth in vivo [243]. In a similar fashion, the noncoding miR-22 was found to enhance the radiosensitivity in human SCLC cell line by targeting WRNIP1, promoting apoptosis and inhibiting cell migration when overexpressed [244]. Another noteworthy observation is that methylation in SCLC cell lines reduced the expression of miR-34 family members, which are known tumor suppressive miRNAS. The transfection of miR-34b/c to SCLC cell lines (H1048 and SBC5) resulted in significantly decreased cell growth, migration, and invasion [245]. The circulating miR-141 from plasm exosomes and serum of SCLC patients was found to be upregulated when compared to healthy volunteers. The ability of miR-141 to promote angiogenesis by targeting KLF12 (Kruppel-like factor 12) was demonstrated [246]. Earlier, miR-126 was found to inhibit SCLC cells proliferation by targeting SLC7A5, whereas the miR-217 was reported to inhibit proliferation and to promote apoptosis in SCLC cells by targeting PCDH8 [247, 248]. The miR-195 promoted SCLC apoptosis by inhibiting Rap2C protein-dependent MAPK signal transduction [249]. Similarly, the miR-26b was reported to promote apoptosis and suppress tumorigenicity by targeting myeloid cell leukemia 1 protein [250]. In another study, miR-485-5p was found to suppress the proliferation, migration, and invasion of SCLC cells by targeting flotillin-2 [251].

3.2 Role of lncRNAs in SCLC

There are five lncRNAs that are known to be involved in SCLC: HOTTIP, HOTAIR, TUG1, CCAT2 and PVT1 [252]. The lncRNA HOTTIP is upregulated in SCLC, is associated with chemoresistance and shorter survival. The HOTTIP acts through HOTTIP/miR-574-5p/EZH1 and HOTTIP/miR-216a/BCL-2 axes [253, 254]. The lncRNA HOTAIR, same as in NSCLC, is upregulated in SCLC, and is associated with lymphatic invasion and chemoresistance in SCLC [255, 256]. The ncRNAs TUG1 and CCAT2 are also upregulated in SCLC, where TUG1 is associated with chemoresistance, clinical stage and shorter survival, whereas CCAT2 is associated with malignant status and poor prognosis [257, 258]. Similarly, the lncRNA PVT1 is

upregulated in SCLC patients and is associated with lymph node metastasis and clinical stage [259]. Recently, Linc00173 was reported to modulate glucose metabolism and multidrug chemoresistance in SCLC [260].

4. Conclusion

Lung cancer remains to be a leading cause of cancer death. The past two decades have witnessed an unprecedented growth in cancer research, and new findings have continuously supported and expanded the notion that ncRNAs play a significant part in all aspects of lung cancer. Future advancements in molecular technology will help researchers answer more complicated questions about this disease, including uncovering the additional ncRNAs and the mechanisms by which these regulated lung cancer development, progression, and response to therapy. The heterogeneity of lung cancer subtypes, variation in mutational burden, sex differences, and different stages of cancer further necessitate a combination of individualized therapies, such as platinum-based doublet chemotherapy, immunotherapy, and targeted therapy to combat lung cancer effects on human health. The relative success of combined therapies underlines the need to further investigate the roles and functions of ncRNAs within cancer processes. A better understanding of tumorigenesis mechanisms and identification of ncRNAs and other participants will play an important role in improving lung cancer diagnosis, prognosis, prevention, and therapy.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

MicroRNAs and Pancreatic ß Cell Functional Modulation

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Abstract

Recent reports of diabetes susceptibility loci located on the non-coding regions of the genome highlight the importance of epigenetic control in health and disease. Specifically, microRNAs have shown to have an important regulatory role in pancreatic ß cell physiology. Human studies implicated that ß cell mass and function are regulated by microRNAs in health and disease. Further, the microRNAs are also implicated in ensuing diabetic complications. Delineating the peculiar role of microRNAs in ß cell physiology and pathophysiology will fill the missing gaps in our current knowledge and help to devise better treatment regimens for diabetes. This chapter will discuss multiple effects of different microRNAs on the ß cell physiology in the context of maintenance and function in Type 2 diabetes mellitus.

Keywords: microRNA, beta-cell, insulin, diabetes mellitus

1. Introduction

MicroRNAs (miRNAs/miRs) are pertinent genetic regulators of embryonic development and differentiation, postnatal growth, and metabolism [1]. Initially discovered in *C. Elegans*, miRNAs are now considered a universal phenomenon for the gene expression regulation from multicellular organisms to mammals [2–6]. The mammalian genome has been reported to encode more than 500 known microRNA genes. microRNA binds with the mRNA (3′ untranslated region 3'UTR) and result either in the translational repression of that specific gene or in the total degradation of the mRNA [6–9]. Apart from 3'UTR, miRNA has been reported to interact with 5'UTR and gene promoters and enhancers [10]. miRNAs are critical for normal animal development. As expression pattern of numerous genes important for the embryonic development and subsequent cellular differentiation have been shown to be regulated by microRNAs [11].

The pathological importance of miRNAs is highlighted by the substantial reports of the association of aberrant expression of miRNAs in many human diseases [12, 13]. As miRNAs are also present in the extracellular fluids and act as a signaling molecule for cell communication, their diagnostic importance as a biomarker in different lethal diseases has also been reported [14, 15] Biogenesis of miRNAs and the mechanisms through which they regulate gene expression and their tissue distribution has been previously discussed in detail [1, 10, 16–18]. The transcriptional activity of miRNA sequences is solely dependent upon RNA polymerase II/III activity [19, 20]. The

transcribed miRNA can act through canonical as well as non-canonical pathways [20, 21]. A complementary match either exact or partial between microRNA and the 3'-UTR (conserved) site of the mRNA will result in either the subsequent degradation of the mRNA or moderate reduction in the mRNA levels resulting in translation repression [22, 23]. The degradation of mRNA will result in complete blockage of protein expression. Whereas specific translation repression results in a reduction in the protein formation from the target genes. This fine-tuning of gene expression and subsequent protein formation by microRNAs is important for normal physiological responses. A single miRNA can target hundreds of different genes and mRNAs thus exhibiting multiple levels of complementarity. It is estimated that more than 60% of protein-coding genes are microRNA targets.

In the canonical pathway of microRNA generation (explained in more detail in [24]) events start with the transcription of microRNA sequence by the RNA polymerase II or III, the microRNA transcript is spliced, capped and polyadenylated in the nucleus and is called pri-microRNA. The nuclear pri-microRNA is cleaved by a nuclear protein complex called microprocessor complex and includes Drosha and DGRC8 proteins. After this cleavage event, the pri-microRNA is exported to the cytoplasm by the help of a protein called exportin 5 (Exp 5). Cytoplasmic pre-microRNA is further cleaved by another protein called Dicer and converting the pri-microRNA into a microRNA duplex of around 22 nucleotides. These duplex molecules are loaded onto another protein called Argonaute (AGO). Duplex microRNAs are unwinded and the passenger strand is removed while the guide microRNA strand remains with the AGO proteins and forms the mature effector complex called RNA-induced silencing complex (RISC). RISC complex is guided by the microRNA complementarity to bind with mRNA targets and 3'end de-adenylation occurs resulting in the mRNA degradation of the translation arrest on mRNA. Interestingly deletion of the components of this canonical pathway like DICER or AGO proteins leads to β cells functional impairment in terms of glucose-induced insulin secretion and dedifferentiation into a progenitor-like state [25–28]. The current chapter will focus on the physiology and pathophysiology of the β cells focusing on the insulin signaling responsible for glucose homeostasis and the influence and interplay of different microRNAs onβ cells functionality in Type 2 diabetes (T2DM).

2. β cells, insulin and macronutrient metabolism

Insulin is the primary endogenous protein responsible for the physiological regulation of metabolism [29]. The regulated secretion of insulin from the pancreas helps to maintain glucose homeostasis in distinct physiological conditions [30, 31]. Endocrine cells that secret specific hormones for the maintenance of glucose levels are present in specialized closed zones in the pancreas called Islets of Langerhans [31–33]. Islets of Langerhans contain numerous cell types with an appropriate amount of vascular and nervous innervation [31]. Four different types of endocrine cells populated Islets of Langerhans namely: alpha (α) cells, beta (ß) cells, delta (δ) cells, and pancreatic polypeptide (PP) cells. ß cells secrete insulin and initiate postprandial glucose metabolism and control the rising blood glucose levels and α cells secrete of glucagon which enhances blood glucose levels during fasting [34, 35]. Insulin is also responsible for lipid and protein metabolism [36–38]. The release of insulin from ß cells is initiated by glucose uptake, a phenomenon known as glucose-stimulated insulin secretion (GSIS) [39–41]. At physiological postprandial levels, glucose initiates insulin gene

transcription by recruiting specific transcription factors (PDX-1, MafA, and NeuroD) and post-transcriptionally improves the insulin mRNA stability thus acting as a major physiologic regulator of insulin [42–44]. Glucose-specific channels/gates are present on the ß cell membrane which are commonly known as glucose transporters (GLUT) [30, 41]. Many types of glucose transporters are present throughout the body [45]. Specifically, GLUT2 is abundant in the pancreas and liver whereas GLUT4 is abundant in skeletal and cardiac muscles as well as adipocytes [45, 46].

Insulin initiates carbohydrate metabolism through phosphorylation of glucose and subsequent formation of glucose-6-phosphate [47, 48]. Insulin phosphorylates glucose through the hexokinase enzyme in muscles and glucokinase (GCK) in ß cells and hepatocytes [49–52]. Insulin binds to the cell surface insulin receptors commonly present in different tissues [53]. The insulin-insulin receptor binding activates cytoplasmic adaptor proteins called insulin receptor substrates (e.g., IRS1, IRS2) [54]. IRS proteins activate phosphoinositide2-kinase enzyme (PI3K) and convert tyrosine phosphorylation signal into the lipid kinase. Activated PI3K recruits ATP molecules which initiates the AKT activation (serine and threonine kinase) [55]. Activation of AKT by insulin proved landmark and explained the conversion of tyrosine phosphorylation into serine/threonine phosphorylation signal. Insulin-induced AKT activation also helped to describe the insulin-induced regulation of key steps in insulin signaling (a) glucose uptake by glucose transporter, (b) phosphorylation and subsequent inactivation of glycogen synthase kinase 3 (GSK3), (c) activation of the mechanistic target of rapamycin (mTOR) and subsequent synthesis of protein and fats, (d) Transcriptional control of gene expression by forkhead family box O (FOXO) transcription factor proteins. Insulin increases glucose uptake via GLUT4 in muscle and adipose tissue thus enhancing the postprandial rate of glucose transport, insulin also inactivates GSK3 and stimulates glycogen synthesis in these tissues [56]. Insulin prevents hepatic glucose synthesis by inhibiting glycogenolysis and gluconeogenesis. Insulin via AKT inhibits FoxO1 which results in the decreased expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose −6-phosphatase (G6PC) genes [57–59]. Insulin regulates lipid and protein metabolism also through AKT. Insulin via AKT induced an increase in mTOR and a decrease in FoxO1 and sterol regulatory element-binding protein (SREBP) inhibits adipocyte lipolysis and helps to lower the plasma fatty acid levels by promoting lipogenesis and enhancing the hepatic formation of very-low-density lipoprotein (VLDL) [60–65]. Insulin increases the protein synthesis in skeletal muscles and the liver by enhancing the amino acid transport inside the cells, accelerating mRNA translation, and reducing protein degradation and urea formation via AKT activated mTOR [37, 66–71].

3. Glucose homeostasis: α and β interplay

β cells exhibit a remarkable degree of plasticity in terms of insulin production. The α and ß cells work in conjunction to stabilize glucose levels in feeding and fasting conditions through the periodic release of insulin and glucagon respectively thus achieving glucose homeostasis [30, 49, 72]. Rising plasma glucose levels after feeding require immediate systemic activation of glucose metabolism. Delayed or decreased activation of glucose metabolism results in abnormally high glucose levels known as hyperglycemia. Higher-than-normal concentrations of glucose incite glucotoxic reactions inside the cells. ß cells are triggered by rising postprandial glucose levels to synthesize and secrete insulin. The resulting increasing insulin levels activate

enzymes that help to metabolize glucose. Glucokinase and hexokinase initiate glucose phosphorylation primarily in hepatocytes and muscle cells respectively. The post-prandial rise in the insulin declines over time because of the decline in the glucose level, a feature of GSIS [39]. Interestingly insulin negatively impacts glucagon secretion [73–76]. Plasma glucose levels decline under fasting conditions. As glucose is the primary cellular source to generate ATP, a minimum threshold of plasma glucose levels must be maintained to avoid hypoglycemia. Glucagon maintains plasma glucose levels, to avoid hypoglycemia during fasting, by initiating hepatic gluconeogenesis/glucogenolysis [34, 77–81].

4. Diabetes mellitus

Diabetes mellitus is metabolic dysfunction that results in a significant decline in the cellular ability to metabolize glucose either because of the lack of insulin or insulin inactivity (insulin resistance) or both [82, 83]. Diabetes mellitus is estimated to affect 700 million people worldwide by the year 2040 [84]. The inability of the cells to metabolize glucose cause an abnormal increase in the cellular and plasma levels of glucose (hyperglycemia). Hyperglycemia causes cellular damage via multiple mechanisms including (a) the increased influx of glucose through the polyol pathway, (b) increased formation of advanced glycation end products (AGEs), and (c) subsequent increased expression of AGE receptors and their ligand [85, 86]. Hyperglycemia initiates the reactive oxygen species (ROS) formation via activated glycation reactions and mitochondrial electron transport chain where ROS acts as the main trigger for the initiation of the polyol pathway, formation of AGEs, and subsequent increased AGE receptor expression [87].

Diabetes mellitus has been categorized in two primary forms: Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM). T1DM has been characterized by a mutation in the insulin gene or immune cell-mediated destruction of ß cell resulting in either the synthesis of abnormal insulin protein that fails to activate insulin receptors or a complete lack of endogenous insulin secretion [88]. T1DM patients are usually diagnosed early in their life. The only possible medical treatment referred to these patients is the multiple daily doses of synthetic insulin. T2DM on the other hand is much more complicated and requires a thorough diagnostic approach [82, 89–91]. T2DM is considered one of the most common metabolic disorders globally. Major risk factors for T2DM include a sedentary lifestyle, lack of exercise, excessive use of a high-carb and high-fat diet, overweight, and obesity [92]. Poor lifestyle and dietary habits have been attributed to the global incidence of Type 2 diabetes in the last 2 decades. Obesity, visceral fat deposition and increased body mass index (BMI) play a central role in the pathophysiology of Type 2 diabetic patients [82].

5. Pathophysiology of Type 2 diabetes mellitus (T2DM)

The development of T2DM is mainly attributed to the progressive decline in insulin secretion over time from ß cells and the progressive inability of insulin-responsive tissues (muscles, fat, and liver) to respond to insulin over time, resulting in hyperinsulinemia and subsequent insulin resistance [93–96]. The inability of the insulin hormone to activate insulin receptors at the cellular level has been attributed to be the major cause of hyperinsulinemia and insulin resistance [97, 98]. *Ex vivo*

analysis of pancreatic islets from T2DM donors in terms of GSIS exhibit disproportional insulin levels where increased glucose concentration fails to elicit appropriate ß cells response in terms of insulin [99–101]. Insulin binding to insulin receptors at the plasma membrane activates a signaling cascade that initiates glucose metabolism inside the cells. Insulin-bound insulin receptors or activated insulin receptors go through internalization at the plasma membrane, a phenomenon known as insulin receptor endocytosis [29, 102]. Following the activation, the endocytosis of the insulin receptor is the primary physiological mechanism through which the duration and intensity of insulin signaling are controlled. Hyperinsulinemia accelerates insulin receptor endocytosis and affects the presence of adequate functional insulin receptors at the plasma membrane resulting in insulin resistance [103]. Apart from accelerated insulin receptor endocytosis, insulin-stimulated insulin receptor kinase activity is also decreased in diabetic patients [104].

Compromised insulin signaling in T2DM fails to activate glucose metabolic enzymes like glucokinase and hexokinase resulting in hyperglycemia. The expression levels of GCK and GLUT2 were found to be lower in human T2D islets as compared to healthy control [105]. As high plasma glucose levels initiate glucose-stimulated insulin secretory (GSIS) response from ß cells resulting in the rise of plasma insulin levels. The rising insulin levels should be normalized over time because of the renal insulin clearance mechanism. But compromised renal insulin clearance rate in diabetic subjects results in abnormally high plasma levels of insulin (hyperinsulinemia) [106, 107]. Hyperinsulinemia and hyperglycemia in theory cannot trigger alpha cells to secrete glucagon. But it has been observed that T2DM patients with insulin resistance, hyperinsulinemia, and hyperglycemia also have abnormally high plasma levels of glucagon [108]. Hinting toward the disturbance in the α and β cell interplay through the inability of the insulin to block glucagon gene transcription [109].

T2D has also been attributed to altering the gene expression of key proteins participating in the processes of insulin secretion and function. Specifically, the insulin receptor gene (*IR*) and genes involved in Ca^{2+} influx (*SUR1*, *TMEM37*), and mitochondrial metabolism (*GPD2, PCK1, FXYD2*) [110–113]. The compromised mitochondrial activity in the T2D β cells through reduced ATP/ADP ratio fails to close the K_{ATP} channels and subsequently impacts Ca^{2+} influx and exocytosis of insulin. ATP also enhances insulin granular priming prior to the exocytosis and thus regulates insulin quantity by initiating the modification of proinsulin into insulin inside the insulin granule. Substantial β cell loss has been considered the hallmark of T2DM which even starts during the prediabetic stage [114]. Up to ~50% loss in the β cell mass has been reported in T2DM patients [115–118]. Apoptosis and dedifferentiation have been attributed as the main reasons for this substantial decline in the β mass. Hyperinsulinemia has been attributed to the activation of caspases, formation of H_2O_2 increased expression of nitric oxide synthase (iNOS) in the β cells resulting in their substantial loss [119–121]. Hyperglycemia-induced production of AGE products and the subsequent activation of AGE receptors further promote the release of cytochrome and caspase activation [122]. Hyperglycemia along with increased levels of free fatty acids (glucolipotoxicity) has also been shown to induce β apoptosis and reduction in their overall mass in T2DM [123, 124]. Over accumulation of lipids and over-activation of lipid signaling pathways inβ cells are of pathological significance as it contributes to β cell loss and further lead to the significant decline in insulin secretion and the onset of T2DM. Glucolipotoxicity induces a cascade of events that starts from mitochondrial dysfunction leading to oxidative stress (ROS) which further leads to ER stress which results in improper protein unfolding response and results in the

loss of GSIS and inflammation and activated autophagy. These above-mentioned cascades of events represent altered cell signaling pathways, lipogenic and pro-apoptotic genes, and proteins, increased expression of cytokines, and accumulation of lipids molecules like di and triacylglycerols, cholesterol, and cholesterol esters.

Apart from the above-mentioned mechanisms, β cell dedifferentiation has recently been shown to be another important contributing factor in the progressive decline in the β mass in T2DM [116, 125, 126]. Dedifferentiation is defined as the ability of a cell to revert to its progenitor-like stage (developmental stage). Interestingly it has been observed that dedifferentiation in β cells not only resulted in progenitor-like cells but an increase in other islets cells type has been observed. Implying the possibility that dedifferentiated β cells have further converted into α and δ cells, which interestingly involves the removal of epigenetic brake on glucagon and somatostatin genes for their expression in the functional β cells. This also explains the abnormally high levels of glucagon observed in T2D patients with hyperglycemia, where glucose fails to down-regulate the glucagon gene expression. The transition or dedifferentiation of β cells is characterized by the downregulation of β specific genes and upregulation of pluripotent genes [127–131]. β cell identity genes like *MafA, Nkx6.1,* and *FoxO1* were shown to be downregulated. Whereas pluripotent genes like *Ngn3, Oct4, Nanog,* and *L-Myc* were upregulated. In fact, it has been shown in T2DM patients that glucagon (α) and somatostatin (δ) positive cells have inactivated (cytoplasmic) FOXO1 and NKX6.1 proteins supporting the notion that trans-differentiation of β cells into other endocrine islet cell types [125].

6. Epigenetic regulation of β cell-specific gene expression profile

Epigenetic mechanisms resulting in the alteration of gene expression have been attributed to the regulation or dysregulation of proper β cell function in health and disease [132–137]. DNA methylation, chromatin modifications, and post-translational modifications of histones have been implicated as the main epigenetic mechanisms. Alteration in the expression pattern of Non-coding RNA sequences like microRNA (miRNA/miR) and long non-coding RNA (lncRNA) has also been shown to regulate the β cell function as well as its cellular identity [138–151].

7. MicroRNAs and β cell regulation

MicroRNA (miRNA/miR) sequences are typically small around ~22 nucleotide non-protein-coding transcripts. Activation of their transcriptional activity results in the formation of miRNAs. Formation or presence of miRNAs has been shown to regulate the expression of a network of genes involved in β cell development, function, and pathogenesis [152–158]. microRNAs have been shown to play a vital role in the compensation process of β cells during pregnancy, obesity and T2D [158–161]. MicroRNAs mediate mRNA silencing and posttranscriptional regulation of gene expression in different physiological states of β cells. These miRNAs induced fine tunned changes in β cell gene expression are considered vital in health and disease. As the inability of microRNAs to fine-tune the gene expression levels lead to β cells decompensation which results in abnormal insulin secretion and ultimately the development of diabetes. β cell decompensation is referred to as two main pathological conditions: (1) impaired (reduced) β cell function in terms of glucose sensitivity

and insulin secretion (Reduced GSIS), and (2) reduced β cell number/mass due to dedifferentiation or apoptosis. Impaired β cell function involves molecular defects in insulin biosynthesis, glucose uptake, and exocytosis of insulin granules. β cell maintenance is regulated by microRNAs through the downregulation of non-β cell specific genes and upregulation of the β cells specific genes thus avoiding the β cell dedifferentiation process which is impaired in T2DM patients [162, 163]. As discussed earlier, different molecular components are involved in the secretion of insulin in response to the rising blood glucose levels. Failure of either one of these molecular components results in the failure of β cells to respond to the systemic demands of insulin for macronutrient metabolism. MicroRNAs are involved in the fine-tuned adjustments of β cells and regulate multiple aspects of glucose homeostasis via insulin secretion.

8. MicroRNAs and insulin synthesis and signaling

The insulin gene transcription results in the generation of mRNA which is translated in preproinsulin and cleaved into proinsulin in the endoplasmic reticulum [164–167]. Cleavage of proinsulin into insulin and C-peptide requires activation of convertase enzymes (PC1/3, PC1) through the change in the granular pH into acidic pH. Insulin gene (*ins1*) expression has been found to be compromised after the deletion or inactivation of *Dicer1* gene [27, 28]. Reduced insulin gene promoter activity and decreased insulin content was observed in isolated islets and cultured β cells after the experimental knock-down of a set of microRNAs including miRNA-24, miRNA-26, miRNA-148 and miRNA-181 [168]. A positive correlation in the insulin mRNA levels and negative correlation in terms of GSIS response and the expression profile of miRNA-127-3p and miRNA-184 was observed in the islets isolated from healthy subjects. Interestingly these correlations were found to be absent in the isolated islets of glucose-intolerant (diabetic) donors [169]. *In vitro* expansion of β cells from adult pancreatic islets were found to be dedifferentiated after the proliferation and levels of miRNA-375 (an islet-specific microRNA) were found to be significantly reduced [170] Experimental overexpression of miRNA-375 in dedifferentiated β cells lead to the activation of the β cell specific gene expression profile. MafA, a key β cell specific transcription factor regulating insulin gene expression, has been shown to be directly targeted by the miRNA-204 [171]. Thioredoxin-interacting protein (TXNIP), a redox regulator intracellular protein, has been found to be upregulated in diabetes and its deficiency protect β cells from diabetes-associated apoptosis. TXNIP induces the expression of miRNA-204 by inhibiting STAT3 activity. miRNA-204 thus blocks insulin production in diabetes by downregulating MafA. microRNA-181c-5p has been shown to induce the expression of INS1, PDX1, NKX6.1, and MafA in human induced pluripotent stem cells (hiPSCs) [172]. microRNA-124a has been shown to be highly expressed in islets of T2DM patients [173]. microRNA-124a has shown to be a negative regulator of insulin by targeting 3'UTR regions of mRNAs of Akt, Sirt1, NeuroD, and Foxa2 proteins. All these proteins are vital in the insulin gene transcriptional activity as well as the insulin signaling via insulin receptors. microRNA-7 has been shown to influence insulin signaling by down-regulating insulin receptor substrate protein genes (IRS1, IRS2) in gestational diabetes in humans [174]. Glucagon-like peptide 1 receptors (GLP1R) are present in the β cells and activation of these receptors by GLP-1 induces GSIS response from β cells [175, 176]. GLP1 receptor expression has shown to be downregulated by microRNA-204 in rat ins-1 cell line and primary mouse and human islets cells by targeting the 3'UTR region of GLP1R mRNA [177].

This is particularly important as GLP-1 receptor agonists are primary drugs used by T2DM patients to increase insulin levels.

9. MicroRNAs and insulin secretion

Several microRNAs have been shown to impact glucose uptake and metabolism by the β cells and the exocytotic process of insulin secretion. As GSIS regulates the key components of the process of β cell insulin release. Islets isolated from T2DM patients have shown decreased expression of proteins involved in K_{ATP} and Ca^{2+} channel regulation, metabolism, and exocytosis of insulin resulting in hyperglycemia [178]. Islets from T2DM patients show increased levels of microRNA-130a, microRNA-130b, and microRNA-152 and were found to be glucose-induced [179]. microRNA-130a, microRNA-130b, and microRNA-152 target *Gck* gene and downregulates ATP synthesis by reducing glucose metabolism in β cells thus affecting insulin exocytosis by not only blocking the closure of K_{ATP} channels but also affecting the insulin granule priming. microRNA-29a, microRNA-29b and microRNA-124 have been shown to suppress the gene expression of a β cell disallowed mitochondrial protein called monocarboxylate transporter (MCT-1) [180]. MCT-1 inhibition allows the β cells to only consume glucose for metabolism. Thus microRNA-29a, microRNA-29b, and microRNA-124 help in preserving the cell-specific identity. Human and mouse islets incubated with high glucose (hyperglycemic conditions) exhibit an increased expression of microRNA-29 in β cells [181]. microRNA-29 has been shown to positively impact the expression of a transcription factor called Onecut2. This transcription factor upregulates the granuphilin protein gene expression. Granuphilin protein blocks the exocytosis of secretory granules in endocrine cells. Apart from Onecut2-granuphilin mediated blocking of exocytosis, microRNA-29 also influences exocytotic protein genes like Syntaxin1A and SNAP25 (members of SNARE complex: a family of proteins involved in membrane fusion during exocytosis) [182].

10. MicroRNAs and lipid accumulation in β cells

The characteristic hallmark of T2D is the dysregulation of lipid metabolism and subsequent obesity. Excess amounts of fats present in the bloodstream of T2D patients have been attributed to β cell mass reduction and decreased insulin secretion. MicroRNA sequences have been shown to be manipulated by the exposure of β cells to circulating fats in T2D patients [183]. β cell-specific genes which encode proteins that are involved in lipid metabolism and subsequent cholesterol homeostasis have been found to be under miRNA control [184]. miRNA-33 has been reported to control cholesterol homeostasis through modulating the expression of sterol regulatory binding protein (SREBP) genes [185] miRNA-34a has been shown to induce β cell lipotoxicity through multiple mechanisms after the *in vitro* and *in vivo* exposure to saturated fatty acids [186–189]. Increased β cell influx of fatty acids through di/triacylglycerol and esterified cholesterol pathways including targeting sirtuin1 (SIRT1). Importantly SIRT1 (NAD^+-dependent deacetylase) regulates the expression of multiple genes encoding proteins like tumor suppressor protein p53 and DNA repair factor ku70 and transcription factor protein including nuclear factor κB (NF-κB) and FOXO family. Exposure of β cells to saturated fatty acids alters the expression profile of important miRNAs which might contribute to β cell lipotoxicity. These miRNAs include

miRNA-146 [184], miRNA-182-5p [190], miRNA-297b-5p [191], and miRNA375 [192]. miRNA-146 target genes which are involved with the inflammatory response (Toll-like receptors) [193]. It has been demonstrated that in cultured human islets, the miRNA-146 level increased after the exposure to pro-inflammatory cytokines and decreased exposure to high glucose concentrations but interestingly miRNA-146 levels were unchanged after the palmitate exposure [194]. miRNA-146 along with miRNA 182-5p have been shown to protect against high-fat diet-induced steatohepatitis in a mice model by decreasing in the expression of IL-1 receptor-associated kinase (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) which results in the reduced cytoplasmic lipid accumulation and inflammation [195]. miRNA-182-5p was found to be increased in INS-1 cells incubated with palmitate and significantly decreased the cell viability and increased palmitate-induced apoptosis. When INS-1 cells were supplemented with miRNA-182-5p inhibitors a significant increase in the cellular viability and palmitate-induced apoptosis was observed [190]. C57BL/6 mouse and β-TC6 cell line exposed to stearic acid and palmitic acid lowers the expression of miRNA-297b-5p. Upregulation of miRNA-297b-5p reduced the stearic acid-induced apoptosis but decreased insulin secretion in β-TC6 cells by inhibiting large-tumor-suppressor kinase 2 (LATS-2) [191] miRNA-375 has been shown to block high-fat diet-induced insulin resistance and obesity in mice by promoting hepatic expression of insulin-responsive genes [196].

11. Conclusion

The important role of epigenetics in the functional regulation of β cells in health and disease has been supported by the increasing number of high levels studies on human and rodent models. Excellent research efforts in the last decades have established that microRNAs help to define the β cell identity and dysregulation of β cell microRNA expression acts as a key event in the pathogenesis of T2DM. As the mechanisms involved in the biogenesis of microRNAs are well understood, it is plausible to assume that microRNAs represent attractive therapeutic targets. Selective inhibition of Dicer molecules by using specific siRNAs or pharmacological tools and selective modulation of a single microRNA. Specific microRNAs involved in diabetes represent a new class of specialized biomarkers for the early detection of diabetes. As microRNAs are biochemically quite stable in the extracellular fluid like blood, they represent a very good biomarker for diagnostics. Further in-depth studies are needed to assess the predictive value of serum levels of microRNAs in relation to the disease progression. Novel therapeutic approaches need to be devised for the prevention and treatment of metabolic disorders.

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Chapter 6

Predicting SNPs in Mature MicroRNAs Dysregulated in Breast Cancer

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Abstract

Breast cancer (BC) is the leading type of cancer among women. Findings have revolutionized current knowledge of microRNA (miRNA) in breast tumorigenesis. The seed region of miRNA regulates the process of gene expression negatively. The presence of SNPs in the seed regions of miRNA dramatically alters the mature miRNA function. Additionally, SNPs in the out-seed region of miRNAs have a significant impact on miRNA targeting. This study focuses on the *in silico* analysis procedure of mature miRNA SNPs and their impact on BC risk. The database annotated SNPs on mature miRNAs was used. Also, target gene alterations, miRNAs function in BC, and the interaction of miRNAs with targets were predicted. A list of 101 SNPs in 100 miRNAs with functional targets in BC was indicated. Under the SNPs allele variation, 10 miRNAs changed function, 6 miRNAs lost targets, 15 miRNAs gained targets, 48 onco-miRNAs remained unchanged, and 21 tumor suppressor miRNAs remained unchanged. At last, a list of 89 SNPs, which alter miRNA function and miRNA-mRNA interaction, were shown to be potentially associated with BC risk. This research theoretically generated a list of possible causative SNPs in the mature miRNA gene that might be used in future BC management studies.

Keywords: mature microRNA, SNP, breast cancer, bioinformatics, *in silico*

1. Introduction

Breast cancer (BC) is the most prevalent cancer among women across the world. The malignant growth begins in the ducts (85%) or lobules epithelium (15%) ("*in situ*"), where it typically causes no symptoms and has a minimal chance of spreading (metastasis). These *in situ* (stage 0) tumors may increase substantially, infiltrating neighboring breast tissue (invasive BC) and expanding to nearby lymph nodes (regional metastasis) or organs (distant metastasis). This cancer is generally deadly due to metastasis spread [1]. BC was diagnosed in 2.3 million women in 2020, with 685,000 fatalities worldwide [2]. This cancer was also found in 7.8 million women living in the last 5 years, making it the world's most frequent malignancy by the end of 2020 [2]. BC affects women at all ages following puberty in every country

worldwide, with incidence increasing with age. Therefore, many researchers and clinicians are focused on the etiology of the disease to enhance current medications and discover novel treatments.

MicroRNAs (miRNAs), short non-coding RNAs of approximately 18–24 nucleotides in length, affect gene expression negatively by directly binding the 3′-untranslated region (UTR) of the target messenger RNA (mRNA) and diminishing its stability and translatability. Numerous miRNAs have roles in cell signaling, including proliferation, death, differentiation, and immunity [3]. It has been proposed that they have a role in the formation and development of human malignancies, making them important markers of cancer [4]. The involvement of microRNAs in complex diseases like BC has focused on recent studies [5]. Various miRNAs have been related to regulating genes that have a role in the development of BC. MiR-21, miR-26a, miR-155, miR-221/miR-222, and miR-495 are some of the onco-miRs involved in tumor proliferation and angiogenesis [6–12]. Various miRNAs (miR-100, miR-125b, miR-126, miR-145, miR-200c, miR-298, and miR-335) have been involved in cell cycle regulation, hypoxia and stress response, and apoptotic induction [13–22]. In addition, McAnena et al. [23] found that circulating miR-332 and miR-195 may be utilized to discriminate between local and metastatic BC. Meanwhile, Sathipati et al. [24] indicated that 34 miRNAs could be employed to classify the early and late stages of BC development. These findings support the concept that a small selection of miRNAs can be considered biomarkers for BC risk prediction or prognosis [25].

The seed region of miRNA, located between the second and eighth positions in miRNA, regulates gene expression negatively [26]. As miRNAs are small, even a single alteration in the mature sequence of miRNAs might impact the development of miRNAs, which leads to the production of new miRNA [27]. A novel repertoire of target genes is produced by the presence of SNPs in the seed regions of miRNA, which dramatically alters the biological activity of the miRNA [28]. Additionally, SNPs in the out-seed region of miRNAs significantly impact miRNA targeting enhancements [29].

Therefore, in this study, the functional effect of SNPs in miRNAs regulating the genes implicated in BC has been predicted by bioinformatics approaches, including (1) screening miRNAs that reveal SNPs in mature sequences, (2) identifying miRNAs' target genes related to BC, (3) predicting miRNAs function in BC, and (4) estimating the degree of interaction between miRNAs and target mRNAs. The results may be beneficial in determining potential SNPs to analyze further and investigate SNPs that are causative to BC. Furthermore, the findings may potentially help generate theories and evaluate therapies for BC.

2. Materials and methods

2.1 SNPs in miRNA mature sequences database

In this study, the list of SNPs in mature miRNA sequences was extracted from the "SNPs in pre-miRNAs" database provided by miRNASNP-v3 [30] (http://bioinfo.life.hust.edu.cn/miRNASNP#W/). Mature miRNA location, positioning, and sequences were obtained from miRbase (www.mirbase.org), release 22 [31]. SNP position were acquired from dbSNP [32] (MAF > 1%) (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi).

2.2 *In silico* target prediction in BC pathways

The miRDB (http://mirdb.org/) was adapted to input both miRNA reference and SNP altered sequences for target prediction [33]. Target prediction was performed for all miRNAs containing reference and SNP alternative sequences. All predicted targets with prediction scores ≥80 that are most likely to be accurate [34], were collected and applied to conduct Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses for the identified target genes in the BC pathway (hsa05224). KEGG is a knowledge base for systematic analysis of gene functions [35] (https://www.genome.jp/pathway/hsa05224). MiRNAs that target genes in the BC pathway are considered to be involved in BC formation. The target with the highest predicted target score from miRDB is considered the gene most likely to affect BC via the miRNA regulation.

2.3 Function prediction of target genes and miRNAs in BC

The target genes with the highest target score from miRDB of each miRNA were further analyzed. The published expression level (Log2FC value) of genes in BC was collected from GENT2. GENT2 is a platform for exploring Gene Expression patterns across Normal and Tumor tissues [36] (http://gent2.appex.kr). From Log2FC value, target genes were considered as oncogenes ($Log2FC > 0$, $P < 0.05$) or tumor suppressors of BC ($Log2FC < 0$, $P < 0.05$).

MiRNAs may contribute to (onco-miRNAs) or repress (tumor suppressor miRNAs) the cancer phenotype by inhibiting the expression of tumor suppressor genes or oncogenes, respectively. Therefore, the role of miRNAs in BC can be predicted based on the role of target genes in BC. MiRNAs are considered oncogenic miRNAs when their target genes act as tumor suppressors. Furthermore, conversely, miRNAs were considered tumor-suppressive miRNAs when they target oncogenes. The presence of SNPs on the seed region can alter the target, thereby changing the role of miRNAs in BC.

2.4 Predicting the interaction between miRNAs and targets

The interaction between mature miRNA sequences, in both reference and alternative allele versions of the SNP, and their target genes were analyzed based on the number of seed binding sites of a miRNA at the 3'UTR by TargetScan 8.0 [37] and the Minimum Free Energy (MFE) of the whole mature miRNA sequence with the 3'UTR by RNAhybrid. The target sequences extracted from TargetScan 8.0 (http://www.targetscan.org/vert_80/). The miRNA sequences were further included in RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid), a tool for finding the MFE hybridization of a long and a short RNA [38]. The MFE values should be between −10 and − 30 kcal/mole. The more negative the MFE value (closer to −30), the stronger the interaction of the mature miRNA sequence with the 3'UTR. A miRNA can bind at multiple sites on the 3'UTR of its target, and each interaction site has a corresponding MFE value. Therefore, the interaction between the 3'UTR and the whole mature sequence of miRNA was determined via the mean value of MFEs from all miRNA binding sites on 3'UTR (Mean MFE).

The level of miRNA inhibition on target genes could be influenced by the presence of an alternative allele SNP, which could be shown through the difference between the mean MFE value of the alternative and reference allele version (ΔG). A non-zero ΔG value means a difference in the interaction degree with the same target between miRNA reference and alteration, indicating the SNP is potentially associated with BC

risk. A ΔG value of 0 indicates no change in the level of interaction between the miRNA reference and the miRNA variation with the same target, suggesting that the SNP is unlikely to relate to BC risk.

3. Results

3.1 SNPs in miRNA mature sequences

This study collected a list of 169 SNPs on mature sequences of 161 miRNAs from miRNASNP-v3. The mature reference sequences of 161 miRNAs were obtained from miRBase. The 169 alternative mature sequences of miRNAs corresponding to positional changes of 169 SNPs identified from dbSNP are described in Supplement 1.

3.2 In silico target prediction in BC pathways

After predicting the target genes of the reference and alternative sequences by miRDB (score ≥ 80) and comparing them in the list of genes on the BC pathway of KEGG, out of 161 miRNAs, there are 100 miRNAs with functional targets in BC (Supplement 2). Thirty-eight miRNAs with 38 in-seed SNPs had altered targets, and 62 with 63 out-seed SNPs did not change targets. The most likely target gene, which has the highest target score, of each miRNA was selected to analyze the possible effect on BC further.

3.3 Function prediction of target genes and miRNAs in BC

The functional target genes in BC were determined from the GENT2 database. Hence, the function of miRNAs in BC was also predicted. Of the 38 miRNAs with altered targets, the targets of 31 miRNAs were functionally altered in BC under the influence of SNPs (**Table 1**). Of these 31 miRNAs, 4 predicted onco-miRNAs were predicted to change to tumor suppressor miRNAs because of their new target working as oncogenes (hsa-miR-4257, hsa-miR-499a-3p, hsa-miR-501-3p, hsa-miR-593-5p), 5 predicted onco-miRNAs were predicted to lost targets in BC (hsa-miR-1178-5p, hsa-miR-4482-5p, hsa-miR-4661-3p, hsa-miR-5589-3p, hsa-miR-7854-3p), 6 predicted tumor suppressor miRNAs were predicted to change to onco-miRNAs because of their new target working as tumor suppressor genes (hsa-miR-1302, hsa-miR-2682-3p, hsa-miR-3117-3p, hsa-miR-4695-5p, hsa-miR-5692b, hsa-miR-6810-5p), 1 tumor suppressor miRNA was predicted to lost targets in BC (hsa-miR-627-5p), 10 miRNAs were predicted to gain onco-miRNAs (hsa-miR-4513, hsa-miR-4707-3p, hsa-miR-4741, hsa-miR-4781-3p, hsa-miR-4804-5p, hsa-miR-5090, hsa-miR-662, hsa-miR-6796-3p, hsa-miR-6826-5p, hsa-miR-6879-3p), and 5 miRNAs were predicted to gain tumor suppressor miRNAs (hsa-miR-1269b, hsa-miR-4467, hsa-miR-6717-5p, hsa-miR-6763-3p, hsa-miR-6777-5p). By altering the function of miRNAs, 31 in-seed SNPs on these miRNAs are likely to be associated with BC risk (**Table 1**).

The remaining 7 of 38 miRNAs with altered targets were predicted to remain functional despite the allele change of the SNPs (**Table 2**). Four (hsa-miR-146a-3p, hsa-miR-4284, hsa-miR-4731-3p, hsa-miR-557) and three miRNAs (hsa-miR-3622a-5p, hsa-miR-449c-3p, hsa-miR-548 t-3p) were predicted to be onco-miRNAs and tumor suppressor miRNAs, respectively.

miRNA	SNP			Reference					Alternative					
	ID	Position	Allele	Target				Role of miRNA	Allele	Target				Role of miRNA
				Gene	Log2FC	Expression trend	Category			Gene	Log2FC	Expression trend	Category	
hsa-miR-1178-5p	rs7311975	chr12:119713688	T	FGF9	−1.22	Down	Tumor suppressor genes	Onco-miRNA	C					
hsa-miR-1269b	rs7210937	chr17:12917329	G						C	DVL3	0.28	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-1302	rs74647838	chr12:112695096	G	ESR1	0.95	Up	Oncogenes	Tumor suppressor miRNA	A	CTNNB1	−0.40	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-2682-3p	rs74904371	chr1:98045291	C	TNFSF11	1.80	Up	Oncogenes	Tumor suppressor miRNA	T	SOS1	−0.43	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-3117-3p	rs12402181	chr1:66628488	G	KRAS	0.31	Up	Oncogenes	Tumor suppressor miRNA	A	SOS2	−0.37	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-4257	rs74743733	chr1:150551992	G	MAPK1	−0.09	Down	Tumor suppressor genes	Onco-miRNA	A	E2F2	1.20	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-4467	rs115101071	chr7:102471476	G						A	IGF1R	0.41	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-4482-5p	rs45596840	chr10:104268396	G	TCF7	−0.47	Down	Tumor suppressor genes	Onco-miRNA	A					
hsa-miR-4513	rs2168518	chr15:74788737	G						A	NCOA1	−0.21	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-4661-3p	rs12335005	chr8:91205534	G	NOTCH2	−0.60	Down	Tumor suppressor genes	Onco-miRNA	T					

miRNA	SNP			Reference					Alternative					
	ID	Position	Allele	Target				Role of miRNA	Allele	Target				Role of miRNA
				Gene	Log2FC	Expression trend	Category			Gene	Log2FC	Expression trend	Category	
hsa-miR-4695-5p	rs79637190	chr1:18883265	C	SHC1	0.21	Up	Oncogenes	Tumor suppressor miRNA	T	MAPK1	−0.09	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-4707-3p	rs2273626	chr14:22956973	C						A	FGF9	−1.22	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-4741	rs7227168	chr18:22933411	C						T	FZD8	−0.70	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-4781-3p	rs74085143	chr1:54054127	G						A	FGF2	−1.44	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-4804-5p	rs266435	chr5:72878605	C						G	WNT2B	−0.96	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-499a-3p	rs3746444	chr20:34990448	A	TCF7L2	−0.68	Down	Tumor suppressor genes	Onco-miRNA	G	WNT4	0.38	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-501-3p	rs149912461	chrX:50009773	A	HEY1	−0.39	Down	Tumor suppressor genes	Onco-miRNA	G	FGF4	0.30	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-5090	rs3823658	chr7:102465754	G						A	FZD4	−1.31	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-5589-3p	rs116796353	chr19:10038396	A	MAP2K1	−0.16	Down	Tumor suppressor genes	Onco-miRNA	G					
hsa-miR-5692b	rs451887	chr21:42951004	T	FGF18	0.34	Up	Oncogenes	Tumor suppressor miRNA	C	EGF	−0.71	Down	Tumor suppressor genes	Onco-miRNA

miRNA	SNP			Reference					Alternative					
	ID	Position	Allele	Target				Role of miRNA	Allele	Target				Role of miRNA
				Gene	Log2FC	Expression trend	Category			Gene	Log2FC	Expression trend	Category	
hsa-miR-593-5p	rs73721294	chr7:128081882	C	SP1	−0.29	Down	Tumor suppressor genes	Onco-miRNA	T	KRAS	0.31	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-627-5p	rs2620381	chr15:42199650	A	DVL3	0.28	Up	Oncogenes	Tumor suppressor miRNA	C					
hsa-miR-662	rs9745376	chr16:770249	G						A	IGF1	−1.13	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-6717-5p	rs117650137	chr14:21023373	G						A	SHC1	0.21	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-6763-3p	rs3751304	chr12:132582046	C						T	WNT4	0.38	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-6777-5p	rs56155608	chr17:17813539	G						A	CSNK1A1	0.13	Up	Oncogenes	Tumor suppressor miRNA
hsa-miR-6796-3p	rs3745198	chr19:40369893	C						G	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-6810-5p	rs62182086	chr2:218341922	A	CSNK1A1	0.13	Up	Oncogenes	Tumor suppressor miRNA	G	FGF19	−1.16	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-6826-5p	rs6771809	chr3:129272155	T						C	MTOR	−0.22	Down	Tumor suppressor genes	Onco-miRNA

miRNA	SNP			Reference					Alternative					
	ID	Position	Allele	Target				Role of miRNA	Allele	Target				Role of miRNA
				Gene	Log2FC	Expression trend	Category			Gene	Log2FC	Expression trend	Category	
hsa-miR-6879-3p	rs74814065	chr11:65018557	C						T	FGF9	−1.22	Down	Tumor suppressor genes	Onco-miRNA
hsa-miR-7854-3p	rs2925980	chr16:81533949	A	AKT3	−0.41	Down	Tumor suppressor genes	Onco-miRNA	G					

Ref = The allele in the reference genome. Alt = Any other allele found at that locus. Target gene with score ≥ 80. Log2FC = Log 2 Fold Change with $P < 0.05$.

Table 1.
Prediction of the effect of in-seed SNPs in 31 miRNAs on BC development.

MiRNA	ID	Position	Reference							Alternative						ΔG
			Allele	Target				Role of miRNA	Mean MFE	Allele	Target				Role of miRNA	Mean MFE	
				Gene	Log2FC	Expression trend	Category				Gene	Log2FC	Expression trend	Category			
has-miR-146a-3p	rs2910164	chr5:160485411	C	FZD4	−1.31	Down	Tumor suppressor genes	Onco-miRNA	−16.55	G	WNT9B	−0.40	Down	Tumor suppressor genes	Onco-miRNA	−18.2	−1.7
hsa-miR-3622a-5p	rs66683138	chr8:27701697	G	E2F3	0.25	Up	Oncogenes	Tumor suppressor miRNA	−22.6	A	SHC1	0.21	Up	Oncogenes	Tumor suppressor miRNA	−19.4	3.2
hsa-miR-4284	rs11973069	chr7:73711334	C	FZD4	−1.31	Down	Tumor suppressor genes	Onco-miRNA	−23.7	T	DLL1	−0.64	Down	Tumor suppressor genes	Onco-miRNA	−20.5	3.2
hsa-miR-449c-3p	rs35770269	chr5:55172296	A	KRAS	0.31	Up	Oncogenes	Tumor suppressor miRNA	−18.2	T	AKT2	0.05	Up	Oncogenes	Tumor suppressor miRNA	−17.6	0.6
hsa-miR-4731-3p	rs66507245	chr17:15251649	T	NOTCH2	−0.60	Down	Tumor suppressor genes	Onco-miRNA	−14.7	A	CTNNB1	−0.39	Down	Tumor suppressor genes	Onco-miRNA	−19.7	−5.0
hsa-miR-548 t-3p	rs73872515	chr4:173268209	A	NRAS	0.21	Up	Oncogenes	Tumor suppressor miRNA	−15.2	C	IGF1R	0.41	Up	Oncogenes	Tumor suppressor miRNA	−16.7	−1.5
hsa-miR-557	rs78825966	chr1:168375591	C	RPS6KB1	−0.05	Down	Tumor suppressor genes	Onco-miRNA	−18.8	T	PGR	−0.85	Down	Tumor suppressor genes	Onco-miRNA	−18.5	0.3

Log2FC = Log 2 Fold Change with $P < 0.05$.
MFE: Minimum Free Energy between miRNA and 3'UTR (kcal/mol).
ΔG: The difference of mean MFE between alterative allele and reference allele.

Table 2.
Prediction of the effect of in-seed SNPs in 7 miRNAs on the degree of miRNA role in BC.

For the 62 miRNAs with 63 out-seed SNPs, the targets were not changed because the seed sequence was preserved between the two alleles of the SNPs (**Table 3**). The result showed that 44 and 18 miRNAs were predicted to function as oncogene and tumor suppressors, respectively. The predictive results do not sufficiently demonstrate an association of these 70 SNPs with BC risk. Therefore, the next step is further to predict the interaction of 69 miRNAs and targets to elucidate the potential association of these 70 SNPs on BC risk.

3.4 Predicting the interaction between miRNAs and targets

Among the 69 miRNAs, the interaction of 12 miRNAs with their targets did not differ under the allele variation of the SNPs ($\Delta G = 0$) (**Table 3**), indicating 12 SNPs in these 12 miRNAs are not likely to be associated with BC risk. The remaining 57 miRNAs have different levels of interaction with their targets under the allele variation of the SNPs ($\Delta G \neq 0$) (**Tables 2** and **3**). It suggested that 58 SNPs in these miRNAs are potentially associated with BC risk (**Tables 2** and **3**).

4. Discussion

In knowledge and improvements in bioinformatics, computational predictions of causative factors are being used as a supplementary approach to support the practical assessment of multifactorial disorders. Although miRNA can modulate up to 92% of mammalian genes, only a few target pairings of miRNAs have been experimentally verified [39]. Numerous technical challenges, such as tissue selectivity, poor expression, 3' UTR selection, and miRNA preservation, make existing methodologies difficult for experimental confirmation of interactions between miRNAs and their mRNA targets [40]. Recognizing functional SNPs in genes and examining their impacts on phenotypes may allow for more in-depth knowledge of the possible consequences of making such changes. Biogenesis, expression level, and biological function are all influenced by SNPs in human miRNA genes. For identifying the possible impacts of SNPs, several useful bioinformatics tools have been developed. All mature microRNAs with SNPs implicated in BC and their target genes were obtained. In addition, bioinformatics strategies for predicting these functional SNPs were presented. All steps and results are summarized in **Figure 1**.

The results showed that 12 SNPs did not appear to be associated with BC risk. Thirty-one in-seed SNPs are likely to be strongly associated with disease risk by altering miRNA function in BC (**Table 1**). 58 SNPs appear to be moderately or weakly associated with BC risk by altering the degree of interaction between miRNAs and their targets (**Tables 2** and **3**). According to the most significant difference between Mean MFE values in the hsa-miR-4731-3p, the effect of T/A replacement can significantly increase the change of interaction ($\Delta G = -5$); thus, it increases the effect of SNP rs66507245 on the risk of BC (**Table 2**). It seems that the association of rs66507245 with BC risk was moderate among the group of 70 SNPs predicted based on the ΔG value. The most negligible difference occurs in hsa-miR-6077, hsa-miR-8060, hsa-miR-4704-3p, and hsa-miR-6868-3p, indicating the effect of the SNPs on BC risk were weak (**Table 3**).

These SNPs were not found in GWASs since GWAS is a whole-genome sequencing approach that does not determine SNPs in non-coding regions. Only four SNPs (rs3746444, rs2910164, rs11614913, and rs4919510) have been investigated for the

MiRNA	ID	Position	Allele Ref./ Alt.	Target				Role of miRNA	Mean MFE		ΔG
				Gene	Log2FC	Expression trend	Category		Ref.	Alt.	
hsa-miR-449b-5p	rs10061133	chr5:55170716	A/G	DLL1	−0.64	Down	Tumor suppressor genes	Onco-miRNA	−25.8	−24.6	1.2
hsa-miR-6801-3p	rs10412196	chr19:52222085	T/C	AKT3	−0.41	Down	Tumor suppressor genes	Onco-miRNA	−20.8	−19.8	1.1
hsa-miR-548ae-5p	rs10461441	chr5:58530093	A/G	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−14.2	−12.9	1.3
hsa-miR-4700-3p	rs1055070	chr12:120723245	T/G	NCOA3	−0.01	Down	Tumor suppressor genes	Onco-miRNA	−18.1	−18.6	−0.5
hsa-miR-4302	rs11048315	chr12:25874055	G/A	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−19.4	−19.0	0.4
hsa-miR-5579-3p	rs11237828	chr11:79422176	T/C	JAG1	−0.21	Down	Tumor suppressor genes	Onco-miRNA	−18.6	−18.6	0.0
hsa-miR-501-3p	rs112489955	chrX:50009781	G/A	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−18.3	−18.3	0.0
hsa-miR-5691	rs112511786	chr11:9090358	G/C	MAPK1	−0.09	Down	Tumor suppressor genes	Onco-miRNA	−19.1	−19.7	−0.6
hsa-miR-4786-5p	rs115063401	chr2:239943064	G/A	AKT3	−0.41	Down	Tumor suppressor genes	Onco-miRNA	−23.7	−23.7	0.0
hsa-miR-580-5p	rs115089112	chr5:36147955	T/C	FZD3	0.31	Up	Oncogenes	Tumor suppressor miRNA	−13.4	−13.9	−0.5
hsa-miR-3124-3p	rs115160731	chr1:248826432	C/A	PGR	−0.86	Down	Tumor suppressor genes	Onco-miRNA	−16.0	−17.0	−1.0
hsa-miR-942-3p	rs115372145	chr1:117094703	C/T	SOS2	−0.37	Down	Tumor suppressor genes	Onco-miRNA	−18.3	−18.3	0.0
hsa-miR-4457	rs115769169	chr5:1309319	C/T	APC	−0.22	Down	Tumor suppressor genes	Onco-miRNA	−16.7	−16.1	0.6

MiRNA	ID	Position	Allele Ref./ Alt.	Target				Role of miRNA	Mean MFE		ΔG
				Gene	Log2FC	Expression trend	Category		Ref.	Alt.	
hsa-miR-3130-3p	rs115772313	chr2:206783285	G/A	E2F3	0.25	Up	Oncogenes	Tumor suppressor miRNA	−17.3	−17.3	0.0
hsa-miR-4514	rs116034786	chr15:80997457	A/G	ESR1	0.95	Up	Oncogenes	Tumor suppressor miRNA	−22.1	−20.4	1.7
hsa-miR-196a-3p	rs11614913	chr12:53991815	C/T	JAG1	−0.21	Down	Tumor suppressor genes	Onco-miRNA	−19.9	−18.9	1.0
hsa-miR-548at-5p	rs11651671	chr17:42494785	G/A	PGR	−0.86	Down	Tumor suppressor genes	Onco-miRNA	−15.3	−13.8	1.5
hsa-miR-3192-3p	rs11907020	chr20:18470681	T/C	NCOA3	−0.01	Down	Tumor suppressor genes	Onco-miRNA	−14.0	−14.0	0.0
hsa-miR-5700	rs12314280	chr12:94561809	T/C	AXIN2	−0.51	Down	Tumor suppressor genes	Onco-miRNA	−12.9	−14.4	−1.5
hsa-miR-4433a-5p	rs12473206	chr2:64340782	C/G	KRAS	0.31	Up	Oncogenes	Tumor suppressor miRNA	−17.0	−16.6	0.4
hsa-miR-6077	rs1280409926	chr1:148388307	C/T	SP1	−0.29	Down	Tumor suppressor genes	Onco-miRNA	−21.7	−21.9	−0.2
hsa-miR-548 l	rs13447640	chr11:94466555	G/A	SP1	−0.29	Down	Tumor suppressor genes	Onco-miRNA	−14.5	−15.9	−1.5
hsa-miR-548ar-3p	rs141659366	chr13:114244551	G/A	FZD4	−1.31	Down	Tumor suppressor genes	Onco-miRNA	−14.4	−12.9	1.5
hsa-miR-4444	rs142357696	chr3:75214531	A/G	E2F1	1.18	Up	Oncogenes	Tumor suppressor miRNA	−24.7	−22.1	2.6
hsa-miR-888-5p	rs143634721	chrX:145994837	C/A	PGR	−0.86	Down	Tumor suppressor genes	Onco-miRNA	−16.2	−15.3	0.9
hsa-miR-892b	rs146806052	chrX:145997215	A/G	DLL1	−0.64	Down	Tumor suppressor genes	Onco-miRNA	−21.0	−24.2	−3.2

MiRNA	ID	Position	Allele Ref./ Alt.	Target				Role of miRNA	Mean MFE		ΔG
				Gene	Log2FC	Expression trend	Category		Ref.	Alt.	
hsa-miR-8060	rs1514422	chr3:96360020	G/A	KRAS	0.31	Up	Oncogenes	Tumor suppressor miRNA	−18.3	−18.5	−0.2
hsa-miR-6887-5p	rs1688017	chr19:35122719	G/A	PIK3R3	0.33	Up	Oncogenes	Tumor suppressor miRNA	−28.7	−25.2	3.5
hsa-miR-1304-3p	rs2155248	chr11:93733700	G/T	NOTCH2	−0.60	Down	Tumor suppressor genes	Onco-miRNA	−18.3	−18.6	−0.3
hsa-miR-3130-5p	rs2241347	chr2:206783257	C/T	TCF7	−0.47	Down	Tumor suppressor genes	Onco-miRNA	−22.3	−22.6	−0.4
hsa-miR-1343-5p	rs2986407	chr11:34941869	T/C	CSNK1A1	0.13	Up	Oncogenes	Tumor suppressor miRNA	−21.7	−20.9	0.8
hsa-miR-5087	rs2992458	chr1:148334528	A/G	GSK3B	0.14	Up	Oncogenes	Tumor suppressor miRNA	−17.2	−16.2	1.0
hsa-miR-5189-3p	rs35613341	chr16:88468999	C/G	JAG1	−0.21	Down	Tumor suppressor genes	Onco-miRNA	−25.6	−28.3	−2.7
hsa-miR-8084	rs404337	chr8:93029770	G/A	NOTCH1	−0.28	Down	Tumor suppressor genes	Onco-miRNA	−12.7	−13.4	−0.7
hsa-miR-548ap-5p	rs4414449	chr15:85825667	G/A	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−14.0	−13.0	0.9
hsa-miR-608	rs4919510	chr10:100975021	C/G	CDKN1A	−0.36	Down	Tumor suppressor genes	Onco-miRNA	−30.7	−31.2	−0.5
hsa-miR-7157-3p	rs56148568	chr2:140586631	T/C	EGFR	−1.44	Down	Tumor suppressor genes	Onco-miRNA	−19.1	−19.1	0.0
hsa-miR-6744-3p	rs56310773	chr11:1256664	C/T	PIK3R1	−0.81	Down	Tumor suppressor genes	Onco-miRNA	−32.5	−30.0	2.5
hsa-miR-6805-3p	rs56312243	chr19:55388234	C/T	MAPK1	−0.09	Down	Tumor suppressor genes	Onco-miRNA	−19.5	−19.7	−0.3

MiRNA	ID	Position	Allele Ref./ Alt.	Target				Role of miRNA	Mean MFE		ΔG
				Gene	Log2FC	Expression trend	Category		Ref.	Alt.	
hsa-miR-6071	rs56790095	chr2:85783659	C/G	WNT3	0.34	Up	Oncogenes	Tumor suppressor miRNA	−17.0	−17.0	0.0
hsa-miR-548ab	rs59323834	chr3:103524093	C/T	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−14.6	−12.1	2.5
hsa-miR-3928-5p	rs5997893	chr22:31160117	A/G	ESR1	0.95	Up	Oncogenes	Tumor suppressor miRNA	−17.7	−15.6	2.1
hsa-miR-596	rs61388742	chr8:1817259	T/C	PTEN	−0.26	Down	Tumor suppressor genes	Onco-miRNA	−19.7	−17.5	2.2
hsa-miR-3922-5p	rs61938575	chr12:104591665	G/A	SHC1	0.21	Up	Oncogenes	Tumor suppressor miRNA	−28.6	−27.3	1.4
hsa-miR-4772-5p	rs62154973	chr2:102432320	C/T	FOS	−1.17	Down	Tumor suppressor genes	Onco-miRNA	−20.3	−20.3	0.0
hsa-miR-646	rs6513497	chr20:60308547	T/G	PIK3R1	−0.81	Down	Tumor suppressor genes	Onco-miRNA	−23.4	−20.9	2.6
hsa-miR-8063	rs7162033	chr15:36972836	C/G	IGF1	−1.13	Down	Tumor suppressor genes	Onco-miRNA	−17.6	−15.9	1.7
hsa-miR-8063	rs7183051	chr15:36972838	G/A	IGF1	−1.13	Down	Tumor suppressor genes	Onco-miRNA	−17.6	−16.9	0.7
hsa-miR-6868-3p	rs7208391	chr17:76098024	C/G	AXIN2	−0.51	Down	Tumor suppressor genes	Onco-miRNA	−18.8	−18.6	0.2
hsa-miR-4679	rs72810954	chr10:89063382	G/A	WNT16	0.12	Up	Oncogenes	Tumor suppressor miRNA	−17.0	−17.0	0.0
hsa-miR-4799-5p	rs72955519	chr4:147782619	G/A	MAPK1	−0.09	Down	Tumor suppressor genes	Onco-miRNA	−18.8	−16.3	2.5
hsa-miR-4999-5p	rs72996752	chr19:8389352	A/G	CTNNB1	−0.40	Down	Tumor suppressor genes	Onco-miRNA	−18.5	−17.4	1.1
hsa-miR-624-3p	rs73251987	chr14:31014677	C/G	GSK3B	0.14	Up	Oncogenes	Tumor suppressor miRNA	−15.5	−13.7	1.9

MiRNA	ID	Position	Allele Ref./ Alt.	Target				Role of miRNA	Mean MFE		ΔG
				Gene	Log2FC	Expression trend	Category		Ref.	Alt.	
hsa-miR-4727-5p	rs73295187	chr17:38825855	A/C	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−21.4	−19.6	1.8
hsa-miR-4739	rs73410309	chr17:79707227	G/C	BAK1	0.49	Up	Oncogenes	Tumor suppressor miRNA	−32.5	−31.6	0.9
hsa-miR-6504-5p	rs74469188	chr16:81611365	T/C	FZD5	−0.56	Down	Tumor suppressor genes	Onco-miRNA	−21.9	−21.5	0.4
hsa-miR-323b-5p	rs75330474	chr14:101056252	C/T	PIK3R3	0.33	Up	Oncogenes	Tumor suppressor miRNA	−18.5	−16.5	2.0
hsa-miR-6841-3p	rs76347846	chr8:24953808	A/G	GADD45A	−0.30	Down	Tumor suppressor genes	Onco-miRNA	−19.1	−15.2	3.9
hsa-miR-4704-3p	rs76595065	chr13:66218307	T/C	GSK3B	0.14	Up	Oncogenes	Tumor suppressor miRNA	−23.2	−23.4	−0.2
hsa-miR-6839-5p	rs7804972	chr7:64679085	G/A	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−19.4	−18.1	1.3
hsa-miR-6885-5p	rs78293125	chr19:6389688	A/G	TCF7L2	−0.68	Down	Tumor suppressor genes	Onco-miRNA	−24.4	−24.4	0.0
hsa-miR-4520-3p	rs8078913	chr17:6655449	C/G	GRB2	0.35	Up	Oncogenes	Tumor suppressor miRNA	−24.2	−21.2	3.0
hsa-miR-548 h-5p	rs9913045	chr17:13543607	G/A	CDK6	−0.67	Down	Tumor suppressor genes	Onco-miRNA	−13.8	−13.8	0.0

Ref = The allele in the reference genome. Alt = any other allele found at that locus.
Log2FC = Log 2 fold change with $P < 0.05$.
MFE: Minimum free energy between miRNA and 3'UTR (kcal/mol).
Mean MFE: the mean value of MFEs from all miRNA binding sites on 3'UTR.
ΔG: The difference of mean MFE between alterative allele and reference allele.

Table 3.
Prediction of the effect of 63 out-seed SNPs in 62 miRNAs on the degree of miRNA role in BC.

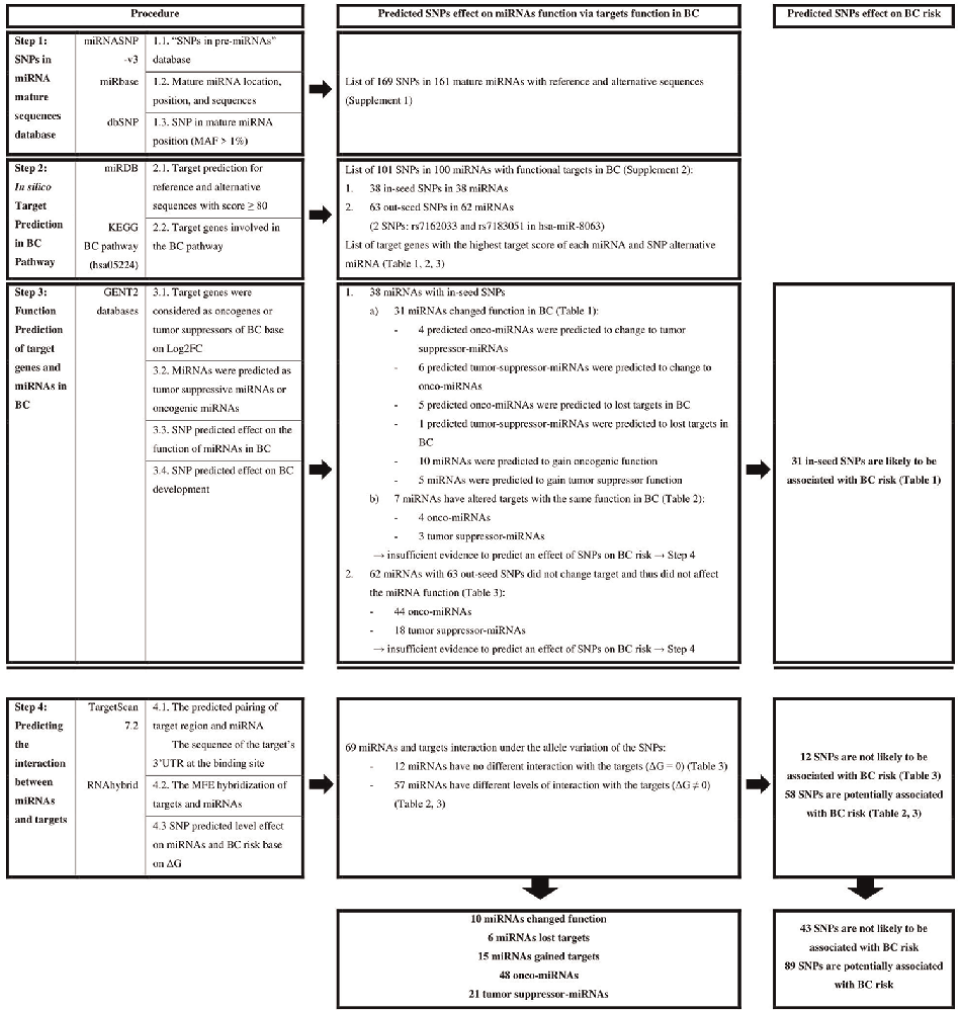

Figure 1.
The methodology and summary results of study.

association with BC risk in case–control studies. The presence of the C allele of rs2910164, an in-seed SNP, in hsa-miR-146a-3p was associated with an increased risk of BC (C vs. G: OR = 1.4, 95% CI = 1.03–1.85, p = 0.03) [41]. Allele C of rs11614913 in hsa-miR-196a-3p was found to be significantly associated with decreased risk of BC (C vs. T: OR = 0.64, 95%CI = 0.49–0.85, p = 0.0019) [42]. Allele G of rs3746444 in seed region of hsa-miR-499a-3p significantly increased BC susceptibility only among Asians (G vs. A: OR = 1.12, 95% CI = 1.00–1.26, P = 0.04) [43]. The G allele rs4919510 in hsa-miR-608 decreased the risk of BC (G vs. C: OR = 0.53, 95%CI 0.30–0.92, p = 0.024) [44]. The remaining 97 SNPs have not been identified for an association with BC risk in any association studies. However, 7 of these (rs7210937, rs12402181, rs2273626, rs2620381, rs35770269, rs10061133, and rs6513497) are associated with the risk of other cancers, including oral and pharyngeal squamous cell carcinoma [45], acute lymphoblastic leukemia [46], leukopenia [47], gastric cancer [48], colon cancer [49], esophageal squamous cell carcinoma [50], and hepatocellular carcinoma [51].

These shreds of evidence suggest that candidate SNPs in this study can be associated with risk in BC and other types of cancer.

At the level of investigating the role of miRNAs in BC, 31 miRNAs and 69 miRNAs were predicted to change and remain unchanged function in the presence of SNP allele changes, respectively. Among them, 7 miRNAs (miR-4513, miR-501-3p, miR-580-5p, miR-3130-3p, miR-196a-3p, miR-8084, and miR-3922-5p) had predicted roles in BC consistent with the results of previous functional studies. Hsa-miR-4513, has-miR-196a-3p, and has-miR-8084 suggested functions as an oncogene in the progression of BC [52–54] were also predicted as onco-miRNAs in this study (**Tables 1** and **3**). Hsa-miR-501-3p, has-miR-580-5p, has-miR-3130-3p, and has-miR-3922-5p indicated tumor suppressor function [55–58] were also predicted as tumor suppressor miRNAs (**Tables 1** and **3**).

Our work gives vital insights into the pathophysiology and progression of BC by presenting essential information on the likely influence of SNPs and distinct regulation patterns on miRNA production and function. Consequently, genetic variations appear to be the appropriate criterion for early diagnosis of BCs in future.

5. Conclusion

In brief, numerous resources were applied following a comprehensive screening of mature miRNAs, including SNPs that play a deciding role on BC to identify SNP's functional effect in the miRNA gene. We conducted a thorough investigation into the influence of the mined SNPs on miRNA function, including target prediction, miRNA-target interaction, and target expression level. Theoretically, this work revealed a list of possible causative SNPs in the mature miRNA gene that may be addressed for further practical research in BC management.

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Conflict of interest

The authors declare no potential conflicts of interest.

Credit authorship contribution statement

Thanh Thi Ngoc Nguyen was involved in conceptualization, methodology, formal analysis, data curation, software, writing—original draft, writing—editing, visualization, project administration, and funding acquisition. Thu Huynh Ngoc Nguyen was involved in investigation, validation, and writing—review. Luan Huu Huynh and Hoang Ngo Phan were involved in investigation and writing—review. Hue Thi Nguyen was involved in resources, conceptualization, methodology, supervision, writing—review, project administration, and funding acquisition.

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Chapter 7

Long Non-Coding RNA in Neural Stem Cells Self-Renewal, Neurogenesis, Gliogenesis and Synaptogenesis

Neetu Singh

Abstract

Evidence reports the key roles of lncRNAs in several regulatory mechanisms of neurons and other brain cells. Neuronal lncRNAs are crucial for NSCs mediated-neuronal developmental stages like neurogenesis, neuronal differentiation, and synaptogenesis. Moreover, multilineage properties of NSCs and their association to specific cell types render them to identify the commonly accepted biomarkers for the brain. It is important to delineate the correlation between lncRNAs and NSCs fate decisions during neuronal development stages. In this review, we will summarize how NSCs fabricate embryonic tissue architecture of the central nervous system (CNS) and act as residuum in subventricular zone (SVZ) nearby the lateral wall of the lateral ventricles and the subgranular zone (SGZ) of hippocampus dentate gyrus (DG) of the adult brain. Additionally, describe the roles and molecular mechanisms of lncRNAs involved in NSCs self-renewal, neurogenesis, gliogenesis and synaptogenesis over the course of neural development. This will help us to better understand neuronal physiology.

Keywords: long noncoding RNAs, neurons, neuronal development, neuronal differentiation, neurogenesis, synaptic activity, synaptic plasticity

1. Introduction

In the central nervous system (CNS), an efficient spatio-temporal regulatory mechanisms play an important role in neural stem cells (NSCs) for the development of neurons and other brain cells. The development and differentiation of the CNS is intricate in nature, and plethora of regulatory factors are involved and express ~40% of lncRNAs [1]. lncRNAs are involved in the early development/differentiation of the nervous system (NS) through NSCs to synaptogenesis [2–4]. Field et al. [5] constituted pluripotent stem cell (PSC)-derived cerebral cortex organoid (CO) cell cultures which recapitulate the cellular organization and gene expression events observed in fetal tissue. PAX6 (neural progenitors), CTIP2 and TBR1 express during early deep-layer neuron and TBR2 expression is in intermediate progenitors in Human COs at

Human cell atlas		ARCHS4	
Week 2-GO-Term enrichment	**Week 5-GO-Term enrichment**	**Week 2-GO-Term enrichment**	**Week 5-GO-Term enrichment**
Fetal brain	Fetal brain	Motor neuron	Motor neuron
Prefrontal cortex	Prefrontal cortex	Prefrontal cortex	Prefrontal cortex
Amygdala	Amygdala	Neuronal epithelium	Spinal cord
Pineal night	Pineal night	Cerebellum	Spinal cord (bulk)
Pineal day	Pineal day	Spinal cord	Cerebellum
Smooth muscle	Cerebellum peduncle	Spinal cord (bulk)	Fetal brain
Occipital lobe	Whole brain	Mid brain	Cingulate gyrus
Uterus	Cardiac myocytes	Fetal brain cortex	Sensory neuron
Prostrate	Cerebellum	Fetal brain	Cerebral cortex
Cardiac myocytes	Occipital lobe	Sensory neuron	Brain (bulk)

Table 1.
The top 10 enriched GO terms from ARCHS4 [6]; based on publicly available RNA-seq data from human and mouse) and Human Cell Atlas ([7]; based on microarrays of human and mouse tissues) ranked by their combined enrichment score [5].

day 35. The ratio of human COs, pluripotency markers such as *OCT3/4* were down-regulated, while early neural stem cell markers, including *PAX6*, were up-regulated during the first week. Subsequently by 5th week *TBR1* were strongly expressed in Deep-layer neurons. Gene Ontology (GO) term analysis showed significant enrichment of lncRNA associated with neuronal development along with prefrontal cortex and foetus brain as described in **Table 1**. On assessing lncRNAs, previously described mammalian conserved lncRNAs *MALAT1*, *NEAT1*, *H19*, *PRWN1*, and *CRNDE* and 79 unannotated lncRNAs were observed among the 920 primate-conserved category. Markedly, in week 2 transient expression of lncRNAs (TREX) originated from *TREX2174* (RP11-314P15) which includes 19 bp insertion overlay at its transcription start site. Importantly, *TREX4039* (extends over AC011306 and *MIR217HG*) peaks at first or second week human COs and disappears by fifth week. At week 2 of human Cos gene expression specific single cells were processed for scRNA-seq libraries and TREX, which have potential roles in early cortical cell fate specifications were identified as depicted in **Table 2**. The clusters identified at week 2 (neuroepithelium (NE) cells-cluster of 1261 cells; early-forming Cajal-Retzius (CR) cells-cluster of 356 cells; the cortical radial glia (RG)-cluster of up 2593 cells were converted into clusters of early neurons (26%), intermediate progenitors (11%), mature radial glial (RG) (26%), immature RG (18%), dividing RG (12%), and cell doublets (8%) at week 5 of organoid cells.

The study showed the importance of cellular specificity of lncRNA function [8]; robust regulatory effects on distal genes upon activation/repression of these TREX lncRNAs was also observed (**Table 2**). In our recent review [9], we have already discussed the mechanisms to identify lncRNA, synthesize lncRNA, transcription of lncRNA and localized processing of lncRNA in nuclear and cellular portions, and their regulatory functions mediated by linking to chromatin alone or by constructing lncRNA-protein-chromatin complexes owing to chromatin modifications and genomic stability, hence influencing pluripotency. LncRNA are also involved in

Name of cells	Gene expression markers	Cluster of single cells	Enrichment of transiently expressed lncRNAs (TREX) at week 2	Activity of lncRNA in trans manner
Neuroepithelium (NE) cells	*HES3* and *NR2F1*	Cluster of 1261 cells	*TREX108* and *TREX8168*	Genes associated with whole brain, superior frontal gyrus, and cerebral cortex suggesting a role in general neural gene networks
Cajal-Retzius (CR) cells	*TBR1*, *EOMES*, *LHX9*, and *NHLH1*	Cluster of 356 cells	*TREX4039*	Induced genes enriched in the ARCHS4 neural epithelium gene set and repressed expression of those associated with superior frontal gyrus and astrocytes
Cortical radial glia (RG)	*SOX2*, *EMX2*, *NNAT*, *PTN*, and *TLE4*	Cluster of 2593cells	*TREX5008*	Induced genes enriched in the ARCHS4 neural epithelium gene set and repressed expression of those associated with superior frontal gyrus and astrocytes

Early-forming Cajal-Retzius (CR) cells expressed TBR1, EOMES, LHX9, and NHLH1, comprising a cluster of 356 cells. The largest cluster strongly expressed cortical radial glia (RG) markers SOX2, EMX2, NNAT, PTN, and TLE4, making up 2593 cells showed discovery of transiently expressed lncRNAs, which have potential roles in early cortical cell fate specifications [5].

Table 2.
10× Chromium 3′ end scRNA-seq on week 2 human COs gene expression based neuroepithelium (NE) cells, were identified by expression of HES3 and NR2F1, forming a cluster of 1261 cells.

transcriptional and post transcriptional regulation and serve as scaffolds, chromatin modifiers and miRNA sponges have also been explained in our recent review [9]. This review aims to explain the roles and molecular mechanisms of lncRNAs focusing on NSCs self-renewal, neurogenesis, gliogenesis and synaptic excitability over the course of neural development.

2. Role of NSCs in designing embryonic tissue architectonics of the central nervous system

Embryonically, the neurons of the brain are organized into layers (cortices) and clusters (nuclei), each having different function and interrelation with other neurons. The embryonic neural tube comprises single cell layer thick germinal neuroepithelium (GNE) which encompasses most proliferative neural stem cells. The GNE continues from the outer edge to the lumen of the neural tube [10], the nuclei of GNE exist at discrete levels, hence giving the impression of numerous cell layers in the neural tube. The nuclei move inside GNE as they move forward to different G0/G1, S and M phases of cell cycle. First and foremost, the S phase or DNA synthesis occurs

when the nucleus is at the outer border of the neural tube, subsequently the nucleus moves towards lumen of the neural tube as the cell cycle proceeds.

However, after a certain time period some of the GNE-cells stop DNA synthesis and mitosis and migrate and differentiate into neuronal and glial cells outside the neural tube [11, 12]. The dividing cells of GNE have been found in the outer cortex of the adult brain, this was explained by radioactive thymidine studies. The published reports suggested neuroepithelial stem cells split "vertically" instead of "horizontally." After vertical division, the daughter cell besides the lumen of the neural tube remains affixed to the ventricular surface (and persists as stem cells in the ventricular zone), while the other daughter cell migrates away [13]. This ultimate vertical division is the terminal division and origination of a neuron happens. The cells which originate late move through GNE and form the most superficial regions of the cortex. Consecutive differentiation is based on the inhabitancy of the new neurons once outside the GNE [14, 15]. The process of division persists i.e., the cells next to the lumen sustain division and are exported outwards to the lumen. In such a manner they form a second layer and sequential addition of more cells derived from GNE thickens and widens the outermost layer and forms mantle (or intermediate zone). The GNE itself acts as a ventricular zone (later known as ependyma that embraces neural stem cells). Further, the cells of the mantle zone differentiates both in neurons and glia. The axons of neurons make connections and move away from the lumen while glial cells form sheath around axons and these are suggested as white matter. While the neuronal cell bodies represent the grey matter.

3. Spinal cord and medulla assembly

In the spinal cord and medulla, three-zone decorum first-ependymal, second-mantle, and third-marginal layers are maintained throughout development. The grey matter (mantle) is a butterfly-shaped structure and is surrounded by white matter; both grey and white matter are enclosed by the connective tissue. Further, on maturation the neural tube is divided into dorsal and ventral halves through a longitudinal groove, the sulcus limitans. The dorsal portion receives input from sensory neurons, whereas the ventral portion is intricated in performing various motor functions.

4. Cerebellar structure

In cerebellum the different neurons which migrate outside the GNE through cell migration, further undergoes differential neuronal proliferation, and selective cell death that creates modifications in the three-zone pattern (ependymal, mantle, and marginal layers).

In the cerebellum, a small quantity of neuronal precursors i.e., neuroblasts derived from GNE either enter the marginal zone to form clusters of neurons called nuclei or migrate to the outer surface of the neural tube to form a new germinal zone. Each single nucleus of the marginal zone works as an individual functional unit, and connects to the outer layers of the cerebellum and other parts of the brain. The new germinal zone forms the outer layer of the developing cerebellum and is also known as the external granule layer-EGL (two cells thick layer. The outer layer of the EGL is composed of dividing neuroblasts, while the inner layer of EGL comprises postmitotic neuroblasts. The latter are the precursors of the preeminent neurons of the

cerebellar cortex and the granule neurons. Granule neurons are the major component of the internal granule layer-IGL that migrates back to white matter of developing cerebellum. Concurrently, the ependymal layer (ventricular zone of GNE) differentiates into neurons and glial cells which include the unique Purkinje neurons. Purkinje neurons interact to form electrical synapses (unlike chemical synapses, there is direct interaction between presynaptic and postsynaptic neurons and also support granule neurons. These neurons secrete a specific morphogen Sonic hedgehog (SHH), which maintains the division of granule neuron precursors (neuroblasts) in the EGL [16].

Each Purkinje neuronal cell body is shaped like a flask and has a large, flat, highly branched thread like extensions creating a dendritic Arbor. This helps in forming hundreds of thousands connections (synapses) with other cells like Bergmann glial cells and granule cells. The axon of each Purkinje neuron connects to neurons in the deep cerebellar nuclei and transmits impulses to the region of cerebellum which controls movement.

The evolution of spatial arrangement of neurons is an essential process for the development and functioning of the cerebellum. Positioning of young neurons is under glial guidance [17, 18]. Importantly, the granule cell precursors (neuroblasts) move forward through adhesion on the long axonal processes of the Bergmann glia [19, 20]. The neural-glial reciprocal interaction between glial cells and neuroblasts [17, 21] is held up by adhesion protein called astrotactin that assists in adhesion of neurons to the glial cells [22, 23].

5. Cerebral structure

The three-zone positioning pattern cerebellum first-ependymal, second-mantle, and third-marginal layers of the neural tube is modified to vertical and horizontal positioning in the cerebrum.

Vertical positioning shows different vertical layers which interact with one another. As in cerebellum, in cerebrum, some of the neuroblasts move outwards from GNE and form the mantle zone and travel under glial guidance along the white matter to produce an additional layer of neurons at the outer layer of the cerebral cortex. This additional layer of grey matter (mantle) is called the neocortex. Neocortex is 2–5 mm thick, present on the surface of brain time-dependently differentiates into six layers (molecular layer, external granular layer, external pyramidal layer, internal granular layer, ganglionic layer and multiform layer) of neuronal cell bodies. Although layers are premature in nature and the maturity is not completed until the middle of childhood. Each vertical layer of the neocortex is unique and holds a specific function based on the types of neurons, and their connections with other neurons. For example, the inputs sent by thalamus are received by the neurons of layer 4, while output back to the thalamus is sent by the neurons of layer 6.

Horizontal positioning of the layers in cerebrum is more complex and they approximately form almost 40 regions that are regulated physiologically and have specific functions. For example, neurons in vertical cortical layer 6 horizontally possess both "visual cortex" and "auditory cortex". Auditory cortex is more anterior in position than the visual cortex. Visual cortex projects axons to the lateral geniculate nucleus of the thalamus (for vision), while auditory cortex projects axons to the medial geniculate nucleus of the thalamus (for hearing).

The vertical and horizontal arrangement in cerebrum depends on numerous multilineage neuroblasts derived from GNE. Further, on terminal mitotic division most of

the neuroblasts of ventricular (ependymal) progress under glial guidance and lead to the formation of cortical plates located at the outer surface of the cortex of cerebrum.

To conclude, the neuroblasts with the early origin derived from GNE form the layer adjacent to the ventricle while neurons of later origin migrate through GNE and set apart to form the more exterior layers of the cortex. This positioning of cells forms an "inside-out" descent of expansion [24]. The neuroblast of the ventricular (ependymal) zone divides into neurons and glial cells in any of the cortical layers [25]. However, the fates of neuroblasts depend on the terminal division. The neuroblasts early in development are in their mid-S phase i.e., are in the way of division i.e., final division is not complete, hence likely become any neuron (for instance neurons of layers 2 or 6), at later stage of development of neuroblasts final mitosis is complete that give rise only to upper-level (layer 2) neurons [26]. The migration of the majority of these young neurons occurs in a radial manner on glial processes [27]. The migration initiates from the ventricular zone to the cortical plate. After neurogenesis, the generated young neurons (~12%) migrate laterally from one region of the cerebral cortex into another [28]. Further, the positioning of young neurons in different cortical areas are designated to unique functional domains. Once the neuronal cells make an appearance at their final designated cortical area, they may produce specific adhesion molecules that sort them together as brain nuclei [29].

6. Role of adult neural stem cells (NSCs) in neurogenesis

The cortical regions are composed of abundant neurons and glial cells produced from the differentiation of GNE-derived neuronal precursor cells/neuroblasts, which are self-sufficient to renew themselves as well as differentiate into multilineage neurons in the brain [30]. The above process of producing neurons and glial cells are termed neurogenesis and gliogenesis, respectively [31]. The largest NSCs/neuronal precursor cells/neuroblasts niches all through the life are essentially located in the adult ventricular-subventricular zone (V-SVZ) adjacent to the walls of the lateral ventricles [32] and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus where new dentate granule cells are produced.

6.1 NSC model in the adult subventricular zone under basal conditions

V-SVZ produces large number of neuroblasts which travel a long distance through the rostral migratory stream (RMS) to the olfactory bulb (OB) in form of a chain, where they finally differentiate into granule cell type local interneurons [33]. Further, once reaching the core of the OB, immature neurons separate from the RMS through radial migration and then migrate towards glomeruli and differentiate into various subtypes of periglomerular (PG) interneurons. PG interneurons interconnect with the apical dendrites (often lack axons) of mitral and tufted cells inside the glomeruli [34]. The majority of interneurons are GABAergic granule cell neurons, which lack axons and form dendro-dendritic synapses with lateral dendrites of mitral and tufted cells in the external plexiform layer. In addition, few number of GABAergic PG interneurons and small percentage of interneurons are dopaminergic in nature. One report also suggested that very low percent of new interneurons are glutamatergic juxtaglomerular neurons in nature [35]. Transcript based markers have been identified for the five developmental stages of adult SVZ neurogenesis of the lateral ventricle and OB [1] activation of radial glia-like cells (GFAP, Vimentin and nestin positive) in the SVZ zone

in the lateral ventricle (LV); [2] proliferation of transient amplifying cells/progenitor cells (Mash1 and low amount of nestin); [3] produce neuroblasts (Dlx2 positive) that migrate to OB; [4] subsequent chain migration of neuroblasts (Dlx2 positive) within the RMS and radial migration of immature neurons (Dlx2 and DCX positive) in the OB; [5] Synaptic interaction through interneurons (NeuN positive) and maturation of GC and PG neurons in the OB (reviewed by [33]) .

6.2 NSC model in the adult hippocampus under basal conditions

Adult SGZ in the dentate gyrus of the hippocampus possess proliferating radial and non-radial neuronal precursor cells that generate intermediate progenitors/transit amplifying cells, which in succession generate neuroblasts.

The multi-lineage radial precursors i.e., radial glia-like cells (RGLs or Type-1 cells) in dentate gyrus of adult hippocampus are identified through expression of transcripts like nestin, GFAP and Sox2. Besides transcript markers a distinguishing feature RGL possesses is radial branch which extends through the granule cell layer. Single RGL undergoes several rounds of self-renewal and differentiation to produce both neurons and astrocytes for a long period, displaying characteristic stem cell properties by each RGLs as evidenced through in vivo clonal assays [36].

Quiescent RGLs once triggered from quiescence stage i.e. G0 phase, RGLs moves towards formation of specific cell types like RGLs, non-radial precursors, proliferative intermediate progenitors (IPCs, or Type-2 cells) and astroglia, excluding the oligodendrocyte lineage. Later on the fate of RGLs may be decided whether they remain in a proliferative state, return to quiescence, or differentiate into an astrocyte.

Non-radial precursors generate new neurons in the adult SGZ and act as primary precursors [37]. They lack any radial process and few cells present as parallel extensions to the dentate granule cell layer. Unlike RGL, non-radial precursors express Sox2, but not GFAP [38] and more proliferative i.e., mostly present in cell cycle [37, 38]. They may or may not show involvement of IPCs and may generate new RGLs or astroglia, while maintaining the precursor state [36].

IPCs of the SGZ region of dentate gyrus rapidly proliferate and possess small tangential processes expressing Tbr2 as transcript marker [39]. They are derived from both radial and non-radial precursors [36, 38]. Afterwards they convert into secondary transient amplifying precursors and start expressing DCX and Prox1, markers of committed immature neurons [39]. IPCs re-enter cell cycle for self-renewal or produce astroglia or maintain as precursors over a long duration remains unclear.

In adult dentate non-neurogenic areas and SGZ region enrichment of another, progenitor population of NG2 cells have been identified [40] Major features of NG2 cells are long wispy processes, expression of specific proteogylcan NG2 as well as PDGF, Sox10 and Olig2 [41] has been observed. Presence of NG2 cells and oligodendrocytes in non-neurogenic areas differentiate them from RGLs in the adult SGZ [36] suggesting that they may have derived from different precursor populations with different embryonic origins. The consensus remains that NG2 cells may produce astroglia during development, and oligodendrocyte generation in the adult nervous system [41].

Astroglia, non-neuronal quiescent precursor cell type as proposed by absence of nestin expression, are a prospective third precursor population in the adult SGZ. Major characteristic features are horizontal or bushy morphology and expression of GFAP, S100β and Aldh1l1 markers [42]. The new astroglia generate from RGLs in the adult SGZ; afterwards they migrate to the hilus or molecular layer [36]. Hence,

neuroblasts from NSCs in SGZ migrate short distances into the granule cell layer and mature into neurons, then integrate into functional circuits [43].

7. The effect of lncRNA on NSCs/NPCs pluripotency

Cell type- and tissue-specific long non-coding RNAs (lncRNAs) incorporate varying classes of transcripts that can control various fundamental molecular and cellular processes in organ development, disease and cancer. Here we will discuss the differential expression and unique function of lncRNAs in human brain development.

LncRNAs are present amply and specifically in different lineages of neurogenic cell-types which plays an important role in neuronal development [8]. For instance, Liu et al. [44] identified cell type-specific lncRNA and mRNA transcript pairs in developing neocortex; (a) radial glia-specific lncRNA *LOC646329*-mRNA *PAX6*, (b) maturing neuron-specific lncRNA *LINC00599-mRNA RTN1*, (c) interneuron-specific lncRNA *DLX6-AS1*-progenitor and differentiated cell-expressed mRNA *NNAT.* Maturing neurons of the cortical plate (CP) were enriched with *LINC00599*. The subpial granular layer interneuron showed predominance of unique *DLX6-AS1* [45, 46].

8. The function of lncRNAs in neurogenesis/neural differentiation

LncRNAs play a pivotal role in regulating epigenetic elements in NPCs or NSCs differentiation and neural development [47, 48]. Spatial and temporal expression of lncRNAs plays a very crucial role in neuronal development in the developing CNS [49]. However, the abundance and specificity of lncRNAs in different neurogenic cell-types or the specific functions show their involvement in development and cellular identity in the nervous system [1, 8].

Recently, a gene expression atlas of embryonic neurogenesis in Drosophila revealed complex spatiotemporal regulation of lncRNAs. This involved a significant set of 13 lncRNAs, on illustration they represented [1] time dependent appearance at subcellular and cellular levels, [2] moderate to excess expression in cytoplasmic or nuclear regions [3] were involved in precise RNA processing (for example splicing and nuclear-cytoplasmic commutation), during critical events in neurogenesis in Drosphila **Table 3** [50].

Interestingly, lncRNAs have undergone specific adaptive functions like selective loss during the evolution of neurogenesis [51]. LncRNA was a key determinant in NSCs or NPCs during cell-fate determination. Additionally, specific lncRNA types are involved in the different stages of NPCs or NSCs differentiation. The neuronal and astrocytic differentiation have been well explained based on a plethora of differentially expressed epigenetically modified lncRNA. It has also been suggested that different lncRNAs have biasness for neuronal differentiation compared to astrocytic differentiation. Although astrocytic differentiation is related more to the sense lncRNAs (lncRNA transcribed from the sense strand of exons possessing coding genes) [52]. Besides, the above functions lncRNAs also participate in synchronizing the fate of NSC differentiation into glia and neurons under physiological and pathological conditions [53, 54]. An overview of lncRNA involved in neurogenesis/neuronal differentiation, gliogenesis or synaptogenesis has been well described in **Table 4**.

CR30009	CR30009 is spliced and primarily exported to the cytoplasm	Increased expression in the early IC and in NBs (4–6 h and 6–8 h) constitutes the earliest neuroblast marker of the glial lineage However, was most highly enriched in glial cells stage 9/10 and stage 13/14	Specific expression in NBs and shows increased expression in glial cells
CR43283 (also known as cherub)	Specifically localized to the cytoplasm throughout embryogenesis and is clearly spliced, but harbors no coding potential and showed dynamic temporal regulation	Expression of cherub was strongly enriched in the earliest neuroblasts at 4–6 h, but enrichment quickly decreased in later neuroblasts (6–8 h); however, over time cherub became specifically expressed being strongly enriched in differentiated neurons and glia by the end of neurogenesis at 18–22 h	Specific expression in NBs and shows increased expression in differentiated neurons and glia
CR32730	Moderately enriched in the nuclear fraction in early and late embryos	CR32730 first detected in 4-6 h neuroblasts and was moderately enriched at 8–10 h in the neuronal, but not in the glial, population	Specific expression in NBs and shows increased expression in early neurons
CR46003	CR46003 was one of the most abundant and did not exhibit clear subcellular enrichment in either early or late embryos	First detected in the ventral column and was most highly enriched in early neuroblasts, but expression persisted in neuroblasts and early neurons	Specific expression in NBs and shows increased expression in early neurons
CR44024	Not predicted to exhibit distinct subcellular localization in early (6-8 h) embryos, but was moderately enriched in the cytoplasm at the end of embryogenesis (18–22 h)	Was first enriched in early neuroblasts and persisted through neuronal differentiation, and is predicted to be excluded from the intermediate and ventral columns and glia	Specific expression in NBs and persistent expression in neurons

Biological materials studied over the time course of neurogenesis: intermediate column (IC); ventral column (VC); neuroblasts (NBs); neurons; glia. Markers specifically used for RNA-FISH was NBs, Pros; neurons, Elav; glia, Repo (4–6 and 6–8 h (IC, VC, NBs); 6–8, 8–10 and 18–22 h (neurons and glia)) [50].

Table 3.
A list of high-confidence set of lncRNAs classified time dependently; distinct subcellular localization patterns and cell-specifically; moderate to overabundance in cytoplasmic or nuclear regions; and involved in highly specific RNA processing in neurogenesis in Drosphila.

9. The function of lncRNAs in neuronal differentiation

LncRNAs are cardinal for neuronal differentiation and for neurogenesis. They are specific to the brain region, especially SVZ, DG or Olfactory Bulb (OB). They exert their functions via interacting with transcription factors or binding to the promoter or enhancer regions of neighbouring/target genes, also act as competing endogenous RNA (ceRNA) against synergistic binding sequences of miRNA or are crucial signaling pathway modulators which regulate chromatin modification, transcription, and post-transcription.

LncRNA name	Mechanism	Biological function	References
Sox2ot	CpG island of Sox2, interacts with transcription factor YY1 and suppresses the expression of Sox2	Prohibit NSCs proliferation and advance neuronal differentiation	[55]
RMST	Target Sox2 promoter region	Promote neurogenesis	[56]
Kdm2b (also known as *Kancr*)	Bind with hnRNPAB and activate Kdm2b gene expression	Causes early neuronal differentiation of cortical projection neurons, hence promotes neurogenesis	[57]
Paupar	Bind with local epi-regulatorygenes-Pax6 and KAP1 through H3K9me3 deposition	Promote neurogenesis in neuroblastoma cells	[58, 59]
Gm21284	Interact with miR-30e-3p, miR-431 and miR-147	Inhibit NSCs proliferation while promote NSCs differentiation	[60]
1604	miR-200c/ZEB1/2 axis	Promote neural differentiation	[61]
Rik-201	Activated by C/EBPβ, miR-96/Sox6	Enhance neural differentiation	[62]
Rik-203	miR-467a-3p/Sox6, miR-101-3a/GSK-3β	Enhance neural differentiation	[62, 63]
MEG3	Act as a negative regulator of miR-128-3p while induced by the cAMP/ response element-binding protein (CREB) pathway	Promotes neuron differentiation	[64]
Malat1	Activate ERK/MAPK, inhibit PPAR/p53	Promote neural differentiation in neuroblastoma-derived Neuro-2a (N2a) cell	[65]
Pnky	Interact with RNA-binding protein (RBP)-PTBP1	Inhibit neural differentiation and neurogenesis	[30, 66]
lncR492	Interact with HuR and activate Wnt signalling	Inhibit neural differentiation of mouse embryonic stem cells	[67]
BDNF-AS	Targeting activating potassium uptake system protein (TrkB) signaling pathway	Inhibit eNSCs-derived neurite outgrowth and neural apoptosis	[68]
UCA1	miR-1 and its target Hes1	Promote NSCs differentiation to astrocyte not to neuron	[54]
lnOPC	lnOPC binds to upstream sequences of OLIG2	Promotes OPCs differentiation and oligodendrogenesis	[69]
lncOL1	Form a complex with Suz12, an oligodendrocyte maturation promoter	Promote oligodendrogenesis by promoting early maturation/differentiation of oligodendrocytes in neural development	[70]

LncRNA name	Mechanism	Biological function	References
lnc158	Promote regulatory transcription factor-nuclear factor-IB NFIB expressio	Promote oligodendrogenesis through enhanced oligodendrocyte-related genes expressions like and enhanced induction of oligodendrocyte lineage differentiation	[71]
Pcdh17it (immature lncRNa)		Oligodendrogenesis marker identified in new-born immature OLs	[72]
OLMALIN/-AS	OLMALINCAS, maps to the first exon of the dominant isoform of OLMALINC	Regulate oligodendrocyte maturation related genes	[73]
Synage (includes three isoforms of Gm2694)	Synage as a sponge for the sponge to microRNA miR-325-3p, act as scaffold for organizing the assembly of the LRP1-HSP90AA1-PSD-95 complex	Regulating synaptic stability in cerebella as distributed in the cytoplasm and synapses of cerebellar cells	[74]
GM12371	Nuclear-enriched	Prolific transcriptional regulator critical for synapse function in hippocampal neurons	[75]
Gm2694 (alias AK082312)	Enriched expression in the mouse cerebellar cortex		[76]
Gm2694 lncRNA (alias linc1582)		Associated with neuroectoderm differentiation	[77]
Gm2694 (alias Trincr1) was documented to	Regulate FGF/ERK signaling	Self-renewal of NSCs	[78]

Table 4.
The roles of lncRNAs on NSCs differentiation/neurogenesis, oligodendrogenesis and synapse stability.

10. Effect on lncRNA target genes expression

LncRNAs regulate neural development by binding to the proximal regions of the protein-coding genes expressions. Most importantly, evolutionarily conserved, nuclearly localized lncRNA Sox1 overlapping transcript (Sox1ot) and Sox2 overlapping transcript (Sox2ot) were identified in the developing brain. Overlapping transcripts Sox1ot and Sox2ot imbricate with Sox1 and Sox2 protein coding transcripts, respectively [79, 80]. Protein coding Sox1 and Sox2 transcripts function as pluripotent transcription factors to maintain the stemness of NPCs and NSCs [81]. Sox1ot and Sox2ot are highly expressed during neural development and are directly proportional to the Sox1 and Sox2 transcription factors abundance respectively [55, 82]. Mechanistically,

overlapping transcript Sox2ot interacts with the transcriptional regulator YY1 and binds to CpG island which is present in the proximity of Sox2 locus and subsequently suppresses the Sox2 expression to restrain stemness of NSCs and NPCs [55]. LncRNA rhabdomyosarcoma 2-associated transcript (RMST) binds with promoter regions of Sox2 and regulates the downstream target genes and is critical for neurogenesis [56]. Another, lncRNA Kdm2b is uniquely transcribed from the bidirectional promoter along with Kdm2b. Epigenetically, lncRNA Kdm2b binds in cis manner with hnRNPAB and manages Kdm2b's transcription. Although lncRNA Kdm2b expression is transient and occurs only during early neuronal differentiation of cortical projection neurons [57]. LncRNA *Paupar*, regulates the function of transcriptional/epigenetic regulatory factors and modulates neuroblastoma cell growth. Most importantly, it directly binds to KAP1, which induces H3K9me3 methylation and modulates the expression of downstream target genes important for neuronal proliferation and differentiation. LncRNA *Paupar* epigenetically modulates (by binding H3K9me3 in cis manner) and controls Pax6 mediated neural differentiation and olfactory bulb neurogenesis [58, 59].

11. lncRNA as competing endogenous RNA (ceRNA) against miRNA

LncRNAs participate in neural development via acting as ceRNA of miRNA and indirectly regulate transcript expression in the cytoplasm [61, 83].

LncRNA Gm21284 promotes differentiation of NSCs to hippocampal cholinergic neurons by binding to miR-30e-3p, miR-431 and miR-147 and on silencing miRNAs impedes NSCs proliferation and enhances NSCs differentiation [60]. LncRNA1604 act as sponge to miR-200c and regulates important transcription factor zinc finger E-box binding homeobox1/2 (ZEB1/2) axis which promotes neural differentiation and on silencing miR-200c repress neural differentiation [61]. LncRNA isoforms also play an important role in neurogenesis. For instance, lncRNA RiK has two variants, Rik-201 and Rik-203. Both these variants get activated by CCAAT/enhancer-binding protein β (C/EBPβ) which are induced during neurogenesis. Mechanistically, Rik-201 triggers C/EBPβ, miR-96/Sox6 axis and [62] and lncRNA Rik-203 induces miR-467a-3p/Sox6, miR-101-3a/Glycogen Synthase Kinase-3β (GSK-3β) [62, 63] to promote neural differentiation. LncRNA MEG3 is also engaged in the process of neuron differentiation. Although it acts as a negative regulator of miR-128-3p it induces the cAMP/response element-binding protein (CREB) pathway [64].

12. LncRNAs as key signalling pathway modulators

LncRNAs moreover can contribute to neural differentiation through NSCs and may function as a key member of the signaling pathway. Neurite outgrowth is an essential act in the early neuronal-differentiation and self-renewal. The lncRNA Metastasis-associated lung adenocarcinoma transcript1 (Malat1) is a requisite for neurite growth in in vitro differentiation of neuroblastoma-derived Neuro-2a (N2a) cell as a model. Knockdown of Malat1 impeded neurite outgrowth and enhanced cell death in N2a cells. This owes to suppression of Mitogen-Activated Protein Kinase (MAPK) and activation of Peroxisome proliferator-activated receptor (PPAR) and p53 signalling pathways [65].

13. LncRNAs mediated repression of neuronal differentiation

As described above, most of the highly expressed lncRNAs promote neuronal differentiation, however, some other neuronal lncRNAs were revealed which blocks neuronal differentiation and plays an important role in brain development.

Nuclear localized lncRNA Pnky, was determined to be involved in neuronal development via inhibiting neuronal differentiation. Specifically, Pnky is expressed selectively in neural tissues that are enriched in SVZ-NSCs which are suppressed into mature neurons. Pnky binds to the pre-mRNA splicing regulator RNA-binding protein (RBP) polypyrimidine tract-binding protein (PTBP1). On silencing either Pnky or PTBP1 alters splicing signature of expressed mRNAs in the cell and subsequently induces neurogenesis in SVZ-NSCs. Hence, inverse correlation has been observed between expression of Pnky-PTBP1 complex and neurogenesis [30, 66]. Another lncRNA lncR492 acts as inhibitor of neuroectodermal differentiation via interacting with mRNA binding protein HuR and activating the Wnt signaling pathway [67]. Zhang et al. reported dose-dependent over expression of lncRNA brain derived neurotrophic factor antisense (BDNF-AS), inhibits neural growth in ketamine-treated mouse embryonic NSC-derived neurons. SiRNA mediated silencing of BDNF transcript expression improved neural apoptosis; inhibited neurite growth in NSC-derived neurons through stimulating potassium uptake system protein (TrkB) signaling pathway [68].

14. Role of lncRNAs in regulation of gliogenesis

Recent reports suggest that radial glial (RG) cells are considered for glial lineage along with a subpopulation of astrocytes. RG as matter of fact act as the NSCs that serve as progenitors for many differentiated neurons and glial cells during development and in the postnatal brain give rise to adult SVZ-NSCs that continue to produce neurons throughout adult life [84]. Importantly, at the inception of cortical development, NSCs or NPCs consecutively give rise to deep layer neurons trailed by surficial layer neurons; at later phase of cortical development, NSCs annihilate neurogenesis and move towards gliogenesis to attain gliogenic capability [85, 86]. Temporal NSCs transition from neurogenesis to gliogenesis is a prerequisite for proper cortical development [86, 87].

Several lncRNAs are considered as key regulators during neuronal-glial fate specification and oligodendrocyte lineage maturation. Time dependent overexpression of human urothelial carcinoma associated 1 (UCA1) was able to decide the direction of NSCs differentiation. Knockdown of UCA1 suggested suppression of NSCs proliferation and differentiation with decreased expression of nestin and the enhanced formation of the neurosphere. Further the silencing of UCA1 repressed NSCs differentiation into astrocytes rather NSCs were directed to differentiate as neurons due to the overexpression of miR-1 expression and decreased expression of its target gene-Hes1. Hence, UCA1 regulated the NSCs proliferation and differentiation through regulating Hes1 expression [54].

Dong et al. screened 5000 lncRNAs and identified lncRNAs that are modulated during oligodendrocyte precursor cell (OPC) differentiation from NSCs and play an essential role in oligodendrogenesis. Lnc-OPC was overexpressed in OPCs and is found to be highly conserved among placental mammals and predicts its role in brain development. Mechanistically, lnc-OPC binds to upstream regulatory elements of

OLIG2 and is directly proportional to the OLIG2 expression. Hence, overexpression of lnc-OPC enhances OPCs differentiation and oligodendrogenesis [69].

LncRNA has also emerged as an important regulator in oligodendrocyte mediated myelination and plays a crucial role in development and function of CNS [88, 89]. This was supported by dynamic co-expression signature of lncRNAs with protein coding genes at different stages of oligodendrocyte growth and myelination. Most importantly, highly conserved chromatin-associated lncRNA-lncOL1 has been identified during oligodendrocyte growth and myelination. Genetic knockdown of lncOL1 causes aberrations in myelination and remyelination processes after injury, while gain of function induces early oligodendrocyte differentiation i.e., maturation in neural development. Mechanically, lncOL1 forms a complex with a by binding to the promoter region of a member of polycomb repressive complex 2 (Suz12), involved in oligodendrocyte maturation [44]. Another lnc158 upregulates in NSCs and stimulates downstream various oligodendrocyte-related genes expressions including DNA binding transcription factor-nuclear factor-IB (NFIB) that regulates oligodendrocyte lineage differentiation [71]. Additionally, immature OL-specific lncRNA-Pcdh17 is a specific marker for newly born immature OLs and has been identified both in developing and adult forebrain of mice [72]. Interestingly, lncRNA oligodendrocyte maturation-associated long intervening non-coding RNA (OLMALINC) and its antisense counterpart, OLMALINCAS, both are equivalently and abundantly expressed in the white matter of human frontal cortex as opposed to grey matter and peripheral tissues and basically take part in modulation of human oligodendrocyte maturation related genes. OLMALINCAS, maps to the first exon of the major isoform of OLMALINC [73].

15. The role of lncRNAs in synaptogenesis

Synaptic stability in the developing and adult nervous system results due to the late phase long-term potentiation i.e., a continuous strengthening of synapses for long lasting increase in signal transmission between two neurons. The role of lncRNAs in modulating synaptic stability is ambiguous. Wang et al. [74], reported that cerebellum of the brain shows increased expression of lncRNA, Synage, to regulate synaptic stability. The lncRNA mediated synaptic stability is either lncRNA acting as a sponge or as a scaffold. As a sponge lncRNA Synage binds to miR-325-3p and alters the expression of downstream cerebellar synapse organizer identified in mouse, rhesus macaque, and human. Additionally, lncRNA Synage serves as a scaffold for rearranging the positioning of the LRP1-HSP90AA1-PSD-95 complex in Parallel fibre (PF)-Purkinje cell (PC) synapses. Knockdown of synage collapses cerebellar phenotype and leads to cerebellar degeneration, death of neurons, decline in synapse density which relates to synaptic pruning, decreased synaptic growth and synaptic plasticity during cerebellar development. Hence, the lncRNA Synage plays a major role in regulating synaptic stability and plays a crucial role during cerebellar development. GM12371 (nuclear enriched [75]) and Gm2694 (cerebellar cortex enriched [76]) (alias AK082312) also acts as a transcriptional regulator of synapse function.

16. Conclusion

Neural development related to NSCs/NPCs which constructs embryonic tissue architecture of CNS as well as exist as remnant in subventricular zone (SVZ) nearby

the lateral wall of the lateral ventricles and the subgranular zone (SGZ) of hippocampus dentate gyrus (DG) of the brain is considered as a complex phenomenon. Advanced large-scale genome-wide RNA sequencing has been performed in various neuronal cells over the course of neural development to understand NSC self-renewal, neurogenesis, gliogenesis and synaptogenesis. This review has described in detail the functional roles of lncRNAs as ceRNA, by regulating proximal protein-coding genes expressions, and epigenetic modulations in regulation of NSCs/NPCs self-renewal, proliferation and differentiation into neuron or glial cells and synaptogenesis. This suggests that lncRNAs might be employed as potential selection biomarkers for identifying or screening suitable NPCs/NPCs. Importantly, a spatiotemporal expression of lncRNA as atlas of embryonic neurogenesis in Drosophila revealed a high- confidence set of 13 lncRNAs will open a new era of lncRNA based NSCs mediated neurogenesis and may help us to better understand the neuronal physiology. However, most of their function remains to be explored, more novel lncRNAs and their molecular mechanisms remain to be found and probed in-depth yet.

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Abbreviations

Aldh1l1	aldehyde dehydrogenase 1 family member L1
AR	androgen receptor
BDNF	brain-derived neurotrophic factor
C/EBPβ	CCAAT/enhancer-binding protein β
ceRNA	ZEB1/2zinc finger E-box binding homeobox1/2
CNS	central nervous system
CO	cerebral cortex organoid
CP	cortical plate
CR	Cajal-Retzius
CREB	cAMP/response element-binding protein
CTIP2	COUP-TF-interacting protein 2
DCX	doublecortin
DG	dentate gyrus
Dlx2	Distal-Less Homeobox 2
EGL	external granule layer
ERK	extracellular signal regulated kinase
ESCs	embryonic stem cells
GSK-3β	glycogen synthase kinase-3β
IGL	internal granule layer
IPCs	intermediate progenitors
IZ	intermediate zone
Kdm2b	lysine demethylase 2B
LncRNAs	long non-coding RNAs
LV	lateral ventricle
GFAP	glial fibrillar protein

Malat1	metastasis-associated lung adenocarcinoma transcript1
MAPK	mitogen-activated protein kinase
MEG3	maternally expressed gene 3
MSNP1AS	Moesin pseudogene 1 antisense
N2a	Neuro-2a
NE	neuroepithelium
NEAT1	nuclear paraspeckle assembly transcript 11
NeuN	neuronal nuclei
NFIB	nuclear factor-IB
NG2	polydendrocytes
Notch	Notch receptor
NPCs	neural precursor/progenitor cells
NSCs	neural stem cells
OB	olfactory bulb
OCT3/4	POU class 5 homeobox 1
Olig2	oligodendrocyte transcription factor
OPC	oligodendrocyte precursor cells
OLMALINC	oligodendrocyte maturation-associated long intervening non-coding RNA
Paupar	PAX6 Upstream Antisense RNA
PAX6	Paired Box 6
PC	Purkinje cell
PDGF	platelet-derived growth factor
Pnky	long intergenic non-protein coding RNA PNKY
PF	parallel fiber
PPAR	peroxisome proliferator-activated receptor
Prox1	Prospero Homeobox 1
PSC	pluripotent stem cell
PTBP1	RNA-binding protein (RBP)-polypyrimidine tract-binding protein
RGLs	radial glia-like cells
RBP	RNA-binding protein
RG	radial glial
RMS	rostral migratory stream
RMST	rhabdomyosarcoma 2-associated transcript
RPS10P2-AS1	ribosomal protein S10 pseudogene 2 anti-sense 1
S100β	S100 calcium-binding protein B
SGZ	sub-granular zone
SHH	sonic hedgehog
Sox10	SRY-related HMG-box 10
Sox1ot	Sox1 overlapping transcript
Sox2ot	Sox2 overlapping transcript
Suz12	polycomb repressive complex 2
SVZ	subventricular zone
TALNEC2	tumor associated lncRNA expressed in chromosome 2
TBR1	T-box brain transcription factor 1
TBR2	T-box brain transcription factor 2
TGF-β	transforming growth factor-β
TREX	transiently expression of lncRNAs
Trincr1	TRIM71 interacting long noncoding RNA 1
TUNA	Tcl1 upstream neuron-associated lincRNA

UCA1	urothelial carcinoma associated 1
Wdr5	WD repeat domain 5
ZEB1/2	Zincfinger E-box binding homeobox ½

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Chapter 8

Interactions of lncRNAs and miRNAs in Digestive System Tumors

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Abstract

Noncoding RNA (ncRNA) includes short (miRNA) and long (lncRNA) that have important regulatory role in different biological processes. One of the important issue in which ncRNA involved is tumor induction and suppression. miRNA and lncRNA were vital players in many tumors including digestive system tumors. This study includes studying the role of 140 hsa-miR including miR-1 to miR-140 and their sponger lncRNA in esophageal and stomach cancers by 249 studies. The review revealed that each miR may play as oncogene only or tumor suppressor via upregulation and downregulation regulatory proteins in cell cycles and activation of physiological cascades. Some of miR have dual role in same type of tumor as oncogene and suppressive miR. Same thing is for lncRNA tacting as oncogenic via sponging some of miR when overexpressed to upregulate oncogenic protein or acting as suppression lncRNA when overexpressed to downregulate some oncogenic proteins activated by miR. The current review concludes the vital role of ncRNA (both miRNA and lncRNA) in some digestive system tumors as oncogene-promoting cancer viability, invasiveness, proliferation, and metastasis or as tumor suppressor inhibiting tumorigenicity or inducing apoptosis.

Keywords: miRNA, lncRNA, sponge, oncogene, tumor suppressor, esophageal tumors, gastric cancers

1. Introduction

Ribonucleic acids (RNAs) are important nucleic acids for cell life and are classified as coding and non-coding RNA (ncRNAs). MicroRNAs (miRNAs) are a class of short noncoding RNAs (ncRNAs) (22 nts) that play important roles in posttranscriptional gene regulation. The majority of miRNAs are transcribed from DNA sequences into primary miRNAs and processed into precursor miRNAs, and finally mature miRNAs [1]. Long noncoding RNAs (lncRNAs) (more than 200 nts) may regulate cell proliferation, apoptosis, migration, invasion, and maintenance of stemness during cancer development [2, 3]. miRNA has a role in many tumors and upregulation/downregulation status of them may influence tumorigenesis, proliferation,

metastasis, and chemoresistance [4, 5]. miRNAs implicated as oncogene of repressors for sets of cancers of liver: focal nodular hyperplasia (FNH) [6], hepatocellular adenoma (HCA) [7], hepatocellular carcinoma (HCC) [8], and cholangiocarcinomas (CCA) [9]. miRNAs were also implicated in pancreatic cancers: pancreatic endocrine tumors (PET) [10], pancreatic ductal adenocarcinoma (PDAC), and pancreatic acinar cell carcinoma (PACC) [11]. Esophageal squamous-cell carcinoma (ESCC) [12], and esophageal adenocarcinoma (EAC) [13], stomach adenocarcinoma (STAD) [14], colon adenocarcinoma (COAD) [15] were also influenced by miRNAs. The current review focused on 140 types of miR (miR-1 to miR-140) as oncogenic or tumor suppressor miR in different digestive system tumors.

1.1 Esophageal cancers

Esophageal cancer is a disease in which malignant (cancer) cells form in the tissues of the esophagus. It is characterized by its high mortality rate and poor prognosis. This disease is the sixth cause of cancer-related deaths and the eighth most common cancer worldwide with a 5-year survival rate of less than 25% [15]. Esophageal cancer occurs as either squamous cell carcinomas (ESCC) or adenocarcinoma (EAC). ncRNA including miRNA and lncRNA has a vital role in esophageal cancers either as oncogenic or as tumor suppressor [16, 17].

1.1.1 lncRNA/miR interaction in esophageal squamous cell carcinoma (ESCC)

Esophageal squamous cell carcinoma (ESCC) is a cancer that forms in the thin, flat cells lining the inside of the esophagus. It accounts for about 90% of esophageal cancers. miRNA can play a vital role in cancer regulation and may be an oncogene or tumor suppressor gene when overexpressed. In esophageal squamous cell carcinoma (ESCC), miR-1, −22, −26, −27, −29, −30, −33, −34, −98, −99, −100, −101, −107, −122, −124, −125, −127, −128, −129, −133, −134, −136, −137, −138, −139, and − 140 were downregulated in ESCC tissues compared with normal one acting as tumor suppressor and targeting different genes [18–43]. In contrast, the expression of miR-7, −9, −10, −16, −17, −18, −19, −20, −21, −23, −24, −25, −28, −31, −32, −92, −93, −96, −103, −105, −106, −126, −130-, and miR-135 was upregulated in ESCC acting as oncogene [44–66], as shown in **Table 1**.

Actually, lncRNA may have oncogenic activity acting as molecular sponge to inhibit activity of tumor suppressor miR. lncRNA opposite in action with tumor suppressor miR will act as oncogenic lncRNA, as shown in **Table 2**. HOX transcript antisense RNA (HOTAIR) expression increased in ESCC and inhibit the activity of miR-1. HOTAIR can be used as diagnostic marker of ESCC. miR-1 exert its suppressive action on ESCC via gene suppression of hepatocyte-growth factor (CCND1, CDK4, and MET). HOTAIR-mediated sponging of miR-1 leads to upregulation of those growth factors leading to ESCC proliferation and propagation [67]. Zinc finger E-box binding homeobox 1 (ZEB1) is transcriptional factor responsible for metastasis in ESCC, which is dysregulated by miR-33 to improve the outcome of ESCC. Differentiation antagonizing nonprotein coding RNA (DANCR) is lncRNA, which downregulates miR-33 and upregulates ZEB1 and so participates in worseness of ESCC [68]. Another lncRNA called MIR31HG acts on miR-34 leading to upregulation of c-Met promoting ESCC [69]. SNHG16 is also lncRNA of miR-98 that directly binds to enhancer of zeste homolog 2 (EZH2) and promotes ESCC propagation [70]. ANRIL is lncRNA of miR-99, which also promotes ESCC proliferation *via* downregulation of

miRNA	Expression in ESCC	Role in ESCC	Target gene	Ref.
miR-1	Downregulated	Tumor-suppressor	NOTCH2, LASP1	[18]
miR-22	Downregulated	Tumor-suppressor	PTEN, C-MYC	[19]
miR-26	Downregulated	Tumor-suppressor	MYCBP	[20]
miR-27	Downregulated	Tumor-suppressor	FBXW7	[21]
miR-29	Downregulated	Tumor-suppressor	FBXO31	[22]
miR-30	Downregulated	Tumor-suppressor	ITGA5, PDGFRB	[23]
miR-33	Downregulated	Tumor-suppressor	ZEB1	[24]
miR-34	Downregulated	Tumor-suppressor	c-MET, FOXM1	[25]
miR-98	Downregulated	Tumor-suppressor	EZH2	[26]
miR-99	Downregulated	Tumor-suppressor	CCND1, CCNA2	[27]
miR-100	Downregulated	Tumor-suppressor	CXCR7	[28]
miR-101	Downregulated	Tumor-suppressor	COX-2	[29]
miR-107	Downregulated	Tumor-suppressor	CDC42	[30]
miR-122	Downregulated	Tumor-suppressor	KIF22	[31]
miR-124	Downregulated	Tumor-suppressor	BCAT1	[32]
miR-125	Downregulated	Tumor-suppressor	p38-MAPK	[33]
miR-127	Downregulated	Tumor-suppressor	FMNL3	[34]
miR-128	Downregulated	Tumor-suppressor	ZEB1	[35]
miR-129	Downregulated	Tumor-suppressor	CTBP2	[36]
miR-133	Downregulated	Tumor-suppressor	COL1A1	[37]
miR-134	Downregulated	Tumor-suppressor	FOXM1	[38]
miR-136	Downregulated	Tumor-suppressor	MUC1	[39]
miR-137	Downregulated	Tumor-suppressor	EZH2, PXN	[40]
miR-138	Downregulated	Tumor-suppressor	NF-κB	[41]
miR-139	Downregulated	Tumor-suppressor	NR5A2	[42]
miR-140	Downregulated	Tumor-suppressor	ZEB1	[43]
miR-7	Downregulated	Tumor-suppressor	HOXB13	[44]
miR-9	Overexpressed	Oncogene	E-cadherin, FOXO1	[45]
miR-10	Overexpressed	Oncogene	FOXO3, KLF4	[46]
miR-16	Overexpressed	Oncogene	RECK, SOX6	[47]
miR-17	Overexpressed	Oncogene	RBL2	[48]
miR-18	Overexpressed	Oncogene	KRAS	[49]
miR-19	Overexpressed	Oncogene	CDC42, RAC1	[50]
miR-20	Overexpressed	Oncogene	RB1, TP53INP1	[51]
miR-21	Overexpressed	Oncogene	TPM1, PTEN	[52]
miR-23	Overexpressed	Oncogene	EBF3	[53]
miR-24	Overexpressed	Oncogene	FBXW7	[54]
miR-25	Overexpressed	Oncogene	PTEN, ZNF512B	[55]
miR-28	Overexpressed	Oncogene	ARF6	[56]
miR-31	Overexpressed	Oncogene	LATS2	[57]
miR-32	Overexpressed	Oncogene	CXXC5	[58]

miRNA	Expression in ESCC	Role in ESCC	Target gene	Ref.
miR-92	Overexpressed	Oncogene	PTEN	[59]
miR-93	Overexpressed	Oncogene	TGFβR2	[60]
miR-96	Overexpressed	Oncogene		[61]
miR-103	Overexpressed	Oncogene	CDH11, NR3C1	[62]
miR-105	Overexpressed	Oncogene	SPARCL1, FAK	[63]
miR-106	Overexpressed	Oncogene	PTEN	[64]
miR-126	Overexpressed	Oncogene	STAT3	[65]
miR-135	Overexpressed	Oncogene	RERG	[66]

Table 1.
Oncogenic/tumor-suppressor miR among ESCC.

lncRNA	Expression of lncRNA in ESCC	Target miR	Target gene	Ref.
HOTAIR	Overexpressed	miR-1	CCND1, CDK4, and MET	[67]
DANCR	Overexpressed	miR-33	ZEB1	[68]
MIR31HG	Overexpressed	miR-34	c-Met	[69]
SNHG16	Overexpressed	miR-98	EZH2	[70]
ANRIL	Overexpressed	miR-99	—	[71]
MALAT1	Overexpressed	miR-101	—	[72]
FAM201A	Overexpressed	miR-101	mTOR	[73]
LINC00152	Overexpressed	miR-107	Rab10	[74]
LINC01296	Overexpressed	miR-122	mTOR	[75]
ZFAS1	Overexpressed	miR-124	STAT3	[76]
HOTAIR	Overexpressed	miR-125	HK2	[77]
NEAT1	Overexpressed	miR-129	CTBP2	[36]
H19	Overexpressed	miR-138	EZH2	[78]
BCAR4	Overexpressed	miR-139	ELAVL1	[79]
SNHG16	Overexpressed	miR-140	ZEB1	[43]
LincIN	Overexpressed	miR-7	HOXB13	[80]
MEG3	Downregulated	miR-9	E-cadherin, FOXO1	[81]
FAM83H-AS1	Overexpressed	miR-10	—	[82]
PART1	Downregulated	miR-18	SOX6	[83]
GAS5	Downregulated	miR-21	RECK	[84]
AC012073.1	Downregulated	miR-93	—	[85]

Table 2.
Oncogenic/tumor suppressor lncRNA among ESCC.

miR-99 [71]. MALAT1 lncRNA may decrease the expression of miR-101 and promote ESCC proliferation and metastasis via upregulation of [72]. Additionally, FAM201A lncRNA acts as a sponge for miR-101 and upregulates mTOR [73]. miR-107 targeting Ras-related protein Rab-10 (Rab10) and decreasing their expression and preventing ESCC propagation. LINC00152 is sponge lncRNA of miR-107, which promotes ESCC *via* overexpression of Rab10 upon miR-107 sponging [74]. Additionally LINC01296 sponge miR-122 and increasing mTOR expression [75]. ZFAS1 lncRNA was found to promote the proliferation, migration, and invasion of ESCC by upregulating STAT3 and downregulating miR-124 [76]. HOTAIR could increase the expression of hexokinase 2 (HK2) in ESCC through sponging of miR-125 [77]. Nuclear paraspeckle assembly transcript 1 (NEAT1) is lncRNA that sponge miR-129 and upregulates their target, CTBP2, promoting ESCC viability and invasion [36]. H19 lncRNA inhibits the effect of miR-138 and promotes EZH2 thereby promoting ESCC [78]. Breast cancer antiestrogen resistance 4 (BCAR4) lncRNA act to sponged miR-139-3p, leading to upregulation of ELAVL1 and promoting tumorigenesis of ESCC [79]. SNHG16 lncRNA squeegee miR-140 and upregulates their target, ZEB1, thereby promoting ESCC [43]. lncRNA LincIN has been reported to be overexpressed and to be involved in the metastasis of breast cancer. LincIN has the potential role in ESCC invasion and metastasis *via* upregulation of HOXB13 and downregulation of miR-7 + enhancement of binding between NF90 on primary miR-7. It seem that no sponge lncRNA were documented for miR-22, −26, −27, −29, −30, −100, −127, −128, −133, −134, −136, and miR-137 in ESCC yet.

lncRNA can act as tumor suppressor counteracting the effect of oncogenic miR. Maternally expressed gene 3 (MEG3) is tumor suppressor lncRNA of miR-9. MEG3 decreased expression was seen in ESCC. Expression level of MEG3 was significantly increased in cancer cells after being treated with the DNA methyltransferase inhibitor 5-Aza-dC, leading to decreased miR-9 and increased E-cadherin and FOXO1 expression [81]. miR-10 can be sponged by lncRNA FAM83H-AS1 in ESCC [82]. Prostate androgen-regulated transcript 1 (PART1) lncRNA was downregulated in ESCC and, when overexpressed, will sponge oncogenic miR-18 leading to upregulation of SOX6 thereby inhibiting ESCC proliferation and metastasis [83]. Growth arrest-specific 5 (GAS5) lncRNA is seen to be elevated in radiation-sensitive ESCC tissues, leading to dysregulation of miR-21 and increasing the level of its target, RECK [84]. lncRNA called AC012073.1 is seen to bind competitively to miR-93 in ESCC [85] as shown in **Table 2**.

1.1.2 lncRNA/miR interaction in esophageal adenocarcinoma (EAC)

Esophageal adenocarcinoma (EAC) is a malignancy classically seen in the distal esophagus. The incidence of EAC is seven times more common in men than in women [86]. lncRNA and miRNA can either be oncogenic promoting tumor proliferation, invasion, and metastasis or acting as a tumor suppressor via targeting specific protein and signaling pathways. It seems few studies were conducted on miR role in tumorigenesis or anti-tumor in EAC, but many studies highlighted the pivotal role of lncRNA. lncRNA called miR205HG was found to have tumor suppressory effect on EAC when overexpressed *via* downregulation of the Hedgehog (Hh) signaling pathway [87]. lncRNA HOTAIR expression was upregulated in EAC tumor acting as oncogene via regulating NTRK2, NP1, CHRDL1, NTRK2, HOXC8, and IL11. Inversely lncRNA CYP1B1-AS1 was downregulated in EAC acting as tumor supressor [88]. Two lncRNA called AFAP1-AS1 and HNF1A-AS1 were shown to be

lncRNA	Expression of lncRNA in EAC	Target miR	Target gene	Ref.
miR205HG	Downregulated	NA	Hedgehog	[87]
HOTAIR	Overexpressed	NA	NTRK2, NP1, CHRDL1, NTRK2, HOXC8 and IL11	[88]
CYP1B1-AS1	Downregulated	NA	NA	[88]
AFAP1-AS1	Overexpressed	NA	NA	[89]
HNF1A-AS1	Overexpressed	NA	H19	[90]
PVT1	Overexpressed	NA	LATS1, YAP1	[91]
MIR22HG	Overexpressed	NA	STAT3	[92]
BDNF-AS	Downregulated	miR-214	JAG1	[92]
ADAMTS9- AS2	Downregulated	NA	CDH3	[93]
LINC00662	Overexpressed	NA	Wnt/β-catenin	[94]

Table 3.
Oncogenic/tumor suppressor lncRNA among EAC.

overexpressed in EAC acting as oncogene [89, 90]. PVT1 lncRNA was also documented as oncogene via upregulation of YAP1 in EAC tissues [91]. Also, MIR22HG lncRNA has same effect on PVT1 (oncogenic) via activation of STAT3/c-Myc/p-FAK pathway. Tumor suppressor lncRNA like BDNF-AS and ADAMTS9- AS2 can exert their suppressive activity via sponging miR-214 and CDH3, respectively [92, 93]. Another oncogenic lncRNA, LINC00662, utilizes its effect *via* activating Wnt/β-catenin signaling (**Table 3**) [94].

1.2 Stomach cancers

Gastric or stomach cancers were also regulated by miR/lncRNA interactions. miR-1 directly targets the MET gene and downregulates its expression acting as tumor suppressor [95]. MET is an oncogene and its activation results from the binding of hepatocyte growth factor (HGF), leading to tumor growth, metastasis, migration, and drug resistance [96]. Targeting MET by miR-1 will deactivate it, and MET can restore its activity by oncogenic lncRNA called LINC00242 when sponging the miR-1 [97]. miR-7 also acting as a tumor suppressor in gastric cancer may be *via* degradation of EGFR mRNA, and thus, the oncogenic lncRNA UCA1 can sponge miR-7, leading to enhancing the expression of EGFR and promoting cell metastasis and migration [98]. miR-9 is implicated as oncogenic *via* downregulation of CDX2 and promotes gastric cancer cell proliferation [99] and may act as tumor suppressor that can be sponged by HULC lncRNA via upregulation of MYH9 [100]. miR-17, −18, −19, and − 20 were documented as oncogenic ncRNA among gastric cancer patients. Oncogenic miR-17 targeting PTEN and EGR2 to enhance proliferation and metastasis of stomach cancer, and this effect can be sponged by lncRNA HOTAIRM1 or LINC01939, which suppressed proliferation and migration of GC cell acting as tumor suppressor lncRNA [101, 102]. Inversely, lncRNA NEAT1 was overexpressed in gastric cancers and positively related with miR-17 *via* activation of GSK3β [103]. By targeting IRF2, miR-18 enhances proliferation and metastasis of gastric cancer [104]. Sponging of miR-19 by lncRNA CASC2 can increase the sensitivity to cisplatin and so it acts as tumor suppressor [105, 106].

<>Oncogenic miR-21 can promote gastric cancer cell viability and progression via downregulation of some tumor suppressor genes like PTEN, RECK, and PDCD4 [107]. Inhibiting miR-21 expression by lncRNA MEG3 can inhibit gastric cancer growth and metastasis [108]. miR-22 acts to suppress gastric cancer via upregulating MMP14, NET1, and Snail while lncRNA CTC-497E21.4 can squeegee miR-22 and promote proliferation and invasion [109]. miR-24 and miR-25 were upregulated in gastric cancer tissues and may promote the occurrence, development, infiltration, and metastasis of gastric cancer. As oncogenic miR-24 inhibits the CDKN1B and CHEK1 and miR-25 targeting FOXO3 [110, 111]. Tumor suppressor lncRNA GATA6-AS acts to inhibit gastric cancer progression by sponging miR-25 [112]. Targeting PTEN, miR-26 acts as oncogenic and upregulated in gastric cancer tissues promoting proliferation and invasion. Additionally, miR-26 can target EZH2 and can be suppressed by tumor suppressor lncRNA TET1–3 [113, 114]. miR-27 is additional oncogenic miR acting on HOXA10 [115]. By inhibiting the phosphorylation of AKT protein in gastric cancer cells, miR-28 acts as inhibiting progression and metastasis and can be sponged by LOC400043 [116, 117]. Low expression was seen in gastric cancer tissues for miR-29 highlighting their role as tumor suppressor targeting CCND2 and MMP2, and can be sponged by lncRNA MEG3 [118, 119]. As tumor suppressor, miR-30 is responsible for inhibiting gastric cancer and increasing sensitivity to anticancer drugs when upregulated [120], and lncRNA PVT1, HNF1A-AS1, and DLEU2 can sponge miR-30 and upregulating Snail protein, PI3K/AKT signaling pathway and ETS2, respectively, promoting gastric cancer cell proliferation and metastasis [121–123].

Conversely, it can be acting as oncogenic miR through P53/ROS-mediated regulation of the mitochondrial apoptotic pathway [124]. miR-31 acts as a vital tumor suppressor ncRNA by inhibiting E2F2s and RhoA expression, also upregulation of miR-31 targeting ITGA5 may suppress tumor cell invasion and metastasis by indirectly regulating PI3K/AKT signaling pathway in human SGC7901 GC cells [125, 126]. lncRNA MIR31HG can encourage gastric cancer cell proliferation and invasion via sponging miR-31 [127]. Another oncogenic miR is miR-32, which augments tumorigenesis of gastric cell cancer by targeting KLF4 and KLF2, and at the same time can be sponged by SNHG5 lncRNA [128]. Gastric cancer suppresivity of miR-33 and miR-34 by targeting CDK6, CCND1, and PIM1 via miR-33 and Bcl-2, Notch, and HMGA2 via miR-34 [129, 130].

Overexpression of miR-43 was seen in gastric cancer tissues that promotes proliferation and metastasis by targeting VEZT [131]. miR-92 suppresses proliferation of gastric cancer and induces apoptosis by targeting EP4, Notch1 [132], and by targeting SOX4 while sponging of miR-92 by lncRNA PITPNA-AS1 and MT1JP can promote gastric cancer migration via upregulation of SOX4 and FBXW7 respectively [133, 134]. Oncogenic miR-93 can promote tumorigenesis by downregulation of IFNAR1 or PTEN [135, 136], and gastric cancer suppressor lncRNA PTENP1, GPC5-AS1, and CA3-AS1 can achieve gastric cancer inhibition by sponging miR93 and upregulating PTEN, GPC5, and PTG3 respectively [136–138]. Dual effect of miR-95 targeting EMP1 as oncogene or tumor suppressor by targeting Slug [139, 140]. Oncogenic miR-96 can target *ZDHHC5*, KIF26A, and FOXO3 promoting gastric cancer cell viability [141, 142]. Downregulation of BCAT1 Treg and CCND2 by miR-98 as a tumor suppressor for gastric cancer [143, 144] and TTTY15 lncRNA can sponge miR-98 and upregulate CCND2 [145]. Tumor suppressor miR-100 can inhibit cell proliferation and induce apoptosis in human gastric cancer via downregulating many proteins, such as BMPR2 [146], ZBTB7A [147], and CXCR7 [148], or acting as oncogene via their antiapoptotic role by inhibiting ubiquitination-mediated p53

degradation [149] and upregulation of HS3ST2 [150]. Contrariwise, oncogenic lncRNA such as HAGLROS [151] and MIR100HG [152] were sponging miR-100 and promote tumorigenesis via activation of the mTORC1 signaling pathway [151], PI3K/AKT/mTOR pathway [153, 154].

Tumor suppressor miR-101 targeting: ANXA2 [155], ZEB1 [156], SOCS2 [157], PI3K/AKT/mTOR [158], EZH2 [159], and AMPK [160] and sponged by LINC01303 and lncRNA XIST by upregulating EZH2 [159, 161], lncRNA SPRY4-IT1 by upregulating AMPK [160], lncRNA SNHG6 by upregulating ZEB1 [162] and lncRNA LINC00943 [163]. Dual oncogenic and tumor suppressive effects of miR-103 were elucidated in gastric cancer. It was found that overexpressed caveolin-1 and RAB10 were targets for suppressive miR-103 [164, 165] while miR-103 acting as oncogenic by downregulation of KLF4 [166]. lncRNA LINC00152 acting as oncogenic by sponging miR-103 and upregulation of RAB10 [165]. miR-105 inhibits gastric cancer cell metastasis, by targeting SOX9 and YY1 [167–169]. miR-106 play an oncogenic role in gastric cancer [170]. lncRNA GPC5-AS1 acting as tumor suppressor for gastric cancer via sponging miR-106 and upregulation of GPC5 [137]. Like miR-103, miR-107 has onogenic and tumor suppressive effects in gastric cancers, oncogenic by downregulation of PTEN [171], NF1 [172], and HIF-1α [173] suppressive by targeting BDNF [174]. lncRNA ZFR and PCAT18 counteracting oncogenic effect of miR-107 by upregulation of PTEN [171, 175]. Suppresivity of miR-122 may be linked to their target downregulation including DUSP4 [176], LYN [177], MYC [178], MMP-9 [179], GIT-1 [180], and VEGFD [181] while their effect can be sponged by lncRNA LINC01296, CRART16, and promoting metastasis by upregulation of MMP-9 and VEGFD respectively [179, 181]. Same thing for miR-124, they inhibit gastric cancer by downregulating many oncogenes like SPHK1 [182], ROCK1 [183], RAC1 and SP1 [184], EZH2 [185, 186], DNMT3B [187], and ITGB3 [188]. lncRNA MALAT1, LINC00511, LINC00240, and HOXA11-AS were overexpressed in gastric cancer as oncogene promoting cancer cell viability, proliferation, and metastasis via sponging miR-124 and upregulating EZH2, DNMT3B and ITGB3 respectively [185–188]. Tumor suppresivity of miR-125 on gastric cancers was attributed to downregulation of many proteins inhibiting proliferation and metastasis: MCL1, BRMS1, VEGF-A, and HER2 [189–192], while lncRNA PVT1 was the only oncogenic ncRNA sponging miR-125 in gastric cancer [193]. Tumor suppressor miR-126 can inhibit proliferation and metastasis of gastric cancers by downregulation of CRKL, VEGF-A, CXCR4, ADAM9, BRCC3, and PIK3R2 [194–199] while TMPO-AS1 and HOTAIR were two oncogenic lncRNA promoting invasion and metastasis by upregulation of BRCC3 and PIK3R2, and sponging miR-126 [198, 199]. As tumor suppressor acting to downregulate MAPK4, WNT7a, SORT1, and MTDH by miR-127 and inhibiting gastric cancer invasion [200–203]. SORT1 and MTDH can be upregulated by sponging miR-127 by lncRNA circ_0110389 and circALPL respectively [202, 203]. Four lncRNA were found to be oncogenic and sponging the tumor suppressor miR-128 and upregulating their targets leading to promoting gastric cancer proliferation and metastasis: lncRNA CCL2 with PARP2, lncRNA HCP5 with HMGA2, lncRNA PCAT1 with ZEB1, and lncRNA LINC01091 with ELF4 [204–207]. Additional 4 oncogenic lncRNA were upregulated in gastric cancer tissues and downregulated miR-129: GACAT2, GACAT3, AC130710, and PCGEM1, which upregulate P4HA2 [208–210]. Oncogenic miR-130 promotes gastric cancer invasion and metastasis by downregulation of TGFβR2, C-MYB, and GCNT4 [211–213] while lncRNA MRPL39 acts to suppress the metastasis by sponging miR-130 [214]. Tumor suppressor miR-132 inhibits

gastric tumor invasiveness and metastasis via downregulation of MUC13, CD44 and fibronectin1 (FN1), KIF21B, and PXN [215–218], while lncRNA XIST can sponge miR-132 and upregulate PXN [218]. miR-134 and their lncRNA can have dual effects as tumor suppressor or oncogene and vice versa. By targeting and downregulation of GOLPH3, YY1 and YWHAZ [219–221] miR-134 acting as gastric cancer suppressor while acting as oncogene to deactivate PTEN while lncRNA circPTK2 can sponge miR-134 to activate PTEN inhibiting proliferation and metastasis [222]. Inversely lncRNA LUCAT1 can act as oncogene sponging suppressor miR-134 and upregulation of YWHAZ [221]. Oncogenic miR-135 can downregulate E2F1 and DAPK2 [223] and upregulate WNT [224] while miR-135 can act as gastric cancer suppressor downregulating SMAD2 [225]. Gastric cancer metastasis and invasion can be promoted by sponging of miR-136 by lncRNA circ_0110389 and circ_100876 leading to upregulation of SORT1 and MIEN1 respectively [202, 226]. Sponging of miR-137 by lncRNA like DSCR8, NCK1-AS1, and circHECTD1 can promote invasion and proliferation of gastric cancer by upregulation of CDC42, NUP43, and PBX3 respectively [227–229]. miR-138 inhibits gastric cancer vitality and progression by downregulation of ITGA2, PLAU, FOXC1, and SIRT2, which can be upregulated after sponging miR-138 by lncRNA UBE2CP3, TRPM2-AS, MCM3AP-AS1, and LINC00152 respectively

miRNA	Expression in ESCC	Role in ESCC	Target gene	Ref.
miR-1	Downregulated	Tumor-suppressor	MET	[95]
miR-7	Downregulated	Tumor-suppressor	EGFR	[243]
miR-9	Overexpressed	Oncogenic	CDX2	[99]
miR-9	Downregulated	Tumor-suppressor	MYH9	[100]
miR-17	Overexpressed	Oncogenic	PTEN, EGR2	[101, 102]
miR-18	Overexpressed	Oncogenic	IRF2	[104]
miR-19	Overexpressed	Oncogenic	NA	[106]
miR-20	Overexpressed	Oncogenic	NA	[106]
miR-21	Overexpressed	Oncogenic	PTEN, RECK, and PDCD4	[107]
miR-22	Downregulated	Tumor-suppressor	MMP14, NET1, and Snail	[109]
miR-24	Overexpressed	Oncogenic	CDKN1B and CHEK1	[110]
miR-25	Overexpressed	Oncogenic	FOXO3	[111]
miR-26	Overexpressed	Oncogenic	PTEN, EZH2	[113]
miR-27	Overexpressed	Oncogenic	HOXA10	[115]
miR-28	Downregulated	Tumor-suppressor	AKT	[116]
miR-29	Downregulated	Tumor-suppressor	CCND2, MMP2	[118]
miR-30	Downregulated	Tumor-suppressor	beclin-1, Snail, and ETS2	[120, 121, 123]
miR-30	Overexpressed	Oncogenic	P53	[124]

miRNA	Expression in ESCC	Role in ESCC	Target gene	Ref.
miR-31	Downregulated	Tumor-suppressor	E2F2s, RhoA, and ITGA5	[125–127]
miR-32	Overexpressed	Oncogenic	KLF4, KLF2	[128, 244]
miR-33	Downregulated	Tumor-suppressor	CDK6, CCND1, and PIM1	[129]
miR-34	Downregulated	Tumor-suppressor	Bcl-2, Notch, and HMGA2	[130]
miR-43	Overexpressed	Oncogenic	VEZT	[131]
miR-92	Downregulated	Tumor-suppressor	EP4, Notch1, SOX4, and FBXW7	[132–134]
miR-93	Overexpressed	Oncogenic	IFNAR1, PTEN GPC5, and PTG3	[135–138]
miR-95	Overexpressed	Oncogenic	EMP1	[139]
miR-95	Downregulated	Tumor-suppressor	Slug	[140]
miR-96	Overexpressed	Oncogenic	ZDHHC5, KIF26A, and FOXO3	[141, 142]
miR-98	Downregulated	Tumor-suppressor	BCAT1, Treg, and CCND2	[143–145]
miR-100	Downregulated	Tumor-suppressor	BMPR2, ZBTB7A	[146–148]
miR-100	Overexpressed	Oncogenic	p53, HS3ST2	[149, 150]
miR-101	Downregulated	Tumor-suppressor	ANXA2, ZEB1, SOCS2, PI3K/AKT/mTOR, EZH2, and AMPK	[155–159]
miR-103	Downregulated	Tumor-suppressor	Caveolin-1, RAB10	[164, 165]
miR-103	Overexpressed	Oncogenic	KLF4	[166]
miR-105	Downregulated	Tumor-suppressor	SOX9, YY1	[167–169]
miR-106	Overexpressed	Oncogenic	NA	[170, 245]
miR-107	Overexpressed	Oncogenic	PTEN, NF1, and HIF1α	[171–173]
miR-107	Downregulated	Tumor-suppressor	BDNF	[174]
miR-122	Downregulated	Tumor-suppressor	DUSP4, LYN, MYC, MMP-9, and GIT-1	[176–180]
miR-124	Downregulated	Tumor-suppressor	SPHK1, ROCK1, RAC1, and SP1	[182–184]
miR-125	Downregulated	Tumor-suppressor	MCL1, BRMS1, VEGF-A, and HER2	[189–192]
miR-126	Downregulated	Tumor-suppressor	CRKL, VEGF-A, CXCR4, ADAM9, BRCC3, and PIK3R2	[194–199]
miR-127	Downregulated	Tumor-suppressor	MAPK4, WNT7a, SORT1, and MTDH	[200–203]

miRNA	Expression in ESCC	Role in ESCC	Target gene	Ref.
miR-128	Downregulated	Tumor-suppressor	PARP2, HMGA2, ZEB1, and ELF4	[204–207]
miR-129	Downregulated	Tumor-suppressor	P4HA2	[210]
miR-130	Overexpressed	Oncogenic	TGFβR2, C-MYB, and GCNT4	[211–213]
miR-132	Downregulated	Tumor-suppressor	MUC13, CD44, fibronectin1 (FN1), KIF21B, and PXN	[215–218]
miR-134	Downregulated	Tumor-suppressor	GOLPH3, YY1, and YWHAZ	[219–221]
miR-134	Overexpressed	Oncogenic	PTEN	[222]
miR-136	Downregulated	Tumor-suppressor	SORT1, MIEN1	[202, 226]
miR-137	Downregulated	Tumor-suppressor	CDC42, NUP43, and PBX3	[227–229]
miR-138	Downregulated	Tumor-suppressor	ITGA2, PLAU, FOXC1, and SIRT2	[230–233]
miR-139	Downregulated	Tumor-suppressor	MYB, MMP11, PRKAA1, ELK1, and SOX4	[234–238]
miR-140	Downregulated	Tumor-suppressor	ATG5, SOX4, ADAM10, and NDRG3	[239–242]

Table 4.
Oncogenic/tumor suppressor miR among stomach cancer.

lncRNA	Expression of lncRNA in Stomach cancer	Target miR	Target gene	Ref.
LINC00242	Overexpressed	miR-1	MET	[97]
UCA1	Overexpressed	miR-7	EGFR	[98]
HULC	Overexpressed	miR-9	MYH9	[100]
HOTAIRM1	Downregulated	miR-17	PTEN	[101]
LINC01939	Downregulated	miR-17	EGR2	[102]
NEAT1	Overexpressed	miR-17	GSK3β	[103]
CASC2	Downregulated	miR-19	NA	[105]
MEG3	Downregulated	miR-21	PTEN	[108]
CTC-497E21.4	Overexpressed	miR-22	NET1	[109]
GATA6-AS	Downregulated	miR-22	FOXO3	[112]
TET1–3	Downregulated	miR-26	EZH2	[114]
LOC400043	Overexpressed	miR-28	AKT	[117]
MEG3	Overexpressed	miR-29	CCND2, MMP2	[119]
PVT1	Overexpressed	miR-30	Snail	[121]

lncRNA	Expression of lncRNA in Stomach cancer	Target miR	Target gene	Ref.
HNF1A-AS1	Overexpressed	miR-30	PI3K/AKT	[122]
DLEU2	Overexpressed	miR-30	ETS2	[123]
MIR31HG	Overexpressed	miR-31	E2F2s, RhoA, and ITGA5	[127]
SNHG5	Downregulated	miR-32	KLF4	[128]
PITPNA-AS1	Overexpressed	miR-92	SOX4	[133]
MT1JP	Overexpressed	miR-92	FBXW7	[134]
PTENP1	Downregulated	miR-93	PTEN, GPC5, and PTG3	[136–138]
TTTY15	Overexpressed	miR-98	CCND2	[145]
HAGLROS	Overexpressed	miR-100	mTORC1 signaling pathway	[151]
MIR100HG	Overexpressed	miR-100	PI3K/AKT/mTOR pathway	[152–154]
LINC01303	Overexpressed	miR-100	EZH2	[159]
SPRY4-IT1	Overexpressed	miR-100	AMPK	[160]
XIST	Overexpressed	miR-100	EZH2	[161]
SNHG6	Overexpressed	miR-100	ZEB1	[162]
LINC00943	Overexpressed	miR-100	NA	[163]
LINC00152	Overexpressed	miR-103	RAB10	[165]
GPC5-AS1	Downregulated	miR-106	GPC5	[137]
ZFR	Downregulated	miR-107	PTEN	[171]
PCAT18	Downregulated	miR-107	PTEN	[175]
LINC01296	Overexpressed	miR-122	MMP-9	[179]
CRART16	Overexpressed	miR-122	VEGFD	[180]
MALAT1	Overexpressed	miR-124	EZH2	[185]
LINC00511	Overexpressed	miR-124	EZH2	[186]
LINC00240	Overexpressed	miR-124	DNMT3B	[187]
HOXA11-AS	Overexpressed	miR-124	ITGB3	[188]
PVT1	Overexpressed	miR-125	NA	[193]
BRCC3	Overexpressed	miR-126	BRCC3	[198]
PIK3R2	Overexpressed	miR-126	PIK3R2	[199]
circ_0110389	Overexpressed	miR-127	SORT1	[202]
circALPL	Overexpressed	miR-127	MTDH	[203]
CCL2	Overexpressed	miR-128	PARP2	[204]
HCP5	Overexpressed	miR-128	HMGA2	[205]
PCAT1	Overexpressed	miR-128	ZEB1	[206]

lncRNA	Expression of lncRNA in Stomach cancer	Target miR	Target gene	Ref.
LINC01091	Overexpressed	miR-128	ELF4	[207]
GACAT2,	Overexpressed	miR-129	NA	[208]
GACAT3	Overexpressed	miR-129	NA	[208]
AC130710	Overexpressed	miR-129	NA	[209]
PCGEM1	Overexpressed	miR-129	NA	[210]
MRPL39	Downregulated	miR-130	NA	[214]
XIST	Overexpressed	miR-132	PXN	[218]
circPTK2	Downregulated	miR-134	PTEN	[222]
LUCAT1	Overexpressed	miR-134	YWHAZ	[221]
circ_0110389	Overexpressed	miR-136	SORT1	[202]
circ_100876	Overexpressed	miR-136	MIEN1	[226]
DSCR8	Overexpressed	miR-137	CDC42	[227]
NCK1-AS1	Overexpressed	miR-137	NUP43	[228]
circHECTD1	Overexpressed	miR-137	PBX3	[229]
UBE2CP3	Overexpressed	miR-138	ITGA2	[230]
TRPM2-AS	Overexpressed	miR-138	PLAU	[231]
MCM3AP-AS1	Overexpressed	miR-138	FOXC1	[232]
LINC00152	Overexpressed	miR-138	SIRT2	[233]
SNHG3	Overexpressed	miR-139	MYB	[234]
CTBP1-AS2	Overexpressed	miR-139	MMP11	[235]
LINC00152	Overexpressed	miR-139	PRKAA1	[236]
Circ-PTPDC1	Overexpressed	miR-139	ELK1	[237]
circ_0000218	Overexpressed	miR-139	SOX4	[238]
CCAT1	Overexpressed	miR-140	ATG5	[239]
TMPO-AS1	Overexpressed	miR-140	SOX4	[240]
SNHG1	Overexpressed	miR-140	ADAM10	[241]
SNHG20	Overexpressed	miR-140	NDRG3	[242]

Table 5.
Oncogenic/tumor suppressor lncRNA among stomach cancer.

[230–233]. Same miR-138, miR-139 is tumor suppressor ncRNA inhibiting of tumorgenicity and their effect can be sponged by lncRNA, which promote tumorigenicity via upregulation of MYB, MMP11, PRKAA1, ELK1, and SOX4 by lncRNA SNHG3, CTBP1-AS2, LINC00152, Circ-PTPDC1, and circ_0000218 respectively [234–238]. Tumor suppresivity of miR-140 in gastric cancer was achieved via decreasing the expression of ATG5, SOX4, ADAM10, and NDRG3. Inverse effect (oncogenic) on gastric cancer can be accomplished by lncRNA CCAT1, TMPO-AS1, SNHG1, and SNHG20, which upregulate previously mentioned proteins respectively [239–242] (**Tables 4** and **5**).

2. Conclusion

The current review concludes the vital role of ncRNA (both miRNA and lncRNA) in some digestive system tumors either as an oncogene-promoting cancer viability, invasiveness, proliferation, and metastasis or as a tumor suppressor inhibiting tumorigenicity or inducing apoptosis.

Conflict of interest

There is no "conflict of interest" for this work.

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Chapter 9

MicroRNAs and Male Infertility

Mohsin Munawar, Irfana Liaqat and Shaukat Ali

Abstract

Spermatozoan production is tightly controlled by the multistep process of spermatogenesis and spermiogenesis. Physiological and molecular disruption in spermatogenesis can lead to various reproductive disorders including male infertility. Male infertility is associated with various etiologies, but mechanism is not determined yet. MicroRNAs (miRNAs) are almost 22 nucleotides long, non-protein coding RNA that play an essential role in posttranscriptional regulations in various biological processes including spermatogenesis. The current review is aimed to summarize the recent literature on the role of miRNAs in male infertility and spermatogenesis and their potential in diagnosis, prognosis, and therapy of the disease. miRNAs have shown tremendous potential to be used as diagnostic and prognostic marker and therapeutic target in diseases related to male infertility. Experimental evidence reveals that aberrant expression of miRNAs affects different cell types and different stages of spermatogenesis, which ultimately leads to male infertility. To exploit the full potential of miRNAs, characterization of unidentified miRNAs is required to understand the miRNA-mediated regulatory mechanism related to male infertility.

Keywords: miRNAs, male infertility, spermatogenesis, spermiogenesis, non-coding RNA, therapeutic agents

1. Introduction

Infertility is the inability to conceive spontaneously in 1 year by a sexually active, non-contracepting couple. Primary infertility, the complete inability to conceive, ranges from 2 to 5%, while secondary infertility, indicating cessation of further fertility, globally has a prevalence rate of 20%. Approximately 15% (48.5 million) of couples cannot conceive worldwide [1].

Among infertile men, the etiology of 30–40% of cases remains unknown, so considered idiopathic male infertility [2]. The possible causes of male infertility are physiological anomalies, immunological factors, some genetic abnormalities [3], and some environmental toxicants, which can disturb the reproductive health of male by disturbing steroidogenesis, spermatogenesis, and histopathological structures [4]. Among all these possible causes of male infertility, several types of small non-coding RNAs, including microRNAs (miRNAs), are expressed in the male germ line and impair the mammalian spermatogenesis [5, 6].

Protein coding genes represent only 1.5%of the genome, which can be increased by up to 2% if untranslated regions (UTRs) are also included. The remaining 98% genome, the non-protein-coding region, has been considered a "black box" until the

characterization of non-coding RNAs (ncRNAs) was done using novel nucleotide sequencing technologies. ncRNAs are present in cellular compartments, participating in multiple biological functions, including the male reproductive system, whereas some are identified in extracellular fluids, correctly named as circulating ncRNAs, where they can be detected in exosomes, bound on lipoprotein and free circulating molecules [7].

Ambros and his colleagues 1993 discovered the miRNA and then revealed the ncRNAs as gene regulators in eukaryotes [8, 9]. *Lin-4* and *let-7* in *Caenorhabditis elegans* were first discovered ncRNAs, later classified as miRNAs. Thus far, almost 38579 entries have been recorded, and among these, up to 1982 miRNAs are only from the human genome [10]. miRNAs code is either located in the intergenic region or introns of genes of the whole genome. In contrast, nearly half of total miRNAs are arranged nearby (within 50kb of another miRNA code) to form clusters. These clusters range from 2 to 46 miRNAs; C19MC is the major known cluster of miRNAs located on chromosome 19 in primates [11]. It is predicted that over 30% of mammalian and 60% of human genes are regulated by miRNAs [12, 13].

miRNAs are small RNA molecules, derived from the genome-encoded-precursor loop, consisting of almost 19–23 nucleotides. They are conserved in species and so regulate the expression of the mRNAs by recognizing the targeted mRNA through base-pair binding to the 3′UTR [14, 15].

2. miRNA nomenclature

MicroRNAs (prefix; “miR" for mature sequence whereas precursor hairpins are labeled as "mir") are named by using the number in sequential order, such as miR-1, miR-2. Despite the discovery of identical miRNAs in different organisms, the same number is assigned. Lettered suffix such as miR-1a and miR-1b is assigned to the sequences whose mature miRNAs sequences differ at one of two positions. Numbered suffix (e.g., miR-1-1 and miR-1-2) is used to label the identical miRNAs that have originated from distinct hairpin loci. The -3p and -5p suffixes refer to the arm from which the mature miRNA sequence originates. Detailed guidelines for naming the miRNAs can be seen in [16, 17].

3. MicroRNAs: biogenesis

miRNAs have a vital role as epigenetic regulators. The enzyme (Dicer protein) used for the biosynthesis of miRNA plays a very crucial role for the developmental processes [18]. During miRNAs biogenesis, following steps will be followed sequentially as elaborated in **Figure 1**:

i. Primarily, a long imperfect ~80 nt hairpin-like structure primary microRNA (pri-miRNA) is transcribed by RNA polymerase (RNA pol) II from intergenic non-coding region or intragenic region intron (untranslated region of protein-coding genes).

ii. Secondarily, pri-miRNA undergoes two steps enzymes (Drosha and Dicer from RNase III superfamily) catalyzed reaction.

   a. Pri-miRNA (~80-nt hairpin structure) is excised to form an intermediate precursor (pre-) miRNA (~65–70 nt) processed by Nuclear *Drosha-DGCR8*

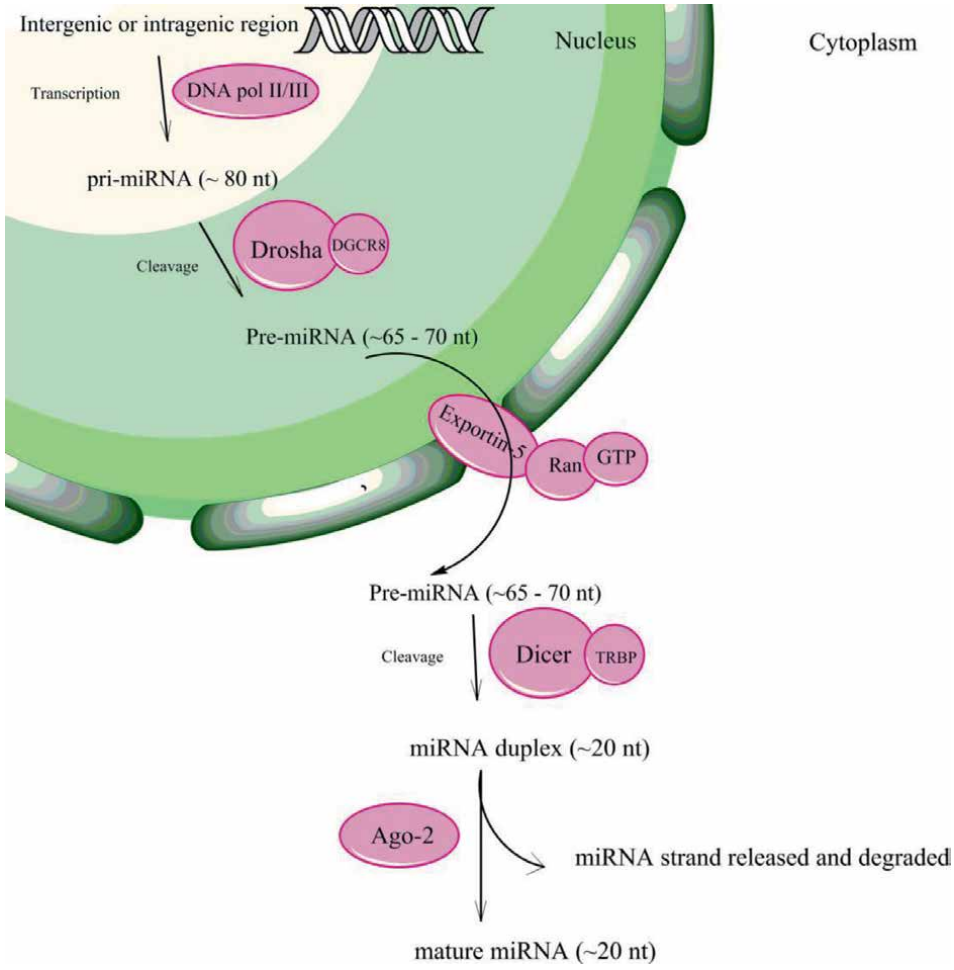

Figure 1.
Mechanism of miRNA's biosynthesis.

(DiGeorge syndrome critical region 8), then transported to cytoplasm through export factor Exportin-5.

b. Pri-miRNA is processed by called *Dicer-TRBP* (trans-activating region binding protein), a second RNase III endonuclease enzyme, to remove the terminal loop of pre-miRNA resulting in ~20 bp miRNA: miRNA* duplex.

iii. The *Dicer-TRBP* processed duplex is loaded onto Ago-2 (Argonaute) protein to form an miRNA induced silencing complex (miRISC). miRISC contains mature miRNA that regulates the gene expression, while another miRNA* will be released and degraded.

iv. Finally activated region (miRNA) of miRISC binds with 3′ UTR of targeted mRNA, ultimately leading to translational repression, deadenylation, or degradation of targeted mRNA [19].

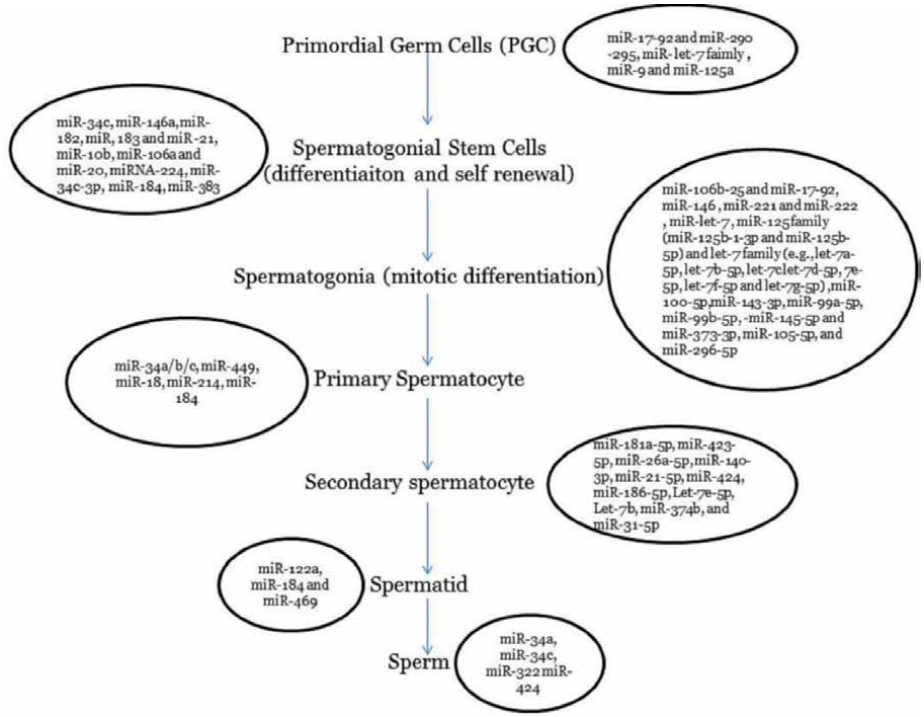

Figure 2.
miRNAs expressed during different stages of spermatogenesis in mammals.

4. miRNAs: regulators of biological processes

miRNAs play a vital role in the regulation of various biological phenomena including cellular differentiation, cell growth, programmed cell death, and embryonic development [20–22], whereas up- or downregulation may lead to serious abnormality. For instance, cardiovascular disease, cancer, diabetes, renal abnormality, schizophrenia, muscular dystrophy, and reproductive disorder especially related to sperm formation and its function have been linked with biosynthesis impairment, mutation, or improper regulation of miRNA [23–29]. miRNA is not solely responsible for the whole regulatory mechanism, but their activities are also dependent on other proteins or complexes depending upon the processor stage [30].

Mature human spermatozoa retain various miRNAs; moreover, variable expression of spermatozoal RNAs has been associated with male infertility [31]. miRNAs, present in primordial germ cells, spermatogonia, primary and secondary spermatocytes, spermatids, and mature spermatozoa, play an epigenetic role in the spermatogenesis and early development as well (**Figure 2**) [32].

5. miRNA as potential biomarker in male infertility

In order to confirm the male infertility, various methods have been adopted, but the most recent way is the use of biomarker. Biomarkers can be used to diagnose the disease before the appearance of physiological symptoms. miRNA is known as the most reliable biomarker due to various known properties.

5.1 Properties of biomarker

A biomarker molecule should be stable, target-specific, and sensitive to detection, noninvasive, and from an easily accessible source. In the field of medical science, protein molecules, which are easily found in serum plasma, have been used as a biomarker for the detection of pathological conditions and their prognosis. However, as a biomarker, protein molecules have some limitations such as reduced diagnostic value due to low sensitivity and specificity of their detection. On the other hand, miRNAs present in the extracellular or intracellular environment appear much earlier and detected at minute concentration, thus allowing early diagnosis. Expressions of miRNAs are often biological-stage-specific [33, 34]. Moreover, miRNA is observed as the homogeneous population of molecules, but protein molecules may become heterogeneous due to different posttranslational modifications.

miRNAs are more stable than mRNA but lesser stable than DNA. Extracellular miRNAs exist in microvesicles, exosomes, and lipoproteins or form a complex with Ago-2 protein, so protected from endogenous RNAse activity, which suggests their reliable use as a noninvasive biomarker for spermatogenic impairment [35–38]. Some miRNAs are stable at room temperature, sustain a repeated freeze-thaw cycle, and got stability under harsh conditions even found in degraded RNA preparations [39–41]. In a study, exosomal miRNAs were found to be stable at 4°C for 2 weeks, at −80°C for 2 months, and at −20°C for 5 year [42]. So their relative stability enhances their usage as biomarker molecules. miRNA (both extracellular and intracellular) can be isolated by using noninvasive procedures and detected with techniques in common routines such as RT-qPCR and miRNA microarray, which is more advantageous than conventional and invasive (e.g., prostate and testicular biopsy) diagnostic procedures [43–47]. Despite all these characteristics of miRNAs as a biomarker, the use of protein as a biomarker may not be completely replaced, but no doubt miRNAs can be labeled as a good candidate for complementary diagnosis tools.

Circulating (serum) miRNAs were firstly used as biomarkers for the detection of diffuse large B-cell lymphoma [48]. In another study, the downregulation of miR-34c, miR-122, and miR-181a was observed in the serum of patients suffering from oligo-asthenospermia [49]. Wang and his colleagues reported a decreased level of various miRNAs in azoospermic patients when compared with control [44]. Many other such published literature suggested that miRNAs are suitable biomarkers for diagnostic purposes [50, 51].

In mammals, sperm deliver genome and epigenome to the oocyte, which includes protein factors, methylation of DNA, and non-coding RNAs [52]. During mammalian spermatogenesis, mRNAs undergo posttranscriptional and posttranslational regulation in differentiating germ cells [53], so the regulatory role of miRNAs during the process of spermatogenesis can be observed clearly.

Many miRNAs as families or individual candidates are found to be very crucial in many systems of humans including the reproductive system. The role of some miRNA's family and individual miRNA candidates is summarized.

5.2 miRNA families

Let-7 **family** is a large family encoded by 12 different loci [54]. A well-known regulatory role of the let-7 family is gamete differentiation and embryonic implantation in humans [55, 56]. Different members of this family were identified in the testis, spermatozoa, and seminal plasma with differential expression [57–59].

miR-30-5p family (five members miR30a/b/c/d/e-5p), encoded by six loci located on chromosomes 1, 6, and 8 in humans. All miRNAs are observed in male reproductive tissue cells [60]. These miRNAs were expressed differentially in spermatozoa and seminal plasma of asthenozoospermic, oligozoospermic, and normozoospermic infertile men [39].

miR-345p/449-5p family with five miRNAs (miR-34a-5p, miR-34c-5p, miR-449a-5p, miR-449b-5p miR-449c-5p) has shown a very important regulatory role in mitosis [61]. These are highly expressed in male reproductive cells but variably expressed in pathologically infertile men especially nonobstructive azoospermic and obstructive azoospermic patients [62]. Their role was also investigated in the process of fertilization, cleavage division, and pre-implantation. These miRNAs are highly expressed in the sperm, as miR-34c-5p is the most abundant human sperm miRNA [52].

MiR-303-3p/370-3p/372-3p and 520-3p family each member of family miR-302-3p regulates over 450 target genes [63]. Members of this family were seen to be differentially expressed in the testis having histopathological impairments and in spermatozoa of patients with asthenozoospermia [62].

miR-99-5P/100-5P family is one of the most ancient families with three members (miR-99a/b/c) located in distinct chromosomal regions (ch 21, 19, and 11 respectively) in the human genome [64]. The member of this family shows differential expression in the testis of NOA patients [57].

miR-888 family (miR-888/ Mir-890, miR-891a/b, and miR-892a/b) is located on the X chromosome of primates only which has specific expression in epididymal tissues [65, 66]. Computational functional analyses predicted that the targets of the miR-888 family were related to morphogenesis of epididymis and sperm maturation [67].

5.3 Individual miRNA

hsa-miR-10b-5p, located on chromosome no 2(1q31.1), plays a pivotal role in gametogenesis as it is highly expressed in the testes. The expression level of hsa-miR-10B-5P, among others, was upregulated in round spermatids of NOA patients [57].

hsa-miR-27b-3p, present on chromosome 9 (9q22.32), was differentially expressed in the mature spermatozoa of infertile men. The expression of miR-27 has correlated with the expression of the CRISP2 (cysteine-rich secretory protein 2) gene, the highly expressed gene in the testis [59]. CGN1-CRISP2 complex has an important role in the development of the sperm tail (sperm motility) and predicts the regulatory role of miR27b-3p in spermatozoa development and motility potential [58].

miR-320a, located on chromosome 8, is highly expressed miRNA in epididymis and spermatozoa so has an important role in multiple reproductive processes [68]. Mmu-miR-320a (hsa-miR-320a) was exclusively expressed in murine Sertoli cells (SCs). The induction of apoptosis elicited miRNA expression was observed in meiotic spermatocytes and haploid spermatids. Forced expression of exogenous miR-320a in SCs may cause oligospermia and defection in sperm mobility, thus compromising the male fertility [69].

6. MicroRNAs: a key player in spermatogenesis

miRNAs play a vital role in the whole spermatogenic process, and their dysregulation ultimately leads to spermatogenic impairments [70]. To determine the role of miRNA, in male reproductive system, various models (mice) have been established

miRNA	Isolation of miRNA	Dysregulation	Cellular processes	Model organism	References
miR-19b and let-7a	Seminal plasma	Up-regulated	Failure in spermatogenesis	Human Oligozoospermia, non-obstructive and azoospermia	[79]
MiR-7-1-3p, miR-141 & miR-429	Seminal plasma	Up-regulated	Spermatogenesis impairment	Infertile men	[80]
hsa-miR-429	Semen	Up-regulated	Biomarker to access male infertility	Human Subfertile and non-obstructive azoospermia	[81]
hsa-miR-34b, hsa-miR-34c-5p & hsa-miR-122	Semen	Up-regulated	Biomarker to access male infertility	Human Subfertile and non-obstructive azoospermia	[81]
miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p and miR-513-5p	Seminal plasma	Up-regulated in asthenozoospermia Down-regulated in azoospermia	Role in infertility	Infertile men (Asthenozoospermia and Azoospermia)	[39]
miR-27a	Semen	Upregulated	Low motility, abnormal Morphology	Infertile men Asthenoteratozoospermia and normozoospermia	[59]
miR-17-92 cluster (miR-17, miR-18a, and miR-20a)	Testes	Upregulated	Testicular atrophy, decreased sperm production	Mice	[82]
miR-17-92 (Mirc1) and miR-106b-25 (Mirc3) cluster	Testes	Knock-out	Reduced testes and mild spermetogenic defect	Mice	[83]
miR-146a			Role in differentiation of spermatogonia.	Mice	[84]
miR-221/222			Maintain the undifferentiated state of mammalian spermatogonia.		[85]
miR-135a	Spermatogonial stem cells	Downregulated	Downregulation of forkhead box protein O 1 (FOXO1) expression.	Rats	[86]

miRNA	Isolation of miRNA	Dysregulation	Cellular processes	Model organism	References
miR-449	Testis			Mice	[87]
MiR-518f	Seminal plasma	Downregulated	Effected sperm quality and testicular environment exposure of Bisphenol A	Human	[46]
miR- 23a/b- 3p	Semen	Upregulated	Oligoasthenozoospermia	Human	[88]

Table 1.
miRNAs, the cellular processes affected by their dysregulation.

by using the *Cre/Lox* system, which enables conditional manipulation of target genes, including knockout, insertion, replacement, activation, or modification of gene expression [44]. To understand the exact function of concerned miRNA *in vivo*, target deletion is the key step; however, in the absence of one miRNA, usually someone other has a compensatory role.

Double knockout of miR-34b/c and miR-449 in mice displayed severe disruption in spermatogenesis, dispensable fertilization, and pre-implantation development. Intracytoplasmic injection of miRNA-dKO sperm led to normal fertilization [52]. Deletion of miR34b/c and miR-449 in mice shows impaired spermatogenesis and spermatozoa maturation resulting in oligoasthenoteratozoospermia (OAT) [70]. Simultaneous inactivation of miR34b/c and miR449 in dKO mice leads to developmental defects and infertility [71, 72].

Salas-Huetos and his colleagues showed that 221 miRNAs were consistently detected in 10 healthy fertile men; among these most expressed, hsa-miR-191-5p was associated with sperm differentiation [73]. The first report on altered miRNA expression in patients with NOA showed that 154 miRNAs were differentially downregulated and 19 upregulated in NOA patients compared with fertile men [74]. Experimentally it is confirmed that specific genes are regulated by miRNAs involved in spermatogenesis. Decreased levels of estrogen-alpha (ERα) and a higher level of miR-100 and let-7b in oligospermia patients compared with fertile men indicated the regulatory role of miRNA over estrogen signaling [75–77]. Phosphatidylinositol-specific phospholipase C, X domain containing 3 (PLCXD3), expressed in spermatogenesis, was downregulated by miRNA34c-3p in severe oligozoospermic patients [78].

Conclusively, these results and some others (as shown in **Table 1**) confirm the regulatory role of miRNAs in male germ and somatic cells, and any change in their expression may lead to reproductive anomalies.

7. miRNAs: therapeutic advancements and infertility

In addition to the potential use of miRNAs as a biomarker, miRNAs have been considered therapeutic services against male infertility. miRNAs overexpression can be inhibited by anti-miR (laboratory designed molecule), or their downregulation might be supplemented with miRNAs mimics [89, 90]. A recent study showed that MRX34 (miRNA mimic of miR-34a) managed to suppress the tumor progression, and anti-miRs their inhibitory activity through binding to a miRNA, thus blocking it [91–94].

In RNA-based therapeutics strategies, the challenge of degradation of the oligonucleotide by RNases in serum or cellular compartments is averted using two distinct approaches, either by altering oligonucleotide or through encapsulations of RNAs for protection (by adding phosphorothioate-like groups or developing delivery vehicles [95].

After several preclinical studies involving miRNA therapeutics, only a small number of miRNAs have moved into clinical development [96]. This is because of the many challenges, including;

1. Difficulty in identifying the best miRNA candidates or miRNA targets for each disease type and the absence of efficient delivery systems in specific tissues. As described below, several novel delivery systems are currently being developed to develop miRNA therapeutics using a systematic approach.

2. miRNA and its target identification by using bioinformatics tools, in silico interaction, and *in vitro* experimentation (cell culture, etc.)

   a. Selection of miRNA chemistry such as 2'-methyl, locked nucleic acid (LNC), or phosphothionate.

   b. Optimization of the delivery system by viral particle encapsulation or through liposomes.

   c. *In vivo* testing by using a model organism so that target localization, the efficiency of delivery, and toxicity level can be reported.

   d. Clinical trial for dose optimization, determination of the mode of drug delivery, and toxicity analysis.

5. Another challenge is to provide stability, accuracy in *in vivo* targeting, avoid toxicity and thwarting off-target complex to a therapeutic miRNA [96].

6. To achieve the efficient therapeutic functioning, correct *in vivo* dose concentration has not been determined yet [97].

8. Conclusion

miRNAs play a vital role in developing an organism and regulating many biological processes in the living organism. This chapter summarized the role of miRNAs in male infertility. Many miRNAs are reported as regulators of many functions, including developing reproductive organs, spermatogenesis, spermiogenesis, sperm mobility, and fertilization. Despite using miRNAs as biomarkers, miRNAs are also investigated as more reliable therapeutic agents for various diseases such as cancer, diabetes, and infertility.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 10

The Role of Noncoding RNAs in the Response of Oil Palm Plants to Abiotic Stresses

Fernanda Ferreira Salgado, Priscila Grynberg and Manoel Teixeira Souza Junior

Abstract

The genus *Elaeis* comprises two species, *E. guineensis* Jacq. and *E. oleifera* (Kunth) Cortés, which are known as the African and the American oil palms, respectively. The African oil palm originated from West Africa and is the predominant species in commercial plantations. This oilseed crop is the number one source of consumed vegetable oil in the World. Several abiotic stressors affect the plant life cycle interfering with growth and productivity. Salinity and drought are abiotic stresses that affect plantations on all continents, resulting in the loss of billions of dollars annually. MicroRNAs (miRNAs) are small endogenous noncoding RNAs that impact almost all biological processes, affecting either the transcriptional or posttranscriptional regulation of gene expression. Here we describe the R&D initiatives on oil palm miRNAs, highlighting the current knowledge on miRNAs' involvement in oil palm response to abiotic stress and postulating possible miRNA-based strategies for the genetic improvement of oil palm salinity and drought stresses tolerance.

Keywords: abiotic stress, tolerance, transcription factor, transcriptome, noncoding RNA, oil palm, drought stress, salt stress

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is known as the most productive oilseed crop in the World, bearing great economic importance due to its large-scale production and high efficiency of the extraction and refining processes to obtain palm oil and palm kernel oil [1, 2]. In 2021/2022, the World consumed approximately 82 million metric tons of palm oil and palm kernel oil, making this oilseed crop the number one source of consumed vegetable oil [3].

The oil palm industry faces criticism due to a series of unsustainable practices (deforestation and consequent biodiversity loss, increased greenhouse gas emissions, and environmental and aquatic pollution), finding itself under pressure to adopt new and innovative procedures that could help this sector reverse this negative public perception [4]. Darkwah and Ong-Abdullah [5] highlighted some of these procedures, such as choice for intensification over extensification, adoption of

precision agriculture technologies, support for smallholder farmers, sustainability certification, and circular economy.

Oil palm plantations are in areas with tropical forests in the equatorial belt, as they need high rainfall throughout the year [6]. In Brazil, for instance, there is an extensive area with favorable conditions for cultivating oil palm outside the Amazon rainforest; however, those areas experience long periods of drought when oil palm does not meet the physiological water requirement to maintain productivity [7], and, consequently, need to be artificially irrigated with proper management to avoid soil salinization. Approximately 30% of the irrigated land area in the World is affected by salt [8], which, to a certain extent, shows a link between drought and salinity stresses.

Studies have shown that the progression of abiotic stresses is limiting global agricultural production, with no sign of reversal shortly [9–11], making it necessary to develop crops that are resilient to different abiotic stresses, such as drought and salinity, ensuring food security. Therefore, it is currently a challenge for plant scientists to develop crops resistant or tolerant to these conditions, capable of withstanding climatic instabilities and environmental stresses, especially combinations of these stresses. So, the research community aiming to develop knowledge and technology to allow oil palm breeding programs in Brazil and elsewhere to breed for superior genotypes must consider that.

Plants respond to environmental stimuli in a complex and highly coordinated manner at biochemical, physiological, and molecular levels [11–13]. When exposed to stress, rapid and effective reprogramming at the molecular level is required to adapt to unfavorable conditions [12, 14, 15]. This reprogramming regulates the expression of stress-responsive genes, especially at transcriptional and posttranscriptional levels [14, 16, 17].

micro RNAs (miRNAs) are posttranscriptional and translational regulators frequently correlated with plant stress tolerance and modulating stress response [15, 18, 19]. miRNAs have enormous potential for crop improvement, being the focus of studies by scientists in recent years [18, 20–22].

Therefore, the main objectives of this review are to describe the R&D initiatives known to date on oil palm (*Elaeis* spp.) miRNAs, to report on their biogenesis and mode of action, to summarize the current knowledge on miRNAs' involvement in oil palm response to abiotic stress, and to postulate possible miRNA-based strategies on the genetic improvement of oil palm's tolerance to abiotic stress.

2. miRNA biogenesis and mode of action

miRNAs are a class of endogenous, noncoding sRNAs transcribed from introns, exons, or intergenic regions [11]. They play a role in posttranscriptional RNA-mediated gene silencing and expression by complementary interaction with their mRNA target site [23, 24]. The biogenesis of plant miRNAs has been reported mainly in *Arabidopsis thaliana* (L.) *Heynh*. (**Figure 1**) [25].

Plant miRNAs range from 21 to 24 nucleotides in length [26]. Similar to protein-coding genes, miRNA genes (MIRs) are commonly transcribed by RNA polymerase II, forming a single-stranded precursor RNA called primary miRNAs (pri-miRNAs) that have imperfect self-complementary folding regions. Still in the nucleus, the 5′ m7G-cap and 3′ polyadenylation are added to promote better

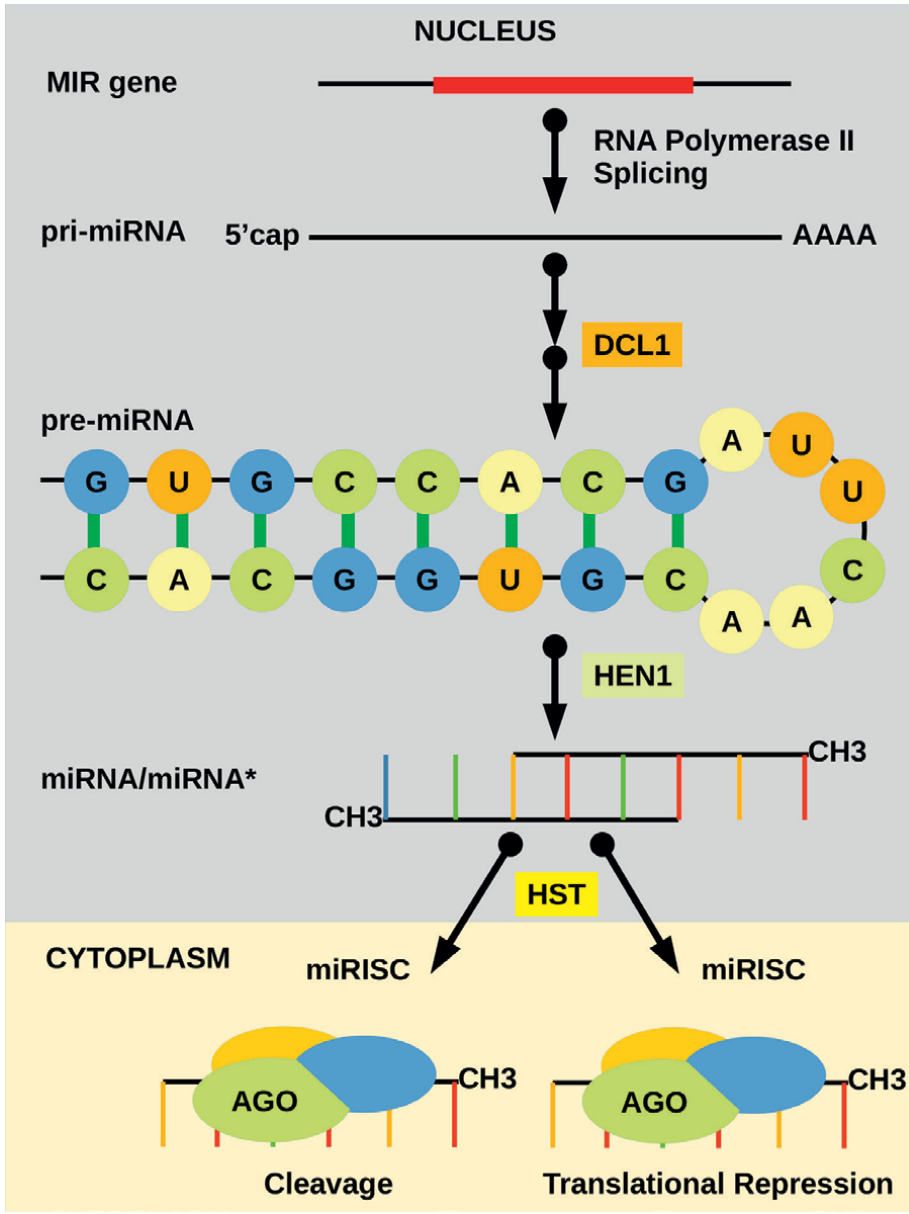

Figure 1.
Biogenesis of plant miRNAs. A miRNA gene is transcribed by RNA polymerase II, giving rise to a primary transcript (pri-miRNA), which is then capped and polyadenylated. DCL1 processes pri-miRNA, perhaps in two or more steps, and HEN1 methylate it to produce the miRNA/miRNA duplex. HST transports the duplex to the cytosol, and the miRNA strand gets incorporated into RISC. The nuclear export of miRNAs may occur before or after RISC assembly. According to the degree of complementarity with the target site, miRISC will either cleave the mRNA or inhibit its translation.*

stability. Then the pri-miRNAs are converted into miRNA precursor sequences (pre-miRNA) by the action of DICER-LIKE 1 (DCL1) along with other associated proteins [15, 27].

The 3′ end of the initial miRNA/miRNA* duplex is methylated by the nuclear protein HUA ENHANCER 1 (HEN1), thus preventing untemplated 3′ polymerization that accelerates miRNA turnover [28]. The mature miRNA is then transported from the nucleus to the cytoplasm by the HASTY protein (HST), then loaded onto AGONAUTE (AGO) proteins and incorporated into an RNA-induced silencing complex (RISC), while often the star miRNA molecules (miRNA *) do undergo degradation. The AGO/miRISC complex search for RNA molecules through sequence complementarity, promoting posttranscriptional gene silencing through endonucleolytic cleavage or translation inhibition [25, 29]. The miRNA mechanisms of biogenesis and function are still unclear; however, studies have shown that they are involved in several cellular processes, including development, differentiation, division, and cell death [11, 26, 30].

An individual miRNA can regulate multiple transcripts, and a single transcript can be acted upon by several distinct miRNAs [11]. The miRNA can regulate the expression of its target gene using three strategies: (a) degradation of a target transcript through almost perfect complementarity; (b) inhibition of translation; or (c) DNA methylation. Regulation via degradation of the target transcript results in the degradation of intron sequences, and these cleaved sequences generate dsRNA molecules with the aid of RDR2 and then produce I-siRNA molecules of 21 and 22 nucleotides. Subsequently, the I-siRNA associated with the AGO-RISC complex directs the cleavage of the target mRNA sequence [31, 32].

Regarding the regulation via translation inhibition, only the roles of AGO1 and AGO10 have so far been understood [33, 34]. On the other hand, the miRNA-mediated inhibition mechanism remains unclear. At last, studies show that miRNAs in *A. thaliana* bind to AGO4, AGO6, and AGO9 to promote transcriptional gene silencing of target genes through RNA-directed DNA methylation (RdDM) [27].

3. miRNAs playing a role in plant response to abiotic-drought and salinity-stress

Abiotic stress, such as deprivation or excess of water, high salinity, low or high temperature, heavy metals, and ultraviolet radiation, is a negative impact caused by nonliving factors on living organisms [35, 36]. They are primary stresses that arise in the plantations limiting seed germination, plant growth, and plant development; and, in some cases, resulting in the death of the plants.

Salinity and drought are two of the most prevalent abiotic stress worldwide, affecting plantations on all continents and resulting in the loss of billions of dollars annually. Plants have developed several mechanisms to combat drought stress, and several genes associated with the response of plants to this stress are known [12, 37]. One of these responses is the positive and/or negative regulation of several transcription factors (TF) related to numerous physiological and cellular functions [38].

The dehydration responsive elements (DREB) are TF related to the activation of genes responsive to drought stress tolerance, and its overexpression can result in tolerance to water deficit in plants. Similarly, the transcription factor MYB regulates negatively in plants during drought stress since it is related to stomata opening [11, 39]. However, the overexpression of other genes associated with drought stress in plants did not result in the expected drought tolerance, demonstrating the complexity of mechanisms responsible for plant tolerance to water stress [40].

Several research groups have identified and reported miRNAs responsive to water stress in several species, such as rice—*Oryza sativa* L. [41], soybean—*Glycine max* (L.) Merr. [42], barley—*Hordeum vulgare* L. [43], and Arabidopsis [44]. Those water stress-responsive miRNAs belong to three classes. The first class includes miRNAs targeting transcription factors that contribute to the gene regulation of the stress response, comprising miR156, miR159, miR165, miR169, miR171, miR172, miR319, and miR396 [21, 44, 45].

In the second class, the miRNAs are involved in the direct response to water stress, some of which are miR167, which is responsible for directing the auxin response factors ARF6 and ARF8 [46]; miR168, which targets the ARGONAUTE 1 (AGO1) mRNA [47]; and miR393 and miR394, which target F-box protein mRNAs that play roles in drought tolerance [45, 48, 49]. The last class contains miRNAs such as miR397 and miR408, which have hydrolase and oxidoreductase genes as their target gene [50, 51].

Zhou and colleagues [41] identified 30 miRNAs differentially expressed in rice under drought stress, 19 were new miRNAs. Regarding their expression, 14 were upregulated (miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026, and miR1125) and 16 downregulated (miR156, miR159, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088 and, miR1126). In soybean, upregulation of miRseq13, miR397ab, miR1513c, miR169-3p, and miR166-5p was observed in sensitive plants, while the same miRNAs were downregulated on tolerant crops [42].

When it comes to salinity stress, several genes are related to the plant's stress response, including those involved in ion channel activation, signal transduction, and modification regulated by plant growth factors, especially the morphological architecture of the root [52]. In *A. thaliana*, studies have shown increased expression of miRNAs miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394, miR396, and miR397 in response to salt stress, while miR398 was downregulated [21].

According to Ding and colleagues, when analyzing corn variants tolerant and sensitive to salt stress, members of the miR396, miR167, miR164, and miR156 families downregulated, while miRNAs miR474, miR395, miR168, and miR162 upregulated in the tolerant ones [18]. In radish (*Raphanus sativus*), 22 new and 49 already known miRNAs appeared under salt stress [53]. In young oil palm plants under saline stress, 27 new and 52 already known miRNAs appeared [15]. When analyzing the expression profile of these miRNAs, 72 of them showed negative differential expression and the remaining seven had no significant differential expression [15].

Recent studies allowed a better understanding of the mechanisms of action of miRNAs and their relationship in response to different abiotic stresses [38, 54, 55]. It is necessary to highlight that miRNAs are species-specific and present different expression levels when analyzed in distinct plant species or within a specific one under the same stress [15, 24].

4. miRNAs in oil palm (*Elaeis* spp.)

Despite many advancements in the prediction and characterization of miRNAs in plants, there are only a few studies where researchers report the identification and characterization of miRNAs in oil palm, either under abiotic stress conditions or not. Currently, there are 10 published studies reporting miRNAs identification

and characterization in oil palm [15, 30, 56–63]. Considering the great economic importance of oil palm, the number of miRNA studies reported on this oilseed crop is relatively low (**Table 1**).

Nasaruddin and colleagues found five potential miRNA encoding sequences in oil palm by a combined homology and structural analysis approach, having roles in regulating the auxin response, floral development, and basal transcription [30]. Mehrpooyan, Othman, and Harikrishna [56] identified in oil palm six paralogs of miR172, a regulator of the APETALA2 (AP2)-like family transcription factors. Their results suggested that the expression of different miR172 precursor paralogs is tissue-specific and showed that each of the two mature miR172 isoforms had different expression patterns during floral development.

Low and colleagues identified 14 miRNAs in contigs assembled from sequences generated from the hypomethylated or gene-rich regions in the genomes of both species from the *Elaeis* genera [57]. Target prediction of these miRNAs identified just one putative target gene, similar to the Rab21-family small GTPase, a small GTP-binding protein of the Ras superfamily. Silva and colleagues identified 57 mature miRNAs in *E. guineensis* and 52 in *E oleifera*, respectively, revealing that majority of them are transcription factors involved in the plant development process [58].

According to Somyong et al. [59], miRNA159 is related to the determination of females in oil palm trees, which is directly related to the higher production of oil palm trees, since palm oil with high number of female inflorescence and of clusters is most favorable. Noting that no expression of EgmiR159a was found in male flowers in their study, confirming its role in female sexual differentiation (**Table 1**). Ho and colleagues identified 15 oil palm-specific miRNA candidates when investigating microRNA expression in female inflorescence at two stages of floral [60].

In Gao et al. [61], the micro RNA EgmiR179 regulates the biosynthesis of metabolites through the negative regulation of its target gene NDT1, increasing oil content in palm oil [61]. Zheng and colleagues identified 452 microRNAs (miRNAs), including 170 conserved miRNAs and 282 new miRNAs, when gaining insights into the oil palm regulatory mechanisms of lipid and fatty acid metabolism. They found 37 fatty acid synthesis-related genes as putative miRNA-target genes and indicated that 22 conserved miRNAs and 14 new ones might be involved in fatty acid metabolism pathways. Tregear et al. [63] reported finding 30 previously unreported oil palm miRNA genes in a molecular study of the process of sexual differentiation in the immature inflorescence of oil palm.

Our research group reported prospecting and characterizing miRNAs in oil palm plants under salinity stress (**Table 1**) [15]. That was the first step in a study prospecting and characterizing miRNAs in oil palm plants under salinity [64] or drought stress and looking for insights on commonalities—miRNAs and putative miRNA-target genes—on the molecular response of young oil palm plants to these two abiotic stresses.

We did find 81 miRNAs—52 known ones and 29 new ones (**Figure 2**)—and 139 differentially expressed putative miRNA-target genes [15, 64]. In Salgado et al. [15], miR166, miR169, miR319, miR396, miR529, and egu-miR24sds showed altered expression profiles in young oil palm plants subjected to salt stress, both targeting TF, indicating a miRNA-dependent posttransitional regulation during the plant's response to the environment.

All miRNAs identification and characterization studies done so far in oil palm allowed the identification of 55 miRNA families or groups of miRNAs that derive from a common ancestor (**Figure 3**).

Technology	Condition	miRNA	Species	Oil palm material	Publication
Small RNA-seq	Normal	miR159a	*E. guineensis*	Female flower	Somyong et al. [59]
3730 sequencing technology/ Blast	Normal	miR2911, miR2916, miR156j, miR2914, miR2910, miR319f, miR167g, miR319e, miR845a, miR845b	*E. guineensis* and *E. oleifera*	Leaf	Low et al. [57]
Blast	Normal	miR156, miR157, miR159, miR160, miR164, miR166, miR167, miR168, miR169, miR171, miR172, miR319, miR390, miR393, miR394, miR395, miR396, miR397, miR398, miR399, miR528, miR529, miR530, miR535, miR815, miR1428, miR1432, miR2118, miR2275, miR5148, miR5179, miR5532, miR5801	*E. guineensis* and *E. oleifera* and *Phoenix dactylifera*	Genome	da Silva et al. [58]
Blast	Normal	miR156, miR157, miR159, miR160, miR854	*E. guineensis*	Genome	Nasaruddin et al. [30]
RNA-seq	Normal	miR5179	*E. guineensis*	Fruit	Gao et al. [61]
Small RNA-seq	Salt stress	miR156, miR391, miR395a, miR536, miR156, miR160, miR166, miR167, miR169, miR171, miR172, miR395, miR396, miR399, miR528, miR156, miR159, miR166, miR319, miR393, miR535, miR156, miR162, miR169, miR529, miR530, miR168, miR319, miR159. egumiR01, egumiR02, egumiR03, egumiR04, egumiR05, egumiR06, egumiR07, egumiR08, egumiR09, egumiR10, egumiR11,egumiR12, egumiR13, egumiR14, egumiR15, egumiR16, egumiR17, egumiR18, egumiR19, egumiR20, egumiR21, egumiR22, egumiR23, egumiR24, egumiR25, egumiR26, egumiR27.	*E. guineensis*	Leaf	Salgado et al. [15]
Small RNA-seq	Normal	miR1432, miR160, miR163, miR166, miR168, miR172, miR1859, miR1873, miR2199, miR2654, miR396, miR4365, miR528, miR535, miR827, miR835, miR858, miR894, egumiR3, egumiR5, egumiR6, egumiR7, egumiR8, egumiR9, egumiR10, egumiR11, egumiR12, egumiR13, egumiR14, egumiR15.	*E. guineensis Tenera* hybrid palms (*Dura* × *Pisifera*)	Female Inflorescence	Ho et al. [60]

Technology	Condition	miRNA	Species	Oil palm material	Publication
Small RNA-seq	Normal	miR156, miR160, miR164, miR172, miR444, miR5179, miR528, miR167, egumiR259, egumiR223, egumiR218, egumiR255, egumiR43, egumiR132, egumiR172, egumiR113, egumiR273, egumiR158, egumiR275, egumiR138, egumiR59, egumiR201, egumiR209, egumiR116, egumiR129, egumiR211, egumiR7, egumiR210, egumiR274.	*E. guineensis*	Mesocarp	Zheng et al. [62]
Small RNA-seq	Normal	miR319, miRN1, miR168, miR482, miR2118, miR159, miR171, miR167, miR397, miR160, miR156, miR535, miR396, miR536, miR169, miR319, miR179, miR166, miR394, miR399, miR172, miR164, miRN4, miR390, miR393, miRN5, miRN6, miR162, miR827, miR398, miRN7, miR395, miRN8, miRN9, miRN10, miR408, miR528, miRN11.	*E. guineensis*	Immature Inflorescence	Tregear et al. [63]
Blast	Normal	miR172	*E. guineensis*	Inflorescence	Mehrpooyan et al. [56]

Table 1.
Summary of miRNA studies reported on oil palm.

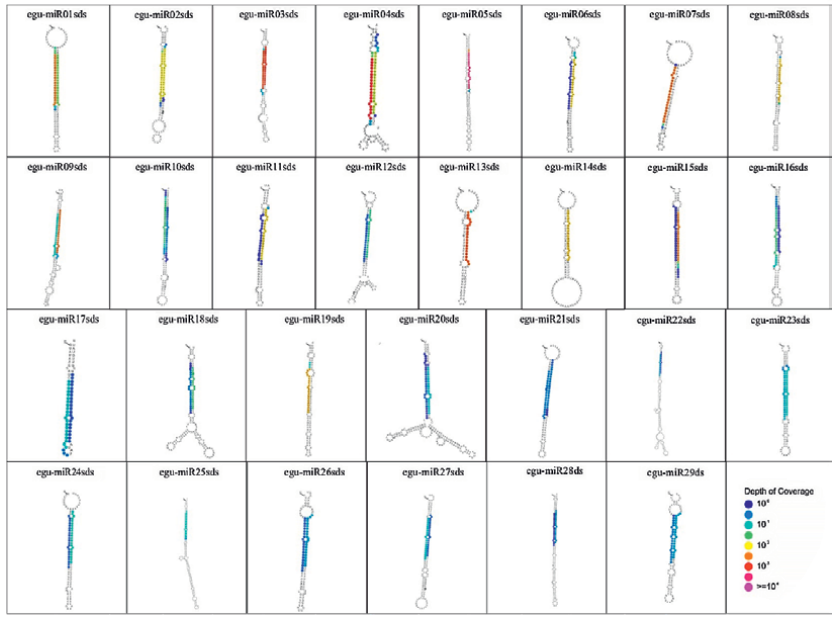

Figure 2.
Structure of the 29 new miRNAs identified in oil palm (Elaeis guineensis) plants exposed to abiotic stresses—salinity and drought (Source: [15, 64]).

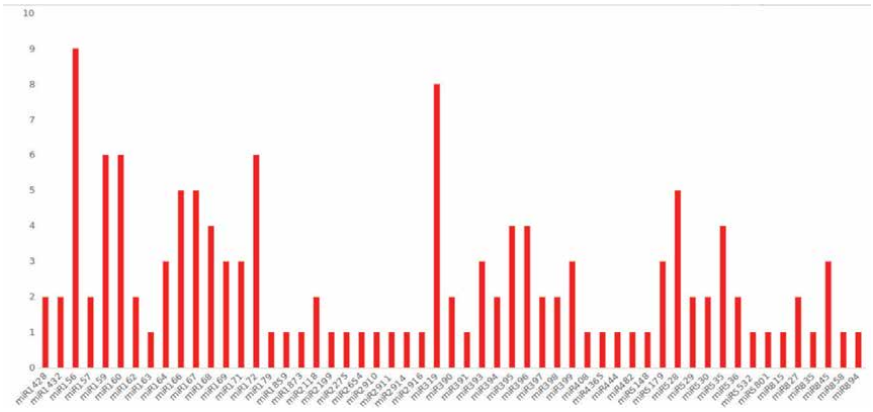

Figure 3.
Families of miRNAs found in oil palm, and the amount of members per family.

5. miRNA-based strategies to improve oil palm tolerance to abiotic stress

In the last decade, due to the recent advances in high-throughput sequencing techniques, vis-à-vis the empowerment of bioinformatics tools, much progress has been made in characterizing and understanding miRNAs mechanism of action, and predicting their target genes [65–68].

The possibility of the involvement of miRNAs in the regulation of genes directly related to the modulation of the impact of abiotic stress on plants has enabled researchers to take the next step by altering the expression levels of miRNAs, either

by overexpression or knocking down strategies. This step attempts to gain further insights that could allow the development of distinct ways to generate superior genotypes harboring tolerance to those stresses, either by vertical or horizontal transfer of genes or even by genome editing [8].

According to Hajyzadeh et al. [69], the overexpression of miR408 in chickpea (*Cicer arietinum* L.) resulted in improved drought tolerance. Plants overexpressing the pre-miR408 from *A. thaliana* were without wilting and drying after 8 days of undergoing the drought treatment, unlike the control plants [69]. Arabidopsis plants overexpressing miR408 showed resilience to multi-stress environments—salinity and drought [70]. Through biochemical analysis and fluorescence imaging with measures of the photosystem efficiency, it was reported tolerance of the transformants compared to control ones [70].

Transgenic lines of creeping grass (*Agrostis stolonifer*a L.) overexpressing rice pre-miR393 showed a better tolerance response to saline stress (at 250 mM for 10 days), drought (15 days), and heat (40°C at day, 35°C at night, 13 days), confirming that miR393 is a potent candidate to confer resistance to multiple stresses [71]. According to Baek and colleagues, the overexpression of miR399f in Arabidopsis promoted greater tolerance to salinity and treatment with abscisic acid (ABA) [72]. Arabidopsis' putative miRNA-target genes—ABF3 and CSP41b—had low expression levels in transgenic plants, and these genes are related to stress due to their participation in ABA signaling [72].

Although the strategy of overexpression of miRNAs is dominant among the studies to obtain plants resistant to different stresses, the inhibition approach is also being promoted through the use of the short tandem target mimic (STTM) approach, causing loss of function [11]. Although there has been an increase in studies on the use of miRNAs to obtain plants resistant to multi-stress, there is still a long way to go.

According to Ferdous and colleagues, the overexpression of Hv-miR827 in barley (*H. vulgare* cv. "Golden Promise") influenced the grain weight and allowed the plants to recover after drought treatment, in addition to providing an improvement in the efficiency of the use of water [73]. Such behavior is also in transgenic Arabidopsis plants overexpressing the miRNA Ath-miR827 [74, 75].

miR319 is one of the oldest and most conserved miRNA families in plants, responsive to several stresses, including drought and salinity, based on high-throughput sequencing [76]. Studies have shown that its overexpression in transgenic creeping grass resulted in tolerance to salt and drought stresses; and it was linked to downregulation of at least four putative target genes of miR319 (AsPCF5, AsPCF6, AsPCF8, and AsTCP14) [77].

So far, no studies are known reporting on successfully horizontally transferring (or editing) miRNAs to or from oil palms aiming at achieving tolerance to abiotic stresses. Our research group prospected stress-responsive miRNAs and putative target genes that are good candidates for such endeavor [15, 64]. miRNAs and their putative target genes responsive to both stresses at once are the priority candidates for further studies.

Among putative target genes responsive to both stresses identified by our group in oil palm, there are some lncRNAs. Several studies have shown that lncRNAs play essential roles and different functions in the biological processes of plants. They can play a role as sRNA precursors to produce sRNAs, such as miRNAs and siRNAs. In response to different stresses, lncRNAs also play a role in the RNA-directed DNA methylation (RdDM) pathway [78–80].

6. Conclusions and future perspectives

Due to a series of unsustainable practices used by the oil palm industry, there is criticism pressuring it to adopt new and innovative procedures, including getting away from the tropical rain forests.

Predictably, primary stresses (abiotic and biotic) will arise once the industry establishes itself in those new areas for oil palm cultivation, forcing it to search for tools to deal in an economically sustainable way with them. Salinity and drought are on the top of such a list of primary abiotic stresses. Many studies report successful attempts to increase plants' tolerance to such stresses by altering the expression levels of miRNAs, either by overexpression or knocking down strategies. Despite the great economic importance of oil palm, we found only 10 published studies reporting miRNAs identification and characterization in this oilseed crop. When considering abiotic stresses, the scenario is even worst; only one published study so far. In this sense, it is clear that further studies are necessary on the role of miRNAs - and their putative target genes - in oil palm's response to abiotic stress. Besides that, as candidate genes become available from those studies, it will be necessary to validate their potential as tools to generate superior genotypes harboring tolerance to those stresses, either by vertical or horizontal transfer of genes or even by genome editing.

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Conflict of interest

The authors declare no conflict of interest.

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The discovery of noncoding RNAs (ncRNAs) is changed our understanding of genome, transcriptome, and interactions with proteome in a cell. Since the discovery of the first microRNA (miRNA) called lin-4 on Caenorhabditis elegans in 1993, our understanding of gene expression has deepened. Further miRNA discoveries have led to the investigation of new ncRNA families and their function, biogenesis, and interactions with other types of molecules. Recent developments in bioinformatics, public databases, sequencing technologies, and biochemical techniques have increased our understanding of the biogenesis, function, and interactions of ncRNAs. This book provides an overview of current ncRNA research dealing with human diseases and cancer and plant miRNAs.

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