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Cervical Cancer

Screening, Treatment and Prevention - Universal Protocols for Ultimate Control

Edited by Rajamanickam Rajkumar

CERVICAL CANCER - SCREENING, TREATMENT AND PREVENTION - UNIVERSAL PROTOCOLS FOR ULTIMATE CONTROL

Edited by **Rajamanickam Rajkumar**

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Notice

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Meet the editor

Rajamanickam Rajkumar is a Professor of Community Medicine and PhD guide at Meenakshi Medical College, Kanchipuram, Tamil Nadu, India. He won a meritorious gold medal for his research on Leprosy Eradication in India, during his MD, which he passed with distinction in 1993. With IARC, he initiated a Rural Population-Based Cancer Registry, in 1996. He was honored with a PhD degree, from Open University of Colombo, for this pioneer work. He received training in Colposcopy and Pre-cancer management, in the UK, Ireland, and Singapore. In 2001, he implemented a large-scale Cervical Cancer screening program, using the village-level health workers/nurses and VIA screening technology. This project of IARC was a great success. In 2011, in collaboration with the Society for Colposcopy and Cervical Pathology, Singapore, and the Ohio State University Medical Center, USA, he formed a society for training doctors and nurses in cervical cancer prevention. In 2012, he received the "Best Teacher and Research Award" from Meenakshi Academy of Higher Education and Research, MAHER, Chennai, India.

In 2016, he formed a network of medical and nursing colleges to undertake cervical cancer and HPV screening programs, among the most underserved and unreached poor women of rural India. From 2017, he has been involved in Primordial Prevention of HPV and guiding PhD research, in Cancer Prevention, at national and international universities. He invites international collaborations in Cervical Cancer Prevention and HPV vaccines and research. For 2018, he has planned for cohort studies to find out the recurrence rate and incidence of invasive cancers in CIN-treated women; this will all be an important study in the field of cervical cancer prevention and control.

Contents

Preface

Cervical cancer is widely known, highly prevalent, largely targeted, successfully prevented, effectively screened, efficiently treated, and eligible for control.

The sheet anchor for the success of cervical cancer prevention and control programs is "screening." However, in most of the programs the participation rate of women for screening is low because of the following reasons. Lack of awareness in the community should be overcome by well-planned, structured, organized, and focused health education programs. "Availability" is ensured by organizing community-based screening camps. "Affordability" should be taken care of by government and non-government sources. "Accessibility" is ensured by arranging camps in local health posts. "Acceptability" is made possible by health care providers who are local, well-trained women.

Other issues, such as precancer treatment with doubtful efficiency, should be subjected to intensive research. Prevention becomes costly with the inclusion of *human papillomavirus* vaccine and this should be addressed by health care planners.

Hence, this book: *Cervical Cancer—Screening, Treatment and Prevention—Universal Protocols for Ultimate Control*.

Authors from different countries have given their valuable contributions, explaining the status of cervical cancer screening in limited resource settings.

The InTech publisher has completed yet another mission along with the authors and Editor in striving towards their universal vision of cervical cancer control.

We sincerely hope that our mission and vision are achieved and women worldwide will have a better quality of life.

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Section 1

Introductory Chapter

Chapter 1

Introductory Chapter: Cervical Cancer - Screening, Treatment and Prevention

Rajamanickam Rajkumar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76907

1. Introduction

This book covers the above topics in a nutshell. The authors from various countries have contributed valuable topics, enriching the contents scientifically and socially. This introductory chapter gives the important and updated details of the topics covered in the book.

2. The uterus

The uterus, anatomically, is a pear-shaped organ, placed between urinary bladder and rectum. The etymology of 'cervix' is that it is from Latin, meaning 'neck' and it opens into the vagina. The invasive cancer occurs in the cervix and is called cervical cancer (**Figure 1**).

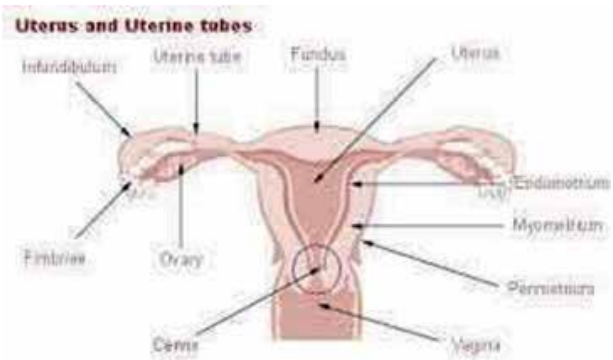

Figure 1. The anatomy of uterus.

3. Natural history of cervical cancer

3.1. Histology

Squamous epithelium and columnar epithelium are both types of epithelium lining in the surface of the cervix.

The squamocolumnar junction is the junction between squamous epithelium and columnar epithelium and it migrates from the periphery of the ectocervix inward towards the external os and finally to the distal cervical canal when age increases.

The process by which the columnar epithelium is replaced by stratified squamous epithelium is termed as squamous metaplasia and the area where this transformation takes place is referred to as the transformation zone (IARC, 2005; WHO, 2006).

3.2. The development of cervical cancer

The cervix is protected by stratified squamous cell epithelium from injuries by toxins and from infections. The human papilloma virus (HPV) primarily targets the squamous cells, and persistent infection by the high-risk strains leads to change of cells to metaplasia and dysplasia, which is the precancer stage and this occurs in the transformation zone—TZ.

3.3. The HPV epidemiology: HPV: The causal factor

HPV16 and 18 are responsible for the development of all the precancers and invasive cancers of the uterine cervix.

HPV types:

High-risk 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59;

Low-risk 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81.

3.4. HPV transmission

HPV transmission occurs through skin and mucous contact during sexual contact, and the cofactors are early sexual exposures and multiple partners.

Persistent HPV infections cause cervical cancers but most of the HPV infections are transient due to the protection from cell-mediated immunity.

4. Classification of precancers and invasive cancers

1. Low-grade squamous intraepithelial lesion (LSIL):
   occurs due to persistent HPV infection;
   cervical intraepithelial neoplasia grade 1 (CIN1);

mild squamous dysplasia;
flat condyloma; koilocytotic atypia; koilocytosis.

2. High-grade squamous intraepithelial lesion (HSIL):
a squamous lesion with high risk of developing into cancer;
cervical intraepithelial neoplasia grade 2 (CIN2);
cervical intraepithelial neoplasia grade 3 (CIN3);
carcinoma in situ (CIS).

3. Squamous cell carcinoma (SCC):

an invasive epithelial tumour composed of squamous cells of varying degrees of differentiation.

4.1. Associated risk factors

Cervical cancer begins with abnormal changes in the cervical tissue. The risk of developing these abnormal changes has been associated with the following factors:
relationship to sexual intercourse;
many partners during lifetime;
frequent intercourse;
early onset of sexual activity;
first pregnancy in teenage years;
multiparity (several children) by mid 20s;
venereal diseases;
genital herpes (herpes simplex virus type 2—HSV-2);
human papilloma virus (HPV);
race: incidence higher in blacks and Hispanics;
low socioeconomic status;
poor genital hygiene;
cigarette smoking;
peak incidence over 40 years.

4.2. Signs and symptoms

post-coital or unexplained vaginal spotting or bleeding;
persistent vaginal discharge;
pelvic pain.

4.3. Five-year survival rates

Adenocarcinomas of the cervix have a worse prognosis than squamous cell cancers.

Squamous cell carcinoma, adenocarcinoma:
Stage 0 = 100%;
Stage I = 60–85%;
Stage II = 40–60%;

Stage III = up to 40%;
Stage IV = <15%.

5. Treatment of cervical intraepithelial neoplasia

Ablation: cryotherapy, laser ablation.
Excision: loop electro excision procedure—LEEP, laser conisation, or cold knife conisation —CKC.

Success rate of all the above modalities is 80–100%.

6. Efforts t o prevent HPV infection

6.1. HPV vaccination

GARDASIL is a quadrivalent vaccine against HPV types 6, 11, 16 and 18 and is given in a three-dose schedule.

CERVARIX is a bivalent vaccine against HPV types 16 and 18 for the prevention of CIN and cervical cancer in females aged 10–25 years.

The efficacy of these vaccines ranges from 0 to 80%.

7. Cervical cancer: prevention and control

7.1. The three-tier system of primary, secondary and tertiary prevention

7.1.1. Primary prevention

HPV infection is the causal factor and it can be prevented by Health Education and Vaccination.

Health education:

genital and menstrual hygiene;

stop tobacco use;

encourage male circumcision;

condom promotion;

safe sex;

Prophylactic HPV vaccines for girls before sexual life exposure;

Two-dose vaccine.

The WHO recommends two-dose vaccine (given at 0 and 6 month or 0 and 12 month) for those starting vaccine before 15 years of age.

7.1.2. Secondary prevention

7.1.2.1. Screening

Screening is a process in which the apparently normal population is subjected to a rapidly applied test to detect an abnormality or a disease condition.

7.1.2.2. Cytology screening

Pap smear screening of women from the age of 25 years can be implemented in the population and the resources need to be planned well to ensure success.

7.1.2.3. HPV testing

HPV testing is a highly sensitive test, but is costly and resource intensive.

7.1.2.4. Visual screening

The most successful and cost-effective methods are as follows:

1. visual inspection with acetic acid (VIA);

2. magnified visual inspection with acetic acid (VIAM);

3. visual inspection with Lugol's iodine (VILI).

7.1.2.5. Colposcopy

Colposcopy is very useful in visual inspection positive lesions to make colposcopic diagnosis, apply a directed biopsy and in guidance of LEEP.

Screen and treat policy for low-resource settings:

Most suited strategy for limited resource settings. A single visit approach has resulted in reduction of incidence rate and mortality rate due to cervical cancer in many countries.

Modalities:

VIA positive—cryotherapy;

VIA positive—colposcopy positive—cryotherapy;

VIA positive—colposcopy positive—biopsy taken—cryotherapy;

VIA positive—colposcopy positive—biopsy taken—biopsy positive—recall for treatment.

In most research settings and in some programmatic settings (e.g., mostly in Asia in countries such as India, Bangladesh and Nepal), colposcopy is used for triaging VIA positives in screen and treat policy.

Criteria to provide cryotherapy:

1. less than 75% of TZ is involved;
2. lesion does not extend to endocervical canal or vagina;
3. no extension of the lesion onto the vaginal walls;
4. lesion adequately covered by cryoprobe;
5. entire squamocolumnar junction is visible;
6. no doubt of invasive cancer.

LEEP:

Ideal to treat CIN 3 lesions and large lesions.

7.1.3. Tertiary prevention

The diagnosis and management of invasive cervical cancer is called tertiary prevention.

7.1.4. Carcinoma of the cervix uteri management according to FIGO staging system

7.1.4.1. Stage description standard treatment

Stage 0: Carcinoma in situ, preinvasive carcinoma.

LEEP, conisation.

Stage I: Invasive carcinoma strictly confined to the cervix.

Stage IA: Invasive carcinoma identified microscopically (all macroscopically visible lesions, even with superficial invasion, should be assigned to stage IB):

Stage IA1: Measured invasion of stroma 3.0 mm or less in depth and 7.0 mm or less in horizontal spread;

Simple hysterectomy or trachelectomy, conisation in selected cases.

Stage IA2: Measured invasion of stroma more than 3.0 mm but not greater than 5.0 mm in depth and 7.0 mm or less in horizontal spread;

Simple or radical hysterectomy and bilateral pelvic lymphadenectomy (or trachelectomy and pelvic lymphadenectomy) depending on local or regional guidelines.

Stage IB: Clinically visible lesion confined to cervix or microscopic lesion greater than stage IA2:

Stage IB1 Clinical lesions of 4.0 cm or less in size;

Radical hysterectomy and bilateral pelvic lymphadenectomy or radiotherapy (or trachelectomy and pelvic lymphadenectomy).

Stage IB2 Clinical lesions more than 4.0 cm in size;

Chemoradiation or radical hysterectomy and bilateral pelvic lymphadenectomy +/− adjuvant radiotherapy or chemoradiation.

Stage II: Carcinoma extending beyond cervix but not to pelvic sidewall; carcinoma involves vagina but not its lower third.

Stage IIA: No parametrial involvement

Chemoradiation or radical hysterectomy and bilateral pelvic lymphadenectomy in selected patients +/− adjuvant radiotherapy or chemoradiation

Stage IIB: Parametrial involvement

Chemoradiation or radical hysterectomy and bilateral pelvic lymphadenectomy in selected patients +/− adjuvant radiotherapy or chemoradiation

Stage III: Carcinoma extending onto pelvic wall; the tumour involves lower third of the vagina. All patients with hydronephrosis or nonfunctioning kidney are included unless known to be result of other causes.

Stage IIIA: Involvement of lower third of the vagina; no extension of pelvic sidewall.

Stage IIIB: Extension to pelvic sidewall and/or hydronephrosis or nonfunctioning kidney.

Chemoradiation or radiotherapy

Stage IV: Carcinoma extends beyond true pelvic or clinically involves mucosa of bladder or rectum. Bullous oedema does not allow a case to be designated as stage IV.

Stage IVA: Spread of growth to adjacent organs:

Chemoradiation or radiotherapy.

Stage IVB: Spread to distant organs:

Palliative chemotherapy or radiotherapy.

Sources: (Benedet, 2000; FIGO, 2009).

8. Conclusion

Cervical cancer, though a highly prevalent cancer, is largely and effectively preventable and treatable. The great advances in science and sociology well contribute towards the global crusade to eliminate cervical cancer, especially among the underserved and unreached poor women in the world. The InTech publishers, editor and authors, dedicate this book towards this noble mission (**Appendix A** and **B**).

Acknowledgements

The author-editor places on record high appreciation and gratefulness to Ms. Marina Dusevic, the Author Service Manager, IntechOpen, for her meticulous care and highly efficient technical guidance, in bringing out this book in a very successful manner. Gratefully, I acknowledge the warmth, love, care and efficient assistance of my daughter Dr. R. Rijula Raj MPT and my son Er. R. Rixon Raj during the edition of this book, I thankfully acknowledge Er. S. Pavithraj for his expert computer assistance. My highest regards to R. Celin Rani for her limitless love, care and support. My deep sense of gratitude and thanks to the IntechOpen publishers, for collaborating with me for fourth book, in succession. May all our efforts bring goodness to the poorest of the poor in the world, by prevention of diseases and promotion of health, true to the wish of God and man.

Appendix

Samples of Health education materials as seen in www.

Appendix A. Cervical cancer—health education pamphlet.

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Appendix B. Cervical cancer—clinical information banner.

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Cervical Cancer

Chapter 2

Microenvironment in Vagina as a Key-Player on Cervical Cancer: Interaction of Polymorphic Genetic Variants and Vaginal Microbiome as Co-Factors

Andreia Matos, Alda Pereira da Silva, Rui Medeiros, Manuel Bicho and Maria Clara Bicho

Additional information is available at the end of the chapter

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Abstract

Current knowledge point to persistence of risk factors for the development of cervical intraepithelial neoplasia. The infection with a high-risk oncogenic Human Papillomavirus (HPV) subtypes, most commonly 16 and 18, is a necessary, although not sufficient, condition for development of invasive cervical cancer (ICC) and its precancerous precursor, cervical intra-epithelial neoplasia (CIN). It has been suggested that CIN disease severity and the diversity of vaginal microbiota are associated and this may determine viral persistence and disease behaviour. Our work focuses on the genetic variability associated to the modulation of genotoxicity induced by vaginal microbiota diversity. Relatively little is known about the mechanisms associated with clearance or persistence of HPV infection, therefore we hypothesized that may be under the influence of the genetic background.

Keywords: factors of persistence, genetic variation, microbiome, onco-microbiota, cervical cancer

1. Introduction

The vaginal microenvironment plays an important role in reproductive health. Human microbiome research has shown commensal bacteria to be a major factor in both wellness and disease pathogenesis. Interest in the microbiome has recently expanded beyond the gut to include a multitude of other organ systems for which the microbiome may have health implications. Here, we review the role of the vaginal microbiome in health and disease, with a particular focus on gynecologic malignancies, specifically cervical cancer. Further research is

required to understand the molecular mechanisms involved in the complex role that bacterial communities can play in the development of cancer.

Cervical cancer is one of the most preventable cancers. However, its progression and above all, the progress towards prevention is often frustrating. Moreover, and despite the continuously growing body of knowledge, the role of factors that affect the human papillomavirus (HPV) persistence are not yet fully understood.

Indeed, the oncogenic HPVs are a necessary cause of cervical cancer; however, they are not a sufficient cause, being other cofactors implicated in the increase of risk. We have also to consider external factors to the host, such as smoking habits, nutritional and behavioural factors (number of partners and their characteristics, age at onset of sexual activity), hormonal therapies-sexual steroids (oral contraceptives and post-menopausal substitution therapy), herpes simplex infections, *Chlamydia trachomatis* or other sexually transmitted infectious diseases and also nonspecific inflammatory diseases. Genetic and immunological factors and other endogenous co-factors may induce initiation and progression associated with genotoxicity, mutagenicity and irreversible cell proliferation [1, 2].

Dysbiosis results from the disruption of equilibrium of the microbiome. Given that the vaginal microbiome composition has been shown to play a role in the HPV infection and the rate of HPV clearance, the vaginal microbiome structure may be associated with the development of cervical cancer secondary to a persistent HPV infection.

Nevertheless, recent and concise data show that composition of the early-life microbiota is critical in the development of the immune system, and how deviations from homeostasis can induce disease later in life [3].

Our group has been presenting data that reflects mainly the influence of genetic, epigenetic and environmental including the vaginal microbiota-derived factors in the natural history of HPV associated lesions leading to cervical cancer as a multifactorial disease process.

In this scenario, the microbiota and its genome (microbiome) fulfils part of the natural history of cervical cancer. In the last years, it has been characterized HPV-genotypes profile, and bacterial vaginosis (BV) leading to its association with the prevalence of HSIL and progression to invasive cervical cancer (ICC) in adult women.

Despite the risk factors status knowledge, we may consider the need of a more proactive behaviour, namely, a strategy for improving the local fora with topic therapy. In this chapter, we will focus in the role of genetic susceptibility associated to the development of cervical cancer. Furthermore, we will discuss opportunities for interventions that modify the microbiome for therapeutic purpose.

2. Factors of persistence

2.1. Vaginal microbiota, HPV and co-infections

More than ever, the association of a disrupted microbiota and the increasing incidence of chronic human diseases have been addressed [4]. Locally, the microbiota affects the functions

and regulates the immunity of epithelial barrier. Therefore, the vaginal microbiome plays an essential role not only in health and dysbiosis, but also in modulation of immune response and, possible, in the carcinogenic process. Additionally, the persistence of risk factors, namely, HPV and other co-infections, may be associated to the disruption of these barriers [5].

The carcinogenic process in cervical cancer results in systemic and persistent damages, with important changes in immune checkpoints of the involved microenvironment [6]. From the key-players involved in this process, the microbiota influences, locally, physiological functions from the maintenance of barrier homeostasis to the regulation of metabolism, hematopoiesis, inflammation, immunity and other functions systematically [4, 7]. This barrier is supported by immune cells, for example, B cells, which produces IgA that helps to neutralize pathogenic bacteria (**Figure 1**) [8]. When this barrier locally fails it is created a favourable environment for carcinogenesis, the dysregulation of the integrity of vaginal epithelial cells will lead to more susceptibility for infections, causing low-grade chronic inflammation that leads to disease.

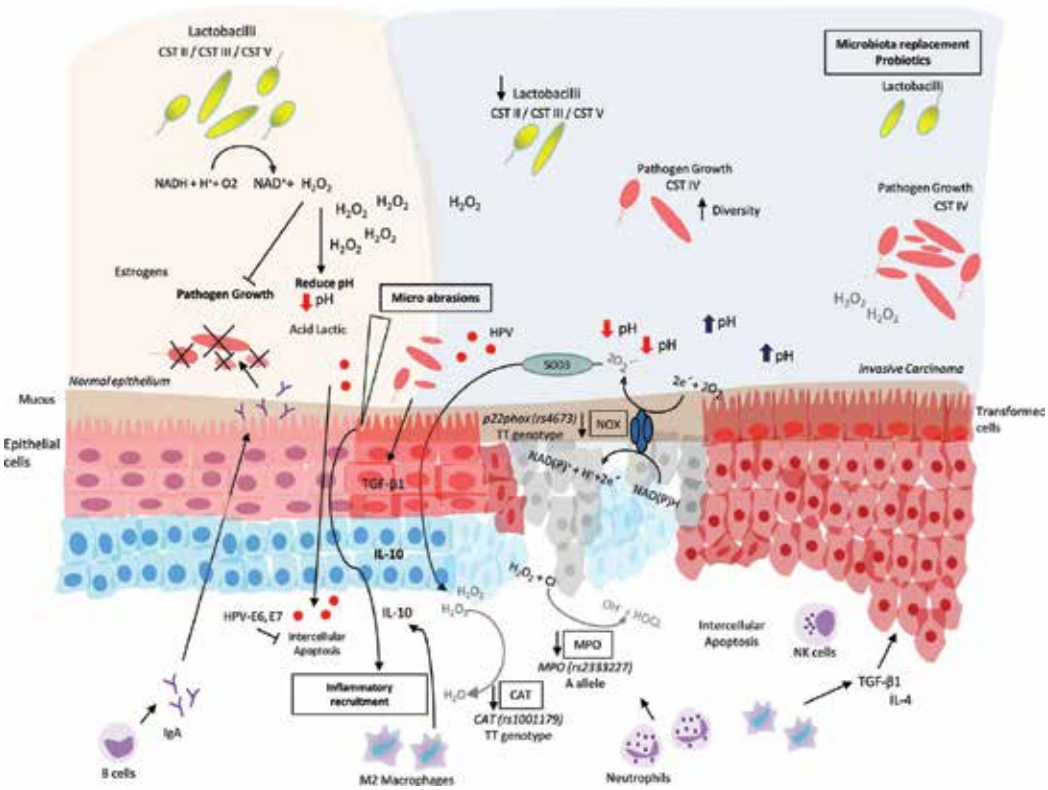

Figure 1. The presence of certain types of lactobacillus lowers pH and induces H_2O_2, which may contribute to the formation of HOCL by myeloperoxidase. CSTs IV are associated to bacterial vaginosis and the other microorganisms, to pre-cancerous lesions and cervical cancer. The types of groups of bacillus may be preponderant for maintaining the vaginal balance. Four species are most important for the balance of the vagina ecosystem: *L. gasseri* (II) *L. crispatus* (I), *L. jensenii* (V) and *L. iners* (III). The inter-individual genetic polymorphic variations should be integrated in a complex model, since a compromise vaginal microflora and inefficient genetic profile may contribute to the development of cervical cancer; CSTs, community state types; CSTs I, *Lactobacillus crispatus*; CSTs II, *Lactobacillus gasseri*; CSTs III, *Lactobacillus iners*; CSTs IV, *Lactobacillus, Sneathia amnii* and *Fusobacterium*; CSTs V, *Lactobacillus jensenii*.

The collection of microorganisms (or microbiota) populates complex ecosystems where genome is called microbiome and the implications on women health, from conception to the next generation, has been recently discussed [9]. A healthy vaginal microbiome is apparently dominated mainly by Community State Types (CST): *Lactobacillus crispatus* (*CSTs I*), *Lactobacillus gasseri (CSTs II), Lactobacillus iners (CSTs III)* and *Lactobacillus jensenii* (*CSTs V*), which, regulate for instance, the balance of reactive oxygen species (ROS) (**Figure 1**) [7]. Although vaginal dysbiosis presents biological plausibly by influencing host's innate immune response, susceptibility to infection, and the development of cervical disease, the underlying cause is not yet well understood. Nevertheless, greater diversity in the vaginal microbiota was associated in women with HPV-positive with cervical intra-epithelial neoplasia (CIN) [10].

There is a strong relationship between infection with HPV, pre-neoplastic lesions and cervical cancer. Moreover, the prevalence of high-risk HPV genital infection (HSIL) and cervical cancer in adult women has been documented. Therefore, the determination of this specific environment correlated with demographic, behavioural and clinical parameters will contribute to a better knowledge of key-players that triggers the carcinogenic pathways in cervical cancer. Other different risk factors including early age at first intercourse, multiple sex partners and low socioeconomic status also have significant role in disease initiation [11].

The HPV infects only epithelial cells, firstly, throughout the basal layer of the epithelium, probably via microabrasions in the epithelial surface, then the viral DNA is released from the capsid and transported into the nucleus as free genetic material or extrachromosomal episomes (**Figure 1**) [12]. Environmental factors might locally influence the initiation of this invasion, among other co-factors, HPV allows potential tumour cells to escape from lactobacilli-mediated control and interfering with intracellular induction of apoptosis [7].

Recently, changes from pro-inflammatory to anti-inflammatory signals - the cytokine milieu - may affect whether or not an infection is cleared [13] and hypothetically an environment favourable or not for tumour growth, might follow a change in regulation of the expression of pro-inflammatory cytokines. After infection takes place, the microbiome changes and its diversity increases. HPV proteins E2, E6 and E7 enhance IL-10 expression secondary to macrophages type 2 presence. These latter are also enhanced in its activity by TGFβ-1 in cytokines expression and its phagocytic efficacy, which is in turn stimulated by the microbiota present. The increase of diversity in the microbiota, through its toxins (FadA from *Fusobacterium* spp.) will promote a metastasis phenotype similar to what happens in cervical cancer [13].

Moreover, polymorphic genetic variants used as surrogate markers might explain the inter-individual variations and the differential immune response causing the persistence and the progression of HPV effects. We had studied some polymorphisms that are associated with genetic susceptibility to cervical infection and increase for risk of acquiring and transmitting HPV infection. These polymorphisms are involved in several pathways, throughout the production of metabolites or other carcinogenic substances, by increasing the susceptibility of the inflamed epithelium or by changing the immune system equilibrium (**Figure 1**).

2.2. Lactobacilli enhance reactive oxygen species and the role of genetic susceptibility in the vaginal microbiome

The ROS, comprise a group of oxygen derivatives from distinct oxidation status of O_2, such as, superoxide radical anion ($O_2^{\cdot-}$), and hydroxyl free-radical (OH^-) and as well as non-radical forms, namely H_2O_2 [14]. The latter has a role as a second messenger molecule in signalling cascades that regulates gene expression and fundamental cellular processes such as proliferation, differentiation and migration [15]. Lactobacilli's H_2O_2 production in the absence of peroxidase may result in toxic concentrations of H_2O_2 and cause damage of the mucosa [16]. The presence of peroxidases guarantees the generating of hypochlorous acid (HOCL) in the vagina inducing a steady removal of excess of H_2O_2 and generation of HOCL [17].

H_2O_2-producing lactobacilli strains, use a NADH oxidase that directly generates H_2O_2 in a two-electron reduction of O_2 (**Figure 1**). Klebanoff et al. proposed that hydrogen peroxide H_2O_2, product of lactobacilli and peroxidase, in the vagina of healthy women might be responsible for the prevention of vaginosis and also might exert an antitumour effect [18]. The antimicrobial effect of H_2O_2-generating lactobacilli is efficiently enhanced in the presence of peroxidases (such as myeloperoxidase and eosinophil peroxidase) and halides [18]. This points to a role of HOCL as superior antimicrobial compound. The vaginal fluid of the majority of healthy women contains sufficiently high concentration of peroxidase to allow biologically significant HOCL synthesis in the presence of H_2O_2-generating lactobacilli [18].

Bauer proposed that peroxidase, which converts H_2O_2 into HOCL, is responsible for creating a microbicial vaginal milieu by maintaining a balanced, non-toxic [18]. The papers of Bauer had highlighted the role of lactobacilli in the vaginal flora of healthy premenopausal women pointing to the beneficial effects for the predominance of microorganisms. Lactobacilli adhere to epithelial cells and thus cause sterile prevention of cell infections with undesirable microorganisms. Lactobacilli cause low pH through production of lactate and also release bactericidal compounds (**Figure 1**) [7]. Others ROS are the highly reactive and toxic by-products of oxygen metabolism, which can damage bacterial nucleic acids, proteins and cell membranes [19].

Recent work of Kruger and Baeur 2017, confirms that the lactobacillus-derived H_2O_2 per se is not likely to be beneficial for the vaginal epithelium, because it causes nonselective lesions in nontransformed as well as transformed cells. The combination of lactobacillus and peroxidase is more favourable. Moreover, the lactobacilli in this system can be completely mimicked in vitro by H_2O_2 generated by glucose oxidase, indicating that its contribution for potential tumour prevention is fully explained by bacterial generation of H_2O_2 [17].

This idyllic scenario is considered for normal cells or untransformed cells, which have a wide antioxidant regulatory defence system that serves to prevent the oxidative stress and the development of neoplasms [20]. Nevertheless, the papillomavirus infected cells (in particular by oncogenic types HPV 16, 33, 31) are resistant to this pathway of apoptosis induction. In transformed cells caused by damages induced by HPV, cells lose control of senescence and p53 activity is abrogated [21].

The combination of the host genome and microbiome increases genetic variation and phenotypic plasticity, enabling the holobiont to increase its overall fitness [22]. Genome-wide association studies identified cervical cancer susceptibility variants across different populations [23]. Therefore, the input of these and other polymorphic variants, may reflect the interindividuality of response in women with cervical cancer. In this chapter, we will focus on some these polymorphisms involved in the modulation of ROS production.

2.2.1. NAD(P)H oxidase (NOX)

The production of $O_2^{\cdot-}$ through NAD(P)H oxidase (NOX) by transformed cells or cervical cancer cells, can be specifically targeted with production of OH^- that induces apoptosis of these cells. The spontaneous dismutation of superoxide anions produce H_2O_2, at low pH, causes mutagenic effects that initiate malignant transformation (**Figure 1**). The high local concentrations of ROS through expression of SOD and catalase, it has also the potential to prevent elimination of transformed cells trough ROS/Reactive Nitrogen Species (RNS)-dependent intercellular apoptosis-inducing signalling [14].

The changes in the gut influences the vaginal microbiome, for instance the expression of NOX is modulated by, for example, the presence of *Helicobacter pylori*, which induces an indirect prooxidative mechanism through recruitment of neutrophils and by assembling of their NOX2 components to the cell membrane [1, 24].

NOX are membrane-associated oligomeric proteins that produce $O_2\cdot$ for host defence and other functions. Generation of extracellular $O_2\cdot$ through NOX is associated with oncogene activation and seems to be required for the control of cell proliferation and maintenance of the transformed state [25]. This protein consists of among other peptides by a regulatory 22-kDa α- subunit (p22phox) and a 91-kDa catalytic β-subunit (gp91phox). The p22phox protein is the NOX element responsible for the regulation of electron transfer to gp91phox [26]. The *p22phox* (*CYBA* gene) polymorphism with rs4673 (C-242 T) causes a functional non-conservative substitution from histidine-72 to a tyrosine residue that decreases its activity [27] (**Figure 1**). Our previous work unravels the association between *CYBA* polymorphism in women with ICC, having been observed a heterosis phenomenon with a protective profile in ICC [14]. This U type curve reflects, on one hand, the homozygote genotype CC leaded to increased ROS production, mainly H_2O_2 resulting from dismutation of O2·, which in turn results in excessive cell growth; on the other hand, the homozygote genotype TT lowers ROS production, mainly decreasing O2· mediated apoptosis cell capacity, resulting in a higher risk for the development of tumours in both cases [14]. Updated data from our cohort, we found that the TT genotype of *ph22phox* polymorphism was a tendency for increased risk in ICC (**Figure 1**) (OR = 3.57, 95% [0.85–13.48], P = 0.057), being age and smoking habits dependent factors.

Women with cervical cancer will have a lower induction of O2· and, consequently, compromising the dismutation by SOD3 of this apoptotic factor into H_2O_2. The continuous modification of vaginal microbiota throughout depletion of lactobacillus or infections with HPV, contribute to increase of pH, influencing the concentrations of ROS in vaginal milieu. Moreover, women with TT genotype of *p22phox* polymorphisms will have a worse response to these important modifications (**Figure 1**).

2.2.2. Catalase

The NOX and catalase (CAT) proteins work in sequence in a metabolic pathway. Transformed cells, spontaneous and enzymatic dismutated O_2^- into H_2O_2 by SOD occurs at right density to allow optimal velocity of the ROS interactions [28]. The CAT is a heme enzyme that plays a predominant role in controlling H_2O_2 and O2· protecting in this way cells from deleterious effects of oxidative stress. In healthy women, this protector effect rises from the conversion of H_2O_2 into H_2O and O_2, but in cervical cancer transformed cells, the ROS signalling is inhibited by a membrane associated catalase and causing control system failure that ultimately results in cell apoptosis failure [26, 29]. Notwithstanding, women with a genetic variant of *CAT* associated with a decrease activity will not contribute to this control system (**Figure 1**).

In humans, the *CAT* gene is located on chromosome 11p13 and its rs1001179 polymorphism (C-262 T) is located on the promoter region and influences transcription and consequent expression of this enzyme and hence the oxidative status of cells and its microenvironment [30]. In a case–control study, we observed a greater risk for developing ICC associated with the homozygote genotype TT of *CAT* polymorphism C-262 T polymorphism of the CAT gene (OR = 3.03, 95% CI 1.46–6.29, P = 0.003) [31]. Similarly to other cancers types, the T variant of this polymorphism is associated to a decreased enzyme activity, generating high levels of ROS [32–34]. The interaction of CC genotype of *p22phox* polymorphism and the TT genotype of *CAT* leads to a higher risk for ICC (OR = 3.95, 95% CI 1.07–14.52, P = 0.032) [31].

Moreover, recent reports have suggested a connection between oestrogen exposure, CAT activity and polymorphism in breast cancer [35]. These findings suggest that CAT genotype modifies the effect of hormone replacement therapy (HRT) use on breast cancer risk and that HRT may affect risk by affecting oxidative stress. This scenario, also might be important in cervical cancer, namely, women with *CAT* TT genotype (associated to a decreased catalase activity), will deficiently protects cells from ROS.

2.2.3. Myeloperoxidase

The oxidative stress conditions are generated by the release of ROS at the infection site by host immune cells such as neutrophils and monocytes. Additionally, resistance of oncogenic papilloma virus-expressing cells to apoptosis induction by the HOCL/hydroxyl anion pathways is likely, as papilloma virus-containing cells are also resistant to intercellular induction of apoptosis [36].

The toxic concentrations of H_2O_2 could be converted by myeloperoxidase (MPO). MPO, a lysosomal enzyme expressed in polymorphonuclear neutrophils, has the potential to kill HPV transformed cells, as a component of an intercellular induced-apoptosis pathway. The MPO was also being pointed as a key-player on controlling of vaginal microenvironment, namely, the H_2O_2-generating *Lactobacillus acidophilus*. In healthy women, this production inhibits the overgrowth of potentially pathogenic organisms; in fact, it can be toxic to other bacteria, fungi, viruses, spermatozoa, or tumour cells [37].

Supposedly, there are no resident neutrophils and macrophages on vaginal microenvironment, nevertheless the persistence of risk co-factors and ROS may lead to the recruitment of

inflammatory cells. The presence of MPO on vaginal milieu is activated. Probably, the persistence of death cells recalls of neutrophils to vagina, being MPO important for the control of excess production of HOCl. In the vaginal microenvironment, the MPO catalyzes the reaction between H_2O_2 and either thiocyanate ions or a halide, such as iodide, bromide or chloride ions, yielding HOCl, which participates in the oxidative burst during the innate host defence [38]. MPO may act in synergy with other proteins. Therefore, an imbalance between oxidants/antioxidants could mean a higher chance for mutations and oncogenesis leading to diseases, including cancer—since MPO produces ROS secondary derivatives can be involved in the neoplastic transformation of cells through this pathway.

H_2O_2, and a halide form a powerful antimicrobial system in phagocytes and tissue fluids, which certain microorganisms can serve as the source of H_2O_2 for this system. The equilibrium of the production of H_2O_2 by *Lactobacilli* in the vagina appears to be a nonspecific host defence mechanism, which can be potentiated by myeloperoxidase that produces HOCl (**Figure 1**).

The production of superoxide through NADPH oxidase from cervical cancer cells, can be specifically targeted with production of OH^- radicals that induces selective apoptosis of these cells [16].

The polymorphism in the *MPO* gene induce a transition G463A (rs2333227), in the promotor region of the gene, where the wild-type G allele promotes the binding of transcription factors leading to a higher transcriptional activity than the A allele [39].

We found that women with the GG genotype had lower risk for cervical cancer than the women who displayed the heterozygous genotype GA (OR = 0.546, 95% CI = 0.315–0.939, P = 0.028, OR = 2.210, 95% CI [1.257–3.886], p = 0.008, respectively). The genotype that leads to a higher concentration of ROS (GG) presents itself as a protection factor in comparison to the homozygous genotype (AA) [39]. Moreover, recently, we observed that the A carriers of MPO polymorphism were about 5-fold of increased risk for cervical cancer (OR = 5.41, 95% CI [2.15–13.64], P < 0.0001) (**Figure 1**), being dependent of age (OR = 3.38, 95% CI [0.85–13.48], P = 0.085) and independent of smoking habits (OR = 3.85, 95% CI [1.33–11.11], P = 0.013). The interaction of HOCl and superoxide of transformed cells will generate apoptosis-inducing hydroxyl radicals.

We suggest that there is an association between the H_2O_2-producing strains found in the vaginal microbial flora and high activity of MPO leading to a clearance of the HPV-infected cells, the relation may also lead to the apoptosis of the transformed cells, producing $O2^{\cdot}$ and $OH^{\cdot}$, acting as a protective factor for a cervical cancer [16, 17, 26].

2.2.4. Reactive nitrogen species

Nitric oxide is generated by nitric oxide synthase (NOS) and presents 3 isoforms: neuronal (NOS1), endothelial (NOS3) and inducible (NOS2). High local concentrations of ROS through expression of SOD and catalase might be associated to prevention of elimination of transformed cells trough ROS/RNS-dependent intercellular apoptosis-inducing signalling [14, 16]. Conversely the excess of NO inhibits the apoptosis induction associated to H_2O_2 and reversely NO-mediated apoptosis induction was inhibited by excess of H_2O_2 [29].

The NOS3 gene is located in the 7q35–36 region of chromosome 7 and the genetic polymorphism with a great clinical relevance is the 27 bp-VNTR 4b/a intron 4 [40]. In addition, NO produced by endothelial and epithelial cells, also modulates the regulation of vascular endothelial growth factor and is possibly associated to increase in processes of invasiveness and metastasis [41]. Preliminary results from our group, although only with a trend, identify that the A variant of NOS3 polymorphism, which is associated with higher activity of this enzyme, predisposes to ICC OR = 7.50, 95%CI [0.88–63.9], P = 0.066).

2.2.5. Catechoestrogens and cytochrome P450 (CYP1A1)

There is a clear association between the excessive and cumulative exposure to oestrogens and the development of cancer in hormone-sensitive tissues, such as the cervix. Therefore, we found that CYP1A1 and Catechol-o-methyltransferase (COMT) work in a metabolic sequence and their interaction could lead to an alternative pathway of oestrogen metabolism with production of 16-OH-estrone that is more proliferative and less apoptotic [42, 43]. The role of oestrogen and the association of *CSTs* favourable for balance vaginal milieu, was previously debated [44].

Aryl hydroxylase (AhR) the transcription factor of CYP1A1 is also associated with immunosuppression after activation of IL-22 pathway, and the maintenance of intraepithelial in innate lymphocytes leading to the mucosal protection from inflammation [45].

Recently, a very interesting work unlighted the mechanism, where the Lactobacillus-derived H_2O_2 suppress host kynurenine metabolism, by inhibiting the expression of the metabolizing enzyme, indoleamine 2,3-dioxygenase (IDO1), in the intestine [46]. Moreover, maintaining elevated kynurenine levels during Lactobacillus supplementation diminished the treatment benefits.

3. Treatment and prevention: microbiota-derived factors

Finally, as suggested by other authors, it may be possible to expand the use of probiotics in the treatment of gynecologic cancers. The study of the role of probiotic bacteria for the prevention of colon and cervical cancer has led to the conclusion the tumour preventive effects of probiotic bacteria might be due to their control of the microbial flora, establishment of beneficial metabolic effects and stimulation of the immune system [37, 47]. Therefore, we can act in the prevention, specifically, the relapses.

Genetic analysis based on single nucleotide polymorphisms identified genetic variants associated with tumour rejection in mice, which could potentially affect ROS production and NK cell activity. That results also supports that B cells play a detrimental role in antitumour immunity and suggest that targeting B cells could enhance the antitumour response and improve the efficacy of therapeutic cancer vaccines [8].

The conventional photon radiotherapy for cervical cancer irradiates parts of the healthy tissue. This treatment perturbs the vaginal microbiome and disrupt the epithelial barrier function, permitting translocation of pathogenic bacteria and causing an inflammatory response [48]. The role of probiotic bacteria for the prevention of colon cancer has led to conclusion of the

tumour preventive potential by the microbes. Additionally, the genetic polymorphism might be related to genetic susceptibility to infections and so, the implementations of probiotics may reinforce the immune system. A better understanding of this line will allow for the development of therapies that can manipulate the microbiome to reinstate homeostasis.

The application of probiotic strains *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 concomitantly with specific anti-infective agents provides more reliable cytological diagnostics, reduces the number of false positive and false negative findings on cervical malignancy and normalizes vaginal microflora in higher percentage of patients with vaginal infections compared with therapy including anti-infective agents only [49].

The use of probiotics, such as L. acidophilus, concomitant with the subsequent use of antibiotics, helps to restore the natural bacteria in the digestive tract that eventually are killed by antibiotics.

Recently, due to changes in the sexual behaviour of the general population, especially in developed countries, there has been an increase in the incidence of HPV infection in other parts of the body, including oropharynx and anus, among others.

In summary, a personalized clinical / therapeutic approach is suggested to avoid unnecessary treatments, based on previous history (onset of sexual activity, number of partners, anovulatory, parity, nutrition, alcohol, tobacco, genetics-immunity, etc.), in vaginal pH, Lactobacillus, in the diagnosis of HPV, viral load, mRNA, HSV, CMV, HSIL, AGC, CINI and CINII / III. *Chlamydia trachomatis, Mycoplasma, Ureaplasma, Neisseria gonorrhoeae*) and in the immunohistochemical study (p16 and Ki-67) of dysplasia [50, 51].

4. Conclusions

The HPV is not a sufficient cause for developing of cervical cancer, therefore other factors may be involved in this susceptibility, namely the microenvironment in vagina and inter-individual genetic polymorphic variations. These variables must be integrated in a complex model that integrates other co-factors, such as, smoking, diet and oral contraceptives. According to this review based on recent data, it seems that a deficiency of an antioxidant mechanism associated to a compromise vaginal microflora and inefficient genetic profile may contribute to the development of cervical cancer. We hypothesis that the genetic background and dysbiosis may contribute to increase risk for gynecologic advanced cancer.

Therefore, the equilibrium of gut/vaginal microbiota and adequate supplementation for a homeostasis of oxidant and antioxidant species may contribute to the regression of the persistence of factors associated with cervical cancer.

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Conflict of interest

The authors declare that they have no competing interests.

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Cervical Cancer Sreening

Chapter 3

Uterine Cervical Cancer Screening

Doris Barboza and Esther Arbona

Additional information is available at the end of the chapter

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Abstract

Cervical cancer is the fourth most common cancer and the third cause of death among women worldwide. More than 85% of the cases occur in developing countries. In Latin America, cervical cancer is the most common cause of cancer deaths among women, primarily in young women with devastating social impact. It is mostly the consequence of lack of a health care infrastructure that allows cervical cancer screening suitable for detecting pre-malignant lesions. With the knowledge that human papillomavirus (HPV) infection is the main cause of cervical cancer, two major preventive interventions have emerged: HPV vaccination and screening, which involve the detection and treatment of cervical dysplasia and early-stage cervical cancer. HPV 16 and 18 cause up to 70% of all cervical cancer cases in Latin America and are covered in all available vaccines. Since tests for high-risk HPV types and HPV vaccines are expensive and they have not been included in immunization programs and given free of charge to eligible women in Venezuela and most less developed regions, screening campaigns with cytology and direct visualization of the cervix with VIN continue to be the major interventions that can prevent cervical cancer in these countries; they need to be implemented in a large scale.

Keywords: cervical cancer screening, human papillomavirus, HPV vaccine, PAP test, Papanicolaou, cytology, acetic acid, oncogenic HPV

1. Introduction

Cervical cancer is a public health problem in adult women in developing countries of South America, Central and Sub-Saharan Africa, meridional and Sub-oriental Asia [1]. It is the fourth most common cancer and the third cause of death among women worldwide [2]. Nine percent (529,800) of new cancer and 8% (275,100) of all cancer deaths in 2008 were caused by cervical cancer. More than 85% of the cases occur in developing countries. Twenty-seven percent (77,100) of all cervical cancer deaths occurred in India, the second most populous country in the world (**Figure 1**).

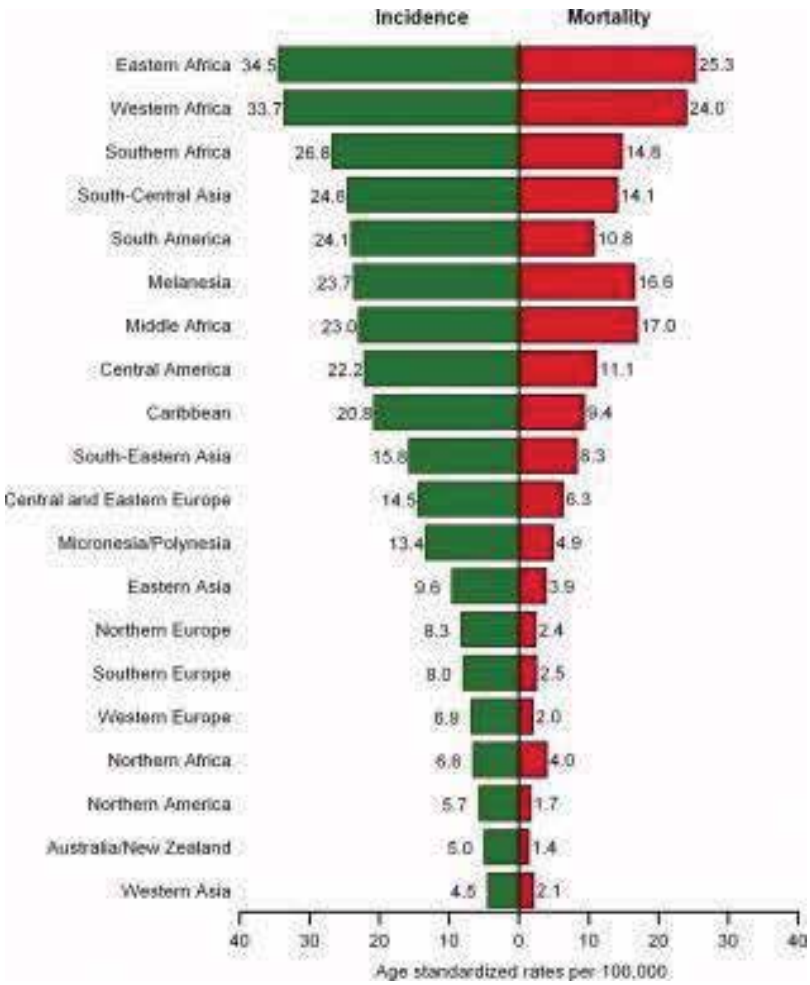

Figure 1. Age-standardized cervical cancer: Incidence and mortality. Rates by world area. GLOBOCAN 2008.

In 2017, the American Cancer Society (Cancer Statistic Center) estimated that there will be 12,820 new cervical cancer patients and 4210 deaths. The incidence rate for cervical cancer from 2009 through 2017 is 7.6 per 100,000 women; the rate of death from 2010 through 2014 is 2.3 per 100,000 women. The incidence and death rates for cervical cancer in Latin America are still high; for example, in Venezuela, the annual average of new cervical cancer cases from 2010 through 2014 was 4019, with a standardized rate on 2014 of 24.88.

In developed countries, most patients are diagnosed in the early stage of the disease or with pre-malignant lesions susceptible to effective treatment. Nevertheless, with the current migratory movement of women, there is an upturn of advanced stage cervical cancer, especially among women who miss their routine gynecologic evaluation or belong to immigrant's groups without suitable medical assistance.

In Latin America, cervical cancer occupies the second position after breast cancer and is the most common cause of cancer deaths among women, primarily in young women. For public

health, the principal importance is that cervical cancer mainly affects young women from low income households, with a devastating impact on them and their families with lot of orphans. Despite it being an easily preventable disease, prevention and screening of cervical cancer are not up to the mark in these regions. If prevention and screening programs do not improve, it is estimated that the annual cases will increase with estimation for 2025 of 126,000 new cases [3].

The highest incidence and mortality of cervical cancer in developing countries and other medically unattended areas is mostly the consequence of lack of a health care infrastructure that allows screening suitable for detecting pre-malignant lesions [4]. The most efficient and profitable screening techniques [5] are cytology-based screening (the Pap test) and HPV DNA screening. A clinical trial in one of India's rural areas with low income households found than 1 round of HPV DNA screening was related with a 50% reduction in probability of developing cervical cancer [6].

Screening programs fail because of substandard quality of pap-smear sampling techniques, methodology errors, limited geographical and population coverage with emphasis on high-risk women, and sub-optimal follow-up.

With the knowledge that HPV infection is the main cause of cervical cancer, two major interventions that can prevent cervical cancer have emerged: HPV vaccination and screening, which involves the detection and treatment of cervical dysplasia and early-stage cervical cancer.

All HPV vaccines currently available cover HPV 16 and 18 that cause up to 70% of all cervical cancer cases in Latin America [7]. Cervarix from GlaxoSmithKline is a bivalent vaccine that covers only HPV 16/18; Gardasil from Merck & Co is a quadrivalent vaccine that covers HPV 16/18 and HPV 6/11, which cause genital warts. Both vaccines prevent primary VPH infection, CNI2 and CNI3 related to HPV 16 and HPV 18, when 3 doses are completed. The 9-valent vaccine (Gardasil 9 from Merck & Co.) covers seven HPV types related to cervical cancer, including HPV 16/18 and HPV 6/11 [8, 9].

HPV vaccine is recommended for girls aged 9–26 years of age to prevent cancers of the cervix, vagina, and vulva related with HPV 16 or 18, or genital warts (HPV 6 or HPV 11), and lesions related with other HPV types, cervical adenocarcinoma in situ, vulvar or vaginal intraepithelial neoplasia [10]. In addition, women must be vaccinated before their first sexual activity, prior to exposure to HPV. HPV vaccination is also recommended for women with weakened immune systems (including people with HIV infection), given their higher risk of having HPV infection.

By mid-2016, 65 countries had introduced HPV vaccines, mostly developing countries, but including an increasing number of middle and low-income countries. Unfortunately, HPV vaccines are expensive and they have not been included in immunization programs and given free of charge to eligible women in Venezuela and most less-developed regions. Thus, screening continues to be the major intervention that can prevent cervical cancer in these countries.

2. Risk factors

Most of the risk factors for developing cervical cancer are associated with a compromised immune response that allows HPV infection, the etiologic agent of nearly all cases of cervical cancer. These factors include the following.

- Early first sexual intercourse; the risk increases if the first sexual activity is before 21 years of age [11, 12], being approximately 1.5% when first sexual activity is at 18–20 years of age and younger.
- Multiple sexual partners.
- High-risk sexual partner, for example, a partner with multiple sexual partners or with HPV infection.
- Squamous vulvar intraepithelial neoplasia or vaginal neoplasia (highly associated with HPV infection) in the past.
- History of sexually transmitted disease (chlamydia, genital herpes) [13, 14].
- Immunosuppression: VIH-positive women have consistently shown to be at an increased risk for high-grade cervical dysplasia. [14]
- Young age at first full-term pregnancy (less than 20 years of age) and high parity are exogenous cofactors associated with an increased risk of cervical carcinoma; these factors are thought to increase the risk through the maintenance of the transformation zone on the exocervix for a prolonged time, which facilitates exposure to HPV [15].
- Low income/socio-economic status is associated with cervical cancer; incidence and mortality are higher in high-poverty communities [16].
- Oral contraceptives: The data analysis of 24 epidemiologic studies [17] found that the risk of cervical cancer increases with increasing duration of oral contraceptive use (5 years or more of using oral contraceptives vs. non-users); the relative risk was 95% and it decreased after use of oral contraceptives has ceased; the same analysis estimated that 10-year use of oral contraceptives that started at 20–30 years of age increases incidence of cervical cancer in middle-age women
- In current smokers, a doubling in risk of developing cervical cancer has been observed, with a positive correlation with the habit intensity; nicotine and smoke derivates from tobacco discovered on cervical mucus suggest a possible biologic mechanism through immunosuppression that favor infectious agent such as HPV; tobacco smoking is associated with squamous cervical cancer [18]
- Some daughters of women, especially young women, who took diethylstilbestrol during pregnancy have developed clear cell adenocarcinoma of the cervix and vagina [19, 20]
- High incidence of cervical cancer is observed in Afro-Americans, Latins, and ethnic groups with low incomes and socio-economic conditions with limited access to effective screening and health system [18]

3. Genetic factors

There is no established model for a genetic base, although population studies have found increased risk in familiar groups. In the past, it was attributed to ambient environmental

exposure and shared risk factors; however, subsequent data comparing sisters and half-sisters far exceed shared environments.

Research has been done to identify genetic alterations that can make women more susceptible to cervical cancer because of less resistance to HPV infection and persistent infection.

To date, results show a large polymorphism diversity in a wide variety of genes, including those regulating immunity and susceptibility [19–21] and generating a large amount of immune mechanism (cytokines production, angiogenesis, tumor suppression pathways, transcription activation) [22–24].

4. Human papillomavirus

The causal role of HPV in all common and non-common histologic types has been firmly established biologically and epidemiologically and has led to a new carcinogenic model for cervical cancer: HPV acquisition, HPV persistence, progression of pre-malignant lesion to invasive cancer [25, 26]. Human papillomavirus is acquired through sexual contact; most population prevalence reaches its peak few years after the median age of initiation of sexual intercourse.

Most HVP infections are transient, lasting no more than 1 or 2 years [27]. Persistent HPV infection for 1–2 years, especially by HPV 16 predicts development of CIN 3 (cervical intraepithelial neoplasia) or malignant changes. The probability of untreated CIN 3 transforming into an invasive cancer is 30%, although 1% of treated CIN 3 transforms into an invasive cancer [28].

There are more than 100 HPV types; high-risk types 16, 18, 31, 35, and 39 are linked to malignant transformation [29]. Type 18 infection progresses with bad prognosis based on recorded survival rates.

High-risk HPV infection may generate some of the following cell biologic alterations leading to malignant transformation. Two of the eight proteins encoded by the HPV genome, E6 and E7, accounts for most carcinogenic effects of high-risk HPV types. They promote carcinogenesis in several ways:

- They interfere with important tumor suppressor pathways; E6 inhibits the p53 tumor suppressor by promoting its proteasomal degradation, while E7 disrupts the retinoblastoma (Rb) pathway [30, 31], or activates oncogenes via EGFR (epidermal growth factor receptor) [32, 33].
- They induce telomerase enzyme activation related with the unlimited potential of neoplastic cells replication [34].
- E6 and E7 abrogate cell cycle checkpoints and induce genomic instability. Both can induce abnormal centrosome numbers and centrosome abnormalities. They also have synergistic effects on centrosome abnormalities and chromosomal instability [35, 36].

Progression of HPV infection to uterine cervical cancer is associated with progressive histologic changes. Cervical intraepithelial neoplasia (CIN) is a histologic change corresponding to dysplasia of cervical squamous epithelium associated with HPV infection and is considered a potential precursor of uterine cervical cancer. They are classified into three grades: CIN grade I, mild dysplasia, or abnormal cell growth confined to the basal 1/3 of the cervical epithelium; CIN grade II, moderate dysplasia confined to the basal 2/3 of the epithelium; and CIN grade 3, severe dysplasia that spans more than 2/3 of the epithelium, and may involve the full thickness.

Historical data demonstrated that the majority (71–90%) of CIN 1 lesions *regress spontaneously* in contrast with persistence and progression rates for CIN 2 and CIN 3, estimated in 57 and 70% respectively [37].

There are mainly four steps implicated in the development of uterine cervical cancer:

1. Oncogenic HPV infection of squamous cells in the transformation zone of the cervix, which is in the union area of the squamous epithelium of the exocervix and the endocervical glandular epithelium.
2. Persistent HPV infection.
3. Progression of persistent HPV epithelial cells infected to a pre-malignant lesion.
4. Development of invasive carcinoma: Tumor cells in the epithelium cross the basement membrane and invade the stroma.

Formal epidemiological evidence of the association between HPV and cervical cancer did not exist until the early 1990s, although molecular characterization of one of the first types of HPV in the 1980s made it possible to develop tests of hybridization to obtain fragments of HPV genes in human tissue. Using hybridization studies based on polymerase chain reaction (PCR), studies have been conducted for the identification of HPV DNA. One of the pioneer studies in Latin America was carried out by the Agency for Research of Cancer, between Colombia and Spain. The results of this study have been considered as the first evidence of the causal association between HPV and cervical cancer. Subsequently, similar studies were carried out in 9 countries (Algeria, Brazil, India, Mali, Morocco, Peru, Paraguay, Thailand, and Philippines) between 1985 and 1988 to evaluate the role of the virus of HPV in the etiology of CIN 3. The DNA was obtained by cytology and was evaluated by Virapap and PCR. In Spain, HPV prevalence based on PCR was detected in 63.2% of the cases and for controls was observed in 47%. In Colombia, HPV DNA was detected in 63.2% of the cases and in 10.5% of the controls. VPH 16 was the most predominant type of virus and showed stronger association with the development of CIN 3. HPV of unknown origin was common in positive cases (18.3% in Spain and 38.0% in Colombia [28]. In 2006, a study was carried out at the gynecologic department of the Padre Machado Hospital, in Venezuela; it included 58 patients with uterine cervical cancer. Typification of human papillomavirus by PCR for types 6, 11, 16, 18, 31,33, and 35 were performed; other variables such as age, stage, and histological type were also analyzed. The purpose of this study was typification of HPV in women with invasive

uterine cervical cancer in Venezuela, identification of the country's most frequent HPV type, and comparison with worldwide incidence of VPH. HPV DNA sequences were associated in 52.3% of the patients, VPH 16 in 24.52%, and HPV 18 in 7.4% of their population. These results suggest the imperative need of large-scale epidemiological studies as these results do not reflect the results reported in other countries [38].

5. Uterine cervical cancer screening

Screening of uterine cervix decreases the incidence and mortality of cervical cancer. Cervical cancer has two main histological types: squamous and adenocarcinoma. Screening can detect precursors and early stage for both types, and treatment of precursors can prevent the development of invasive cancer. Currently, in addition to screening, test for high-risk human papillomavirus types, which form the foundation of uterine cervical cancer pathogenesis, has been included. In view of the high incidence and mortality of cervical cancer, its significance as a global public health problem, and the difficulties involved in establishing effective screening in different regions of the world, the American Society of Oncology (ASCO), in the year 2013 [39], released a world guide for cervical screening and follow-up of positive cases, as well as guidelines for treatment for pre-malignant lesions. The main recommendation was screening for cervical pre-cancers for all women in appropriate age groups and establishing consistent minimum standards for screening considering and based on resource levels and health systems infrastructure.

Based on the results of a large clinical trial in India that demonstrated that cervical cancer screening with acetic acid (vinegar) could prevent thousands of deaths each year in developing countries [39], initial visual inspection with acetic acid (vinegar) was incorporated in the global screening guideline.

Cancer of the cervix is a highly preventable disease; low-income countries lack large-scale screening and vaccination programs against HPV. As a result, more than 85% of the world's cervical cancer diagnoses and deaths occur in less developed regions. Access to programs of detection and treatment of cervical cancer varies not only between countries but also within them. Standards were established in four different areas of health: basic, enhanced, and maximum limited. These levels correspond not only to the financial resources of a country or region, but also the strengths of the health care including personnel, infrastructure, and access to health systems.

ASCO's guideline builds upon WHO's recommendations by providing a minimum set of standards across all countries based on their existing resources, and by accounting for the 2013 VIA study and other recent data. HPV DNA testing is recommended in all resource settings and VIA may be used while HPV testing becomes available. If VIA, as a primary screening, gives abnormal results, women should receive treatment. After a positive HPV DNA testing result, VIA is recommended for follow-up in basic and limited settings. For other settings, HPV genotyping and/or cytology may be used for triage. Women with abnormal triage results should receive immediate treatment in basic and limited settings, or colposcopy in

all other settings. Screening is recommended for women of ages 25–65 years every 5 years and for ages 30–65 years, and if two consecutive tests are negative at 5-year intervals, then every 10 years. In the context of limited setting, screening is recommended for ages 30–49 years, every 10 years and for basic settings, for ages 30–49 years, one or more screens in a lifetime. When a precursor lesion is diagnosed, the recommended treatment includes LEEP, or ablative treatments (cryotherapy, cold coagulation) with a 12-month post-treatment follow-up for all settings. For women who are HIV positive, those who had recently given birth, and those who have undergone a hysterectomy, separate screening recommendations have been provided.

Screening methods include Papanicolaou (PAP) test (cytology) and tests for high-risk human papillomavirus types. Cervical cancer screening detects precancerous lesions in the early stages and their treatment decreases uterine cervical cancer incidence and mortality. In the United States, PAP was adopted in 1950 and in the mid-1980s [40], the incidence of cervical cancer had decreased to 70% [41]. The benefit of screening is that it decreases mortality and the incidence of cancer of the cervix, but information provided by the PAP must be evaluated since infection can be transient and dysplasia can regress spontaneously, especially in young women [42]. Major adverse outcomes of screening are derived for furthers consequence to methods used for treatment of injuries. The effects on the reproductive system include stenosis, loss of pregnancy in the second trimester, premature births, and rupture of the membrane [43].

Most episodes of HPV infection and many cases of CIN 1 and CNI2 are transient and fail to develop into CIN 3 or cancer. Potential problems associated with positive screening tests are stigmatization of a sexually transmitted disease and inconvenience associated with additional diagnostic and treatment procedures [44]. Getting a positive test at any time of life may contribute to the perception that one is at an increased risk of cancer and a desire for more tests with the consequent possibility of another positive test, the monetary costs involving the control procedures after a positive result, and the higher cost, from the health perspective, of developing cancer [45]. Although any false-positive test has the potential to induce anxiety, quality of life test is usually not included in screening trials. As a result, the number of colposcopies related to CIN 3 and cancer has been regulated. Cervical cancer is rare in young women and adolescents and may not be prevented by cytological screening. The incidence has not changed in developed countries, but in low-income countries, it presents in earlier ages [46].

Screening in adolescents leads to an unnecessary evaluation and treatment of lesions with high potential of spontaneous regression with reproductive long-term problems. Cancer prevention programs in adolescent should focus on massive vaccination for HPV [47].

Among the 21 to 29 year-olds, screening is recommended with PAP every 3 years. For women aged 21 to 29, with two or more consecutive negative cytologic findings [48, 49], there is no evidence that supports a greater interval for detection (3 or more years). For women less than 30 years of age, HPV screening is not recommended, given high chance of transient HPV infections. Positive predictive value of these tests limits the usefulness of them as screening methods. Randomized studies have shown that HPV testing for women less than 30 years of age [48–50] results in high detection of transient infections by HPV and the women undergo unnecessary colposcopies [51].

For women older than 30 years, PAP is recommended every 3 years with co-tests (PAP and HPV) every 5 years if both initial tests were negative. For women older than 30 years, HPV infection has a greater chance of being persistent; it also has uncertain clinical significance. Any other determination of HPV test increases the probability of positive results, with largest number of colposcopies with uncertain results [52].

In women older than 65 years, tests are not recommended if they meet the following criteria:

- No risk factors: No history of abnormal test; not a habitual smoker, or currently smoking; no disease related to HPV; not new couples; not immunocompromised; no exposure to diethylstilbestrol in utero
- Optimal screening: Two consecutive negative tests, co-tests, or three PAP tests in the last 20 years, latest during five previous years [53, 54]
- No history of high grade dysplasia or more

There are some clinical conditions where increased risk of developing CIN and cervical cancer are observed, as in human immunodeficiency virus (HIV)-infected women. This conclusion is based on several trials including the study of Wright et al. [55] where the definition of cervical intraepithelial neoplasia (CIN) prevalence, validity of PAP tests, and the association of risk factors in women infected with HPV virus, demonstrated that these patients are more likely to have a persistent infection with the virus, increased rate of high grade cervical dysplasia, and higher risk of developing cervical cancer.

Immunosuppressed women: Patients with immunosuppressive therapy (organ /bone marrow transplants, prolonged treatment with steroids, systemic disease), infected with HIV present greater persistence of infection with minor ability to regress spontaneously and therefore, they have higher rates of cervical dysplasia and cancer. Information about immunosuppressed women are based on the results of screening women with systemic lupus erythematosus (SLE). High grade dysplasia and subtype of high-risk HPV persistence rates are significantly higher in women with SLE who receive immunosuppressive therapy, than immunosuppressed patients treated for other conditions, or patients with minor SLE receiving treatment [56, 57].

At present, for this group of patients, who are immunocompromised or HIV-infected, it is recommended to start screening at age 21, or PAP and HPV tests should be done at the age when participating in the first sexual relationship.

Women with total hysterectomy, no history of CIN or cervical cancer, operated for benign pathologies have a very low risk of developing cervical cancer and need not undergo screening for cancer of the cervix [58, 59]. Women with sub-total hysterectomy probably share the same risks as patients with preserved cervix and must follow the general guidelines. For those women with hysterectomy and a history of CIN 2/3 or adenocarcinoma in situ, if the diagnosis was made prior to surgery or hysterectomy, the ACOG recommends screening at least 20 years after treatment [60]. The most recent summary of recommendations [61] includes the following:

- Start screening no sooner than age 21, regardless of the age of onset of sexual activity or other risk factors. Between 21 and 29 years of age, PAP smear must be done every 3 years. Between 30 and 65 years, co-testing (cytology more than an HPV test) every 5 years is preferable; if not possible, single cytology every 3 years is acceptable. After the age of 65 years, screening can be discontinued if previous screening has been done and found negative and not CIN 2 (+) during the previous 20 years.
- Screening can be discontinued if there is total hysterectomy (with removal of cervix) and a history of CIN 2 (+).

These suggestions are valid for developed countries that allow the implementation of adequate screening campaigns with all the resources available. However, for developing countries with limited resources, cytology and direct visualization of the cervix with VIN are valid methods.

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Chapter 4

Great Role in Gynecological Cancer Prophylaxis of a Unique Health Check-Up Institute, Ningen Dock in Japan (Review)

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Additional information is available at the end of the chapter

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Abstract

In Japan, there are unique facilities (namely Ningen Dock) for health check-up that provide asymptomatic participants with a health examination, including cancer screening activities, at their own expense. The most advanced examination equipment and examinations do not only provide high accuracy, but they also reduce stress on the body of the client. Usage of the medical equipment and diagnostic techniques allows us for successful detection of many diseases in their early stages of development. This early detection leads to quicker response for the disease. On the other hand, gynecological cancer screening is a relatively simple, low cost, and noninvasive method. In this chapter, we introduce a major role of Ningen Dock in gynecological malignancy prophylaxis. Ningen Dock attendances are associated with extremely low positive gynecology cancer screening incidence (0.03%). The level of knowledge and attitude toward screening may be related to multiple factors such as ethnicity, place of residence, income, and social-economic status. Not paying attention to cancer screening may be the risk factors for non-attendance to health check-up. These findings are of importance for improving the gynecological cancer screening practices of the lower screening attendance in Japan.

Keywords: health check-up, Ningen Dock, gynecological cancer, attitude toward screening, cancer screening, cervical cancer

1. Introduction

Health and medical check-ups aim to discover problems that may be harmful to the future health of the examinees, providing proposals for health promotion support solutions. Health

check-ups focus on comprehensive assessments regarding the whole body even without disorders, while medical examinations include a specific disease or organ. In many countries, including Japan, a series of systemic routine health examinations and preventive medicine development in response to client needs undergo on a voluntary basis.

In Japan, there are unique facilities (namely Ningen Dock) for health check-up that provide asymptomatic participants with a health examination, including cancer screening activities, at their own expense [1]. Japan is indeed a country in the world with the most advanced medical devices. For example, about half of the CT scans and about one-third of the MRI scans are owned by medical facilities in Japan [2]. The most advanced examination equipment and examinations do not only provide high accuracy, but they also reduce stress on the body of the client. Usage of the medical equipment and diagnostic techniques allows us for successful detection of many diseases in their early stages of development. This early detection leads to quicker response for the disease.

The "OMOTENASHI" services provided by staffs, including nurses, technologists, and doctors, is supporting the popularity. With the careful client support underpinned by the Japanese culture of hospitality, the Ningen Dock in Japan is popular in neighboring countries. The number of people from another country is rapidly increasing, to visit Japan, to receive the medical services of Ningen Dock. These situations prompted us to introduce a major role of Ningen Dock in gynecological malignancy prophylaxis.

2. Gynecological examination flow

In general, there are three Ningen Dock programs, a half-day course, one-day course, and two-day course. Depending on the selection of the course, different diagnostic and procedural options are available. The cost is not covered by the social insurance. Asymptomatic women, aged from 18 until ~90 undergo medical evaluations, including a medical history, physical examination, blood sampling, urine sampling, and radiological imaging, as part of a routine health check-up and cancer screening (see **Table 1**). The popular plan for women is a gynecological cancer screening. Gynecologic examinations include uterine cytology (Papanicolaou test), transvaginal ultrasonography, and pelvic examination by a gynecologist.

Cervical and endometrial smears are performed using a speculum and/or brush. The cytology findings divided into seven groups: high-grade squamous intraepithelial lesions (HSIL), low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells of undetermined significance (ASC-US), squamous cell carcinoma, atypical glandular cells (AGC), cervical adenocarcinoma, and normal. The cytological findings of endometrium are classified into four categories: suspected endometrial carcinoma, atypical endometrial cell, benign endometrial abnormality, and normal endometrium. When inadequate for classification, smears were again taken from examinees, and their smear samples are retrospectively reviewed if needed.

Abnormal cytologic and/or ultrasonographic findings introduce all examinees to the medical facilities for further managements. Even though no additional information are provided regarding their detailed examination outcomes, the present findings obtained from asymptomatic women may indicate annual gynecologic check-up and adequate follow-up programs

Basic examination (1-day course)	
Life Habits Check	Investigation of lifestyle through medical questionnaire, physical check-up, and advice on how to prevent the development of diseases and how to treat them.
Lungs	Chest X-ray to screening pulmonary disorders such as lung cancer, tuberculosis, and emphysema.
Heart	Screening for high blood pressure and cardiac disorder by electrocardiogram.
Digestive organs	Upper GI tests, abdominal ultrasonography, blood tests, and stool analysis to screen gastrointestinal diseases such as cancer, ulcer, polyp, and dysfunction of liver and pancreas by investigating esophagus, stomach, duodenum, liver, pancreas, and gallbladder.
Eyes	Screening for cataract, glaucoma, and visual change by fundus photography and intraocular pressure measurement.
Breast	X-ray and ultrasonography
Gynecology	Screening for gynecological disorders such as uterine cancer and ovarian tumors through pelvic examination, cervical cytology and ultrasonography. Tumor markers (CA125, CA72-4, CA19-1, and SCC) are optional.
Others	Screening tests for hearing, infections such as hepatitis virus and syphilis and determining blood type.
Optional	
This course is arranged for those who want to take an opportunity to refresh and receive the screening in a more relaxed manner. The courses contain optional examinations that can be added upon the request.	

Table 1. Test items of Ningen dock.

against symptom-free population, and this can cause remarkable reduction in the probability of malignant disease. The study sample is derived from the representative population of high-income and high-attitude toward health maintenance, providing most of our observations as important implications in terms of public health.

If anything abnormal is found, the participants are provided the most appropriate advice, by determining whether follow-up observations would be sufficient, or if medical treatment is required, what kind of medical treatment should be provided, and what facility would be appropriate for a particular treatment.

3. Incidence of positive gynecological cancers in examinees of Ningen Dock

Table 2 shows the cytologic and ultrasonographic findings of all subjects who visited the Ningen Dock in our institute between 2002 and 2016 [3, 4]. Of the cytology from cervix, 140 cases (0.8%)

were found as abnormal. Among them, 127 cases were classified as low-grade cervical smear abnormalities: LSIL and HSIL were seen in 105 cases, ASC-US was seen in 22. Suspected malignancy of squamous cell was detected in five cases within this study period, while case of cervical adenocarcinoma was not found. No cytological abnormality categories were clustered in any specific age group. Endometrial smear showed hyperplasia suspicious in 2.7% cases.

Uterine enlargement was the most frequently detected gynecologic finding, with a peak reaching approximately 25% in 40–49 years age group. The uterine abnormalities had a tendency to decrease in those aged over 60 years. Ovarian tumor (including solid and cystic enlargement) was detected in 5.2–8.0% of those in the age groups of 30–49 years, while those aged over 60 years had less frequency. In 91.3% participants, no gynecologic abnormality was detected.

The abnormal cytologic findings, including dysplastic changes and cervical cancer, are observed to be very low compared with other studies performed in developed countries (3.4–9%) [5–10]. Our findings based on 2011–2016 Ningen Dock records are similar to those of the former observations, and most of participants (95.6%) revealed no gynecological cytology

Age group (years)	No. (%)	Cytology					Uterine tumor and abnormalities	Ovary tumor and abnormalities	Others*
		Cervix				EM			
		LSIL	HSIL	ASC-US	SCC	Other than normal			
<19	12 (<0.1)	1 (<0.1)	0	0	0	0	0	2 (0.1)	0
20–29	794 (4.8)	9 (0.6)	0	5 (0.3)	1 (<0.1)	0	6 (0.4)	18 (1.3)	13 (0.9)
30–39	3172 (19.2)	26 (1.8)	4 (0.3)	3 (0.2)	0	1 (<0.1)	80 (5.6)	74 (5.2)	68 (4.7)
40–49	6217 (37.6)	37 (2.6)	6 (0.4)	3 (0.2)	1 (<0.1)	2 (0.1)	361 (25.2)	114 (8.0)	139 (9.7)
50–59	4615 (27.9)	22 (1.5)	4 (0.3)	9 (0.6)	2 (0.1)	35 (2.4)	164 (11.4)	42 (2.9)	95 (6.6)
60–69	1464 (8.9)	4 (0.3)	0	2 (0.1)	1 (<0.1)	0	44 (30.7)	8 (0.6)	16 (1.1)
70–79	228 (1.4)	0	0	0	0	0	5 (0.3)	1 (<0.1)	6 (0.4)
> 80	18 (<0.1)	0	0	0	0	0	0	0	0
Total 16,520 (100)		99 (0.6)	14 (<0.1)	22 (0.1)	5 (<0.1)	37 (0.2)	660 (4.0)	259 (1.6)	337 (2.0)
						1433 (8.7)			

Between January 2002 and December 2016, 16,520 asymptomatic women, aged 18–85, visited the Ningen Dock in Matsunami General Hospital for their gynecological health check-up. *Including vaginosis, leukoplakie, Bartholin cyst, posthysterectomy, cervical polyp, and prolaps/ptosis. LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; SCC, cervical squamous cell carcinoma; AGC, atypical glandular cells; EM; endometrium. Modified from our previous reports [3, 4].

Table 2. Gynecologic findings of participants distributed by age group.

and ultrasonographic abnormalities. Gynecologic cancer is detected in 0.03%, all of which were at the early stages (so-called CIN3). The very low incident is in good agreement with the primary report in some Ningen Docks [1, 11].

HPV stands for human papilloma virus, which is a group of more than 200 viruses. Most people will get a HPV infection during their lifetime, usually from sexual activity. Most of these infections do not need treatment, but they can cause genital warts. In some, however, HPV infection causes changes in the cervix that can develop into cervical cancer. HPV can infect the cells on the surface of the cervix and damage them, causing their appearance to change and lead to abnormalities in these cells over a number of years. These abnormalities are known as cervical intraepithelial neoplasia (CIN). These changes are classified according to their severity. The mean time between the virus infection and invasive cancer takes about 15 years, and within 2–4 years of detection 15.5–25.5% of low-grade epithelial lesions that become high-grade lesions. In some cases, these more severe changes can develop into cervical cancer. The progression of mild and severe changes to cancer takes many years so these abnormalities are known as precancerous [12–14]. HPV infection is most common in people in their late teens and early 20s [15, 16]. A study in Jordan, one of the most conservative and religious country, found that 0.8% of 1176 women aged 18–70 years are classified as ASC-US and 0.2% as LSIL. In our unique system Ningen Dock in Japan, symptom-free women undergo medical check-up at their own expense. Their educational tradition and high concern on sex-transmitted infection, such as HPV, may restrict the likelihood of multiple sexual partners. This may be the most plausible explanation for extremely low incidence of dysplastic changes and cervical cancer found in our study group of women.

As uterine enlargement, uterine myoma with or without adenomyosis are found in 20–25% of reproductive-age women, indicating that they are one of the most frequent women's lower abdominal tumor [17–19]. The women with myoma do not necessarily complain of symptoms, and even large ones may go undetected by the patient, particularly if she is obese. Myoma-linked symptoms (abdominal distention, vaginal bleeding, constipation, and peritoneal irritation) depend on their location, size, and state of presentation; symptoms are present in 35–50% of patients with myomas. Ovarian tumors, cystic or solid, also seldom cause symptoms. Although the ovarian enlargement is frequently undetected by the patients, the diagnosis of these tumors is not usually difficult by ultrasonographic examination at physical check-up. Our subjects showed lower frequency of uterine enlargement and ovarian tumors.

Many previous trials demonstrated a reduction in the average overall mortality among ovarian cancer patients screened with an annual sequential, multimodal strategy that tracked biomarkers CA125 over time, where increasing serum CA125 levels prompted ultrasound [20–23]. A critical factor which could contribute to false negatives is that many aggressive ovarian cancers are believed to arise from epithelial cells on the fimbriae of the fallopian tube, which are not readily imaged. In addition, because, only a fraction of metastatic tumors may reach an imaging device-detectable size before they metastasize, annual screening with imaging diagnosis may fail to detect a large fraction of early stage ovarian cancers [24, 25]. The ability to detect ovarian carcinomas before they metastasize is critical and future efforts toward improving screening should focus on identifying unique features specific to aggressive, early

stage tumors, as well as improving imaging sensitivity to allow for detection of tubal lesions. So far, multimodal screening strategy in which blood-based assay is positive, and subsequent imaging examination may prove useful in detecting early stage cases [20–22, 25].

4. Gynecological cancer screening intervals

In many countries, undergoing cancer screening is not mandatory but voluntary. Many women are advised to annual gynecological screening for more than a decade. Recently, recommendations of many developed countries include one Pap smear every 3 years after two annual negative results from the age of 18 until 69 years [26]. According to the current American Cancer Society guidelines, adequate negative prior screening and no history of CIN 2 of higher recommend that cervical smear test stops at age 65 [27]. On the other hand, annual screening continues among women of 65 years of age and older, even among those with less than a 5-year life expectancy due to poor health [28]. Likely, as clinical practice continues to change around the screening pelvic examination, consequent changes in utilization of reproductive health services among young adolescence to postmenopausal.

First care visit volume is a key step for continuous use of an extended screening interval, with women who report to first gynecologic care visit during the last year being over 10 times more likely to report current use of a 3-year screening interval than those with three or more visits. It is not possible to separate which come first of less-frequent care seeking and an extended gynecological cancers (including uterine and ovarian malignancies) screening interval. Clearly, some women are screened on 3-year intervals by default; however, others who purposefully follow an extended screening interval may have no perceived need to seek care during a given year.

The continuous screening preference of Japanese women may reflect long-held beliefs about the importance of annual cervical smear examinations and pelvic ultrasonographic examination with limited awareness of the potential harms associated with this practice. The level of knowledge and attitude toward screening are related to multiple factors such as ethnicity, place of residence, income, and social-economic status [29]. From an examiner perspective, annual gynecologic cancer screening has facilitated regular contact with examinees. In general, women are invited by their gynecologists for the examination. The cytologic screening time interval depends on the doctor's personal judgment [30]. If he feels that the test will benefit their patients, the likelihood of performing the test increases. Some systemic review found a positive correlation of educational level, financial status, and an awareness of the mortality rates associated gynecological cancer with gynecological cancer attendance [26, 31, 32]. The level of knowledge and attitude toward health check-up are related to multiple factors such as ethnicity, place of residence, income, and social-economic status [33–37].

5. Discussion

Uterine cancer, in particular cervical cancer, is preventable. More than half of the women diagnosed with cervical cancer have not attended screening in the past 3 years. A community-based screening strategy is one of the greatest success stories in cancer prevention, and widespread

screening reduces the cervical cancer incidence worldwide [38–42]. The mean time between the virus infection and invasive cancer takes about 15 years, and within 2–4 years of detection 15.5–25.5% of low-grade epithelial lesions become high-grade lesions. In some cases, these more severe changes can develop into cervical cancer [5–10]. A routine screening test includes cytology smear test used for the detection of early cervical abnormalities (precancerous dysplastic changes) of the uterine cervix [5–10]. The screening is a relatively simple, low cost, and noninvasive method. Concurrent transvaginal ultrasonography for detection of ovarian and uterine tumors, the cervical and endometrial cytology smear tests attenuate the probability of developing gynecological malignant diseases.

Ningen Dock check-ups provide an occasion to realize preventive medicine. An important aim of gynecological health check-up is to provide support in improving the risk factors that accelerate the risk of outbreak of a malignant disease at an early stage, before subjective symptoms become apparent. Additionally, meticulous educational guidance is provided to match individual living patterns, education level, and ways of thinking. Ningen Dock can also conceive of time in the future when more appropriate and effective educational advice could be continuously provided according to a participant cultural background and lifestyle habits, via collaboration with health-related public services.

Qualitative evaluation of Ningen Dock Facilities consists of documentation and an inspection. These are administration of the facility, satisfaction and safety of examinees, and quality of check-up and follow-up [1]. Recently, the usefulness of Ningen Dock has greatly increased not only in the primary, but also in the secondary prevention of non-communicable diseases due to advances in diagnostic medical technology and therapeutic medicine. However, one of the problems is that relatively large numbers of Ningen Dock examinees who require a second, more detailed examination do not have the examination that has been recommended. For instance, only 61% of the Ningen Dock examinees who required total colon fiberscope as a second, detailed examination due to a positive fecal occult blood test underwent it. Similar tendencies were recognized for almost all Ningen Dock examinations [11]. The reason why Ningen Dock examinees who need second, more detailed examinations do not have them may be that most of them do not understand the importance of such examinations for the early detection of non-communicable diseases and their risk factors because we do not adequately explain the need for more detailed examinations to examinees. Therefore, better education of examinees may be urgently needed in order to further increase the usefulness of Ningen Dock.

In Japan, there are also free physical check-up programs of cancer screening, by which asymptomatic participants undergo a medical examination at public expense. Takagi et al. [43] reported similar data using records of the public expense-covered free examination, and suggested that active gynecologic check-up and adequate follow-up programs even against symptom-free population can reduce in the probability of malignant disease development. Their findings from representative population of high-attitude toward screening, but non-high income, may give new insight into the terms of public health.

The present data are from subject to the limitations of any analysis of self-covered health check-up survey data from participants of Ningen Dock in Japan. Although data are weighted to reflect the Japanese population, the extent to which results are generalizable is no known. Future studies, extended to non-Asian, should attempt to oversample racial minorities and include a detailed assessment of gynecologic cancer screening history and follow-up treatment.

Women attitudes and beliefs related to screening frequency may differ if they reflected truly informed preference and may be related to less screening. The present chapter introduced the extremely low positive gynecology cancer screening incidence in Ningen Dock participants, providing the active strategy in the gynecological cancer screening practices of the lower screening attendance in Japan. However, strategies may be needed to encourage examiners to adopt recommended screening intervals and to educate women about the reasoning behind less-than-annual testing, including explicit discussions about the meaningless and potential harms associated with excess screening.

Disclosure statement

The authors declare no conflict of interest.

Author's contribution

AI designed the study and drafted the manuscript. AI managed all data and performed the analyses. All authors participated in the gynecological examinations at Ningen Dock and commented on various drafts and approved the final version of the manuscript.

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Cervical Pre Cancer

Chapter 5

Secondary Prevention of Uterine Cervical Cancer

Seiya Sato and Hiroaki Itamochi

Additional information is available at the end of the chapter

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Abstract

Secondary prevention by cervical cytology has clearly improved the mortality rate of uterine cervical cancer (CC) by enabling early detection and treatment of high-grade squamous intraepithelial lesion (HSIL) or cervical intraepithelial neoplasia (CIN), which is a precancerous lesion. In the past two decades, HPV-DNA testing, including HPV typing, has clearly brought about positive effects on secondary prevention of CC. However, in practice, CC remains a fatal disease and is the second leading cause of cancer deaths in women aged 20–39 years. Although elucidation of the mechanisms of HPV carcinogenesis and development of a prophylactic vaccine have made CC a preventable disease, eradication of CC is expected to take several decades. Therefore, primary screening to decrease the mortality rate of CC will remain important for a while. In addition, the clinical application of simple biomarkers to stratify HPV-positive women is important for maintenance of medical economy and avoidance of overtreatment in women in the reproductive age. Therefore, the development of an inexpensive therapy or vaccine that can be used worldwide is necessary to overcome cancer deaths due to CC.

Keywords: uterine cervical cancer, cervical intraepithelial neoplasia, secondary prevention, human papillomavirus, carcinogenesis, biomarker, therapeutic vaccine

1. Introduction

Secondary prevention with the use of cervical cytology has clearly improved the mortality rate and early treatment of uterine cervical cancer (CC) by enabling early detection of high-grade squamous intraepithelial lesion (HSIL) or cervical intraepithelial neoplasia (CIN), which is a precancerous lesion [1]. In practice, however, CC was estimated to have 12,820 newly diagnosed cases and 4210 women dying of the disease in 2017 [2]. Moreover, according to the United States data in 2014, CC is the second leading cause of cancer deaths in women aged 20–39 years [2]. Therefore, improvement of screening efficiency remains an important issue.

The etiology of CC is persistent uterine cervical infection with the high-risk human papillomavirus (hrHPV). Therefore, HPV-DNA testing or HPV testing, has become widely used for primary screening of CC. Compared with conventional cytology, HPV testing has higher sensitivity and reproducibility in detecting lesions [3]. However, the specificity of HPV testing is low, with an increase in the number of false-positives, especially in women in their twenties who are highly sexually active [3, 4]. Therefore, HPV testing has been adopted in cancer screening for women over 30 years old. In fact, in the United States (US), the guidelines created by the American Cancer Society, American Society for Clinical Pathology, and American Society for Colposcopy and Cervical Pathology suggested CC screening by cytology starting at the age of 21 and every 3 years until 30 years old; beyond the age of 30, combined HPV testing and cytology for every 5 years was recommended [5]. Based on data from large-scale, longitudinal, randomized-controlled trials in European countries, HPV testing has been adopted as the primary screening tool for CC in women aged 30 years or older [6–9]; in those who are tested positive for HPV, cytology is used as the triage test. In ASC-US cases, HPV testing is performed for triage in the management of CIN, based on the results of available large-scale clinical studies [10–13]. Furthermore, HPV typing has already been used as a biomarker for decisions on therapeutic interventions and subsequent follow-up of CIN [14–19]. Both the US and European guidelines recommended HPV testing to confirm the completion of treatment of CIN.

HPVs are classified according to carcinogenic potential. In general, the frequently reported high-risk types are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 [20]. Among these, HPV 16 and 18 are the most common types that are related to carcinogenesis worldwide; both HPV types are controllable by prophylactic vaccines that contain virus-like particles with antigenicity [21, 22]. Bivalent vaccines for HPV 16 and 18 are commercially available, but quadrivalent vaccines are also available for HPV 6, 11, 16, and 18. Although these vaccines have some cross-protective effects [23, 24], these are basically ineffective for infection by all HPV types. To overcome these limitations, a nonavalent vaccine containing HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58 has been launched [25, 26].

As mentioned above, hrHPV testing has clearly brought about positive effects on early detection of CIN and prevention of CC in the past two decades [27–30]. Several researchers all over the world continue to pursue efforts to eradicate CC. **Figure 1** shows the schema of the natural

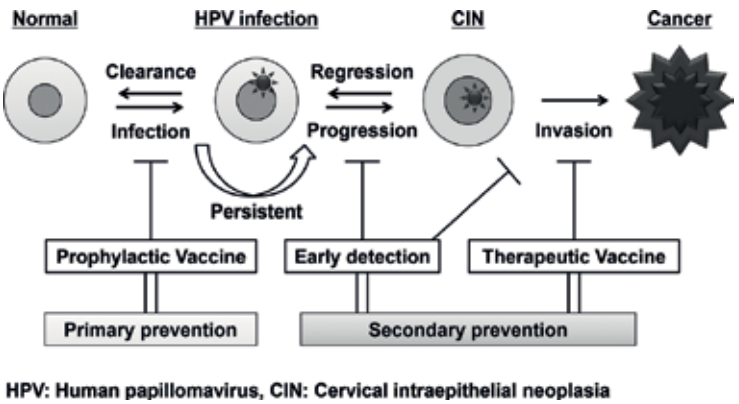

Figure 1. Natural history of HPV and prevention of cervical cancer. Persistent infection of the cervix with high-risk HPV causes cervical cancer (CC), which begins as cervical intraepithelial neoplasia (CIN). Primary prevention of CC can be achieved by prophylactic HPV vaccination. Secondary prevention consists of early detection of CIN and therapeutic vaccination to inhibit progression from CIN to CC.

history of HPV and CC prevention. In this chapter, we will describe the recent developments in secondary prevention of CC.

2. Biology and carcinogenesis of HPV

HPV is a virus with a double-stranded circular DNA in the icosahedral capsid. The genome size is about 8000 bases and contains eight protein-coding genes and a noncoding, regulatory long control region [31]. The early genes (E1, E2, E4, E5, E6, and E7) encode nonstructural proteins involved in replication, transcription, and transformation; whereas the late genes (L1 and L2) encode viral capsid proteins. Among these genes, E6 and E7 play a central role, particularly in carcinogenesis. Notably, a recent whole-genome sequencing study that assessed the risk of viral genetic variation showed that strict preservation of the 98 amino acids of E7, which destroys the function of the retinoblastoma protein (pRB), was critical for HPV 16 carcinogenesis and development of CIN and CC [32].

HPV can infect the epithelial cells of the human mucosa and skin at least once in most women's lives. In other words, HPV infection is a common sexually transmitted disease. Because prophylactic vaccines prevent only initial infection, its value in women is most effective before the first sexual contact [33]. In the early stages of HPV infection, the host is asymptomatic, and in most cases, the virus is eliminated by the immune system within a few years [34]. However, HPV infection can persist in some patients. The reported risk factors for progression of cervical HPV infection to CIN or CC include persistent hrHPV infection, immunosuppression, age over 30 years, and smoking [35].

Persistent hrHPV infection of the cervix is divided into three stages: latent, permissive, and transforming [36–38]. First, HPV invades the epithelial basal cells via minor breaches of the epithelium [39] and become latent as a nuclear episome; the infected cells usually die after virus multiplication. The E6 and E7 genes are rarely integrated into cellular DNA and cause HPV growth in the cells; however, this property also allows continued expression of E6 and E7 proteins at high levels. The expression of E6 and E7 oncogenes in basal cells is tightly controlled; therefore, HPV-infected cells can escape a host's immune defense. In fact, in a small percentage of HPV-infected women, HPV-specific antibodies and T cells are detected at low levels [40, 41]. Recently, it was suggested that the programmed death 1/programmed death 1 ligand (PD-1/PD-L1) pathway might be involved in the mechanism of this immune evasion [42–44].

When infected cells begin to differentiate in the epidermis, the E6 proteins degrade the tumor suppressor protein p53, while the E7 proteins inhibit the function of the pRB; these processes reactivate DNA synthesis and replication of the HPV genome. The cells with integrated E6 and E7 genes will have uncontrolled cell cycles because p53 and pRB are major cell cycle regulators. Furthermore, apoptosis and the tumor suppressor pathway are repressed. During this process, accumulation of genetic mutations and genomic instability ensue [45–50]. As a result, a large number of clones with intratumor heterogeneity are produced, some of which might be able to avoid the host antitumor response [51–54]. Ultimately, with the addition of external factors, these cells will be immortalized and can become cancerous [55].

3. Biomarker for early detection and triage

HPV testing has been introduced for primary screening for CC; it is highly sensitive, but its false-positive rate is high due to the low specificity. Therefore, the need to stratify HPV-positive women with or without abnormal cytology has become a very important issue [56]. At present, HPV-positive women undergo cytology tests and HPV retesting, with colposcopy and tissue biopsy correlations at frequent intervals; however, the precision of this process remains unclear. More objective indicators are required to prevent unnecessary procedures and treatment. As the understanding of the molecular mechanisms of cervical carcinogenesis by HPV has progressed, various biomarkers that predict patient outcomes have been developed not only for early detection but also for triage.

As mentioned earlier, persistent HPV infection of cervical cells leads to tumor formation through several stages. Since HPV infection is often transient, detection of the stage when HPV infection shifts from permissive to transforming is clinically important for cancer screening. Similarly, the histopathologic and molecular diagnostic processes for CIN focus on detection of malignant transformation in HPV-infected cells [57, 58]. The function of HPV-transformed cells is critically dependent on E6 and E7 oncogenes and related molecules such as $p16^{INK4a}$ [59, 60]. Therefore, E6/E7 mRNA and $p16^{INK4a}$ are important targets for early detection and triage. In addition, genetic or epigenetic changes in HPV-transformed cells have been attracting attention as biomarkers for screening of CC, in the triage of HPV-positive women, and as targets of treatment. Because, such new biomarkers can be analyzed from preserved liquid-based cytology (LBC) specimens, their use may be further expanded [61].

3.1. HPV typing

HPV 16 and 18 account for 70% of the causes of CC. The other reported HPV types related to CC are 31, 33, 35, 45, 52, and 58 [62]. Furthermore, the risk of developing CC has been reported to differ according to the type of hrHPV [63]. A cohort study to estimate the risk of disease progression among the HPV genotypes in 570 Japanese women with cytologic low-grade squamous intraepithelial lesion (LSIL) and histologic CIN1/2 showed that the cumulative probability of CIN3 within 5 years was higher in HPV 16, 18, 31, 33, 35, 52, and 58 than in the other hrHPV types [64]. Another Japanese cohort study on cytologic abnormalities, including ASC-US, LSIL, and HSIL (≤CIN2), reported that infection with HPV types 16, 18, and 33 posed a high risk of developing CIN3 [65]. The Japanese gynecologic guideline 2017 recommended HPV typing to evaluate the risk of disease progression for patients with histologically proven CIN1/2 (**Figure 2**). Taken altogether, HPV typing in CIN patients is useful for risk assessment of disease progression [66].

3.2. $p16^{INK4a}$

The $p16^{INK4a}$ is a cyclin-dependent kinase inhibitor that blocks the phosphorylation of various cyclins that control the cell cycle. In many human cancers, including colon and breast, the function of the $p16^{INK4a}$ gene is lost by gene deletions, mutations, or epigenetic silencing. In CC,

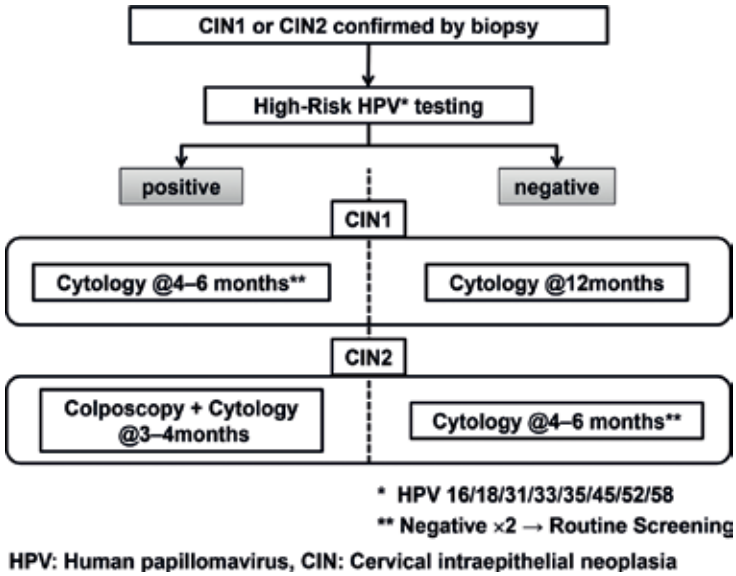

Figure 2. HPV typing for CIN1 and CIN2 in Japan. The Japanese gynecologic guidelines in 2017 recommend HPV typing to evaluate the risk of disease progression in patients with histologically proven CIN1/2. Patients who are positive for high-risk HPV receive more intensive management compared with negative patients.

however, a high level of intracellular E7 expression eliminates the inhibitory methylation mark encoding the CDKN2A gene promoter from $p16^{INK4a}$, resulting in overexpression of the $p16^{INK4a}$ protein [67]. In addition, since E7 inactivates pRB, there is proliferation of cells that highly express $p16^{INK4a}$. In other words, high expression of $p16^{INK4a}$ reflects the high expression of E7, which is a good indicator of CIN3 and CC. For this reason, $p16^{INK4a}$ is widely accepted as a valuable surrogate biomarker for the transforming properties of HPV infection [68]. Based on this fact, a therapeutic peptide vaccine using $p16^{INK4a}$ as the antigen has been developed [69]. Moreover, $p16^{INK4a}$ has been used for dual-staining with p16/Ki-67 cytology (p16/Ki-67); this would complement the low sensitivity of cytology and the low specificity of the HPV test for secondary prevention of CC [70–72].

In Europe, p16/Ki-67 was compared with Papanicolaou (Pap) cytology and HPV testing for screening high-grade CIN (CIN2+) in 27,349 women aged 18 years or older; the p16/Ki-67 had high sensitivity and comparable specificity for CIN2 detection, compared with the other tests [73]. This suggested the utility of p16/Ki-67 as a screening method in young women with high HPV infection rates. Other studies showed the effectiveness of p16/Ki-67 as a triage test for CIN2+ detection in Pap-negative and HPV-positive women ≥30 years old [74]. In addition, the usefulness of p16/Ki-67 for follow-up of patients after CIN treatment was suggested [75]. In Germany, a recent study revealed that combined HPV 16/18 testing and p16/Ki-67 resulted in lower cost and clinically efficient CC screening, compared with conventional annual Pap cytology [76]. As described earlier, several evidences on the utility of p16/Ki-67 have accumulated; therefore, p16/Ki-67 will definitely play an important role in the secondary prevention of CC.

3.3. HPV E6/E7 mRNA

The usefulness of HPV E6/E7 mRNA testing in secondary prevention of CC has already been established [77, 78]. Combined testing of LBC cytology and APTIMA® HPV (AHPV) has already been used in the US and Europe. HPV E6/E7 mRNA testing is useful because it can detect HPV infection and the transformation properties of cervical cells. In fact, HPV E6/E7 mRNA testing was reported to detect high-grade CIN with high sensitivity and specificity [79–81].

ASC-US and LSIL on cytology are mainly caused by low-grade cervical lesions that often resolve spontaneously [82]. Currently, the HPV test is used for triage of women with ASC-US and LSIL; however, its low specificity has increased the number of unnecessary examinations and treatment [83]. On the other hand, HPV E6/E7 mRNA testing enables stratification of the risk of developing CIN2+ in women with both hrHPV-positive and hrHPV-negative cytology [84]. A recent meta-analysis to confirm usefulness of HPV E6/E7 mRNA testing for triage revealed that a positive HPV E6/E7 mRNA testing in women with mild cytology findings, such as ASC-US and LSIL, necessitates immediate colposcopy and intensive follow-up because the risk of carcinogenesis is high [85].

3.4. Epigenomic alterations

Epigenetic events in the host and in viral genomic regions and genes are necessary during HPV-mediated cellular transformation and carcinogenesis [86]. DNA methylation is a typical epigenetic change and characterizes the molecular, cellular, and clinical features of HPV-associated neoplasia. Because hypermethylation is a stable and reversible process, detection of methylation marks is used for diagnosis. In addition, new targeted therapies with demethylating compounds have been developed [87].

Combined testing with DNA methylation and hrHPV is one of the promising screening options for CC. The Triage and Risk Assessment of Cervical Precancer by Epigenetic Biomarker (TRACE) study was conducted to examine the usefulness of human epigenetic biomarker testing in the primary prevention of CC. In this study, methylation of the POU4F3 promoter, which is a promising marker for CIN3, showed significantly higher sensitivity and similar specificity for detecting CIN3+, compared with LBC [88]. This finding suggested that detection of POU4F3 methylation is useful for early detection of CIN3. Another study assessed the correlation between CpG methylation of the HPV16 L1 gene and CC in 145 HPV 16-infected Uyghur women who were divided into five groups, as follows: transient infection (n = 32), persistent infection (n = 21), CIN1 (n = 21), CIN2–3 (n = 33), and CC (n = 38) [89]. After quantifying each CPG methylation by pyrosequencing, results revealed that methylation increased at 13 CpG sites in advanced lesions and that high methylation levels were associated with the risk of developing CIN2+ [89]. These findings may be applied to CC screening.

4. Therapeutic vaccine for CIN

Currently, the standard therapy for CIN is surgical excision such as conization or LEEP. Although these treatments are very effective from the viewpoint of removing HPV-induced

precancerous lesions, these can cause infertility and menstrual disorders secondary to stenosis of the cervix. In addition, the existing CC prophylactic vaccines are ineffective for HPV-infected women and nontargeted HPV types. Therefore, development of a therapeutic vaccine using immunotherapy as a nonsurgical treatment for CIN is an important strategy for the prevention of CC.

The development of therapeutic vaccines has been mainly targeted for HPV E6 and E7 [90], because these proteins are essential for the malignant transformation of HPV-infected cells and are permanently expressed in CIN. In order to induce an E6 or E7 antigen-specific T cell immune response, several kinds of vaccines have been developed; these include adoptive transfer of tumor-specific T cells, chimeric virus-like particle vaccines, dendritic cell, DNA vaccines, peptide vaccines, protein vaccines, and viral or bacterial vector vaccines. Among these, protein vaccines are the most common therapeutic vaccines for HPV 16 because of the simplicity of the method and the lack of HLA restriction [91]. However, there are currently no available therapeutic HPV vaccines against CIN.

Recently, a randomized, double-blind, placebo-controlled phase 2b trial on CIN2/3 patients showed promising results on the efficacy and safety of VGX-3100, which is a synthetic plasmid targeting human HPV 16 and HPV 18 E6 and E7 proteins (ClinicalTrials.gov Identifier: NCT01304524). In the study, the primary endpoint for efficacy was regression to CIN1 or normal pathology at 36 weeks after the first dose. This study enrolled 167 people who were randomized (3:1) to the VGX-3100 group (n = 125) and the placebo group (n = 42); the rate of histopathologic regression was significantly higher by 18.2% [95% CI 1.3–34.4] in the VGX-3100 group, compared with the placebo group (48.2% vs. 30.0%; p = 0.034). The incidence of erythema at the injection site was significantly higher in the VGX-3100 group than in the placebo group (78.4% vs. 57.1%, p = 0.007). On the other hand, there was no significant increase in the number of severe side effects that could interfere with the performance of vaccine therapy [92]. Therefore, this vaccine might be a nonsurgical therapeutic option for CIN2/3, but further research and development are needed in this field. A clinical trial on HPV therapeutic vaccines was detailed in a recent review [93].

5. Discussion

The mathematical model by a German group estimated that the incidence and mortality of CC will drastically decrease in the next 30 years due to the increasing number of screening participants since the 1990s [94]. Furthermore, even at a vaccination rate of only 50%, more than 40% of CC is considered to be preventable in the next 100 years [94]. Nevertheless, more effective primary prevention is necessary to eradicate CC. Currently, an effective vaccine can be used to inhibit a part of the hrHPV infection process that leads to cancer. However, several individuals cannot receive vaccination due to economic or geographical problems. In order to solve this problem, international cooperation and national policy are necessary to construct a CC prevention system. One possible problem in the future would be the changes in the distribution of hrHPV types due to an increase in the number of vaccinated cohorts; this would

likely decrease the efficiency of the current screening system. Therefore, it may be necessary to monitor the distribution of hrHPV in each country and region, and to develop screening methods that are suitable for each situation.

Secondary prevention remains important because vaccines only prevent infection with a limited number of HPV types. In order to reduce the mortality rate of CC, the coverage of a screening program needs to be increased and include patients with advanced CC. To address this issue, the usefulness of self-sampling for HPV testing has been studied [95, 96]. The US National Health and Nutrition Examination Survey in 2007–2010 on women aged 18–59 years revealed a 41.9% prevalence of genital HPV infection [97]. Multivariate analysis in this cohort revealed that HPV infection was related to age, number of sexual partners, smoking, educational level, income, and insurance status [97]. Similar results on the risk of persistent HPV infection have been confirmed in other studies [98, 99]. Therefore, populations with these risk factors require more rigorous and continuous monitoring for effective prevention; in these cases, self-sampling may be particularly useful. Importantly, the hrHPV detection rate by continuous self-sampling of vaginal fluid for 28 days was reported to be consistent regardless of the hormonal cycle [100]. In other words, hrHPV detection by self-sampling can be adapted to all women, even those in the nonmenstrual period, including menopause. A recent meta-analysis of 37 studies including 18,516 women revealed that HPV-DNA sampling screening was highly accepted compared with clinician's sampling. In the future, the importance of self-collection method will increase, especially from the viewpoint

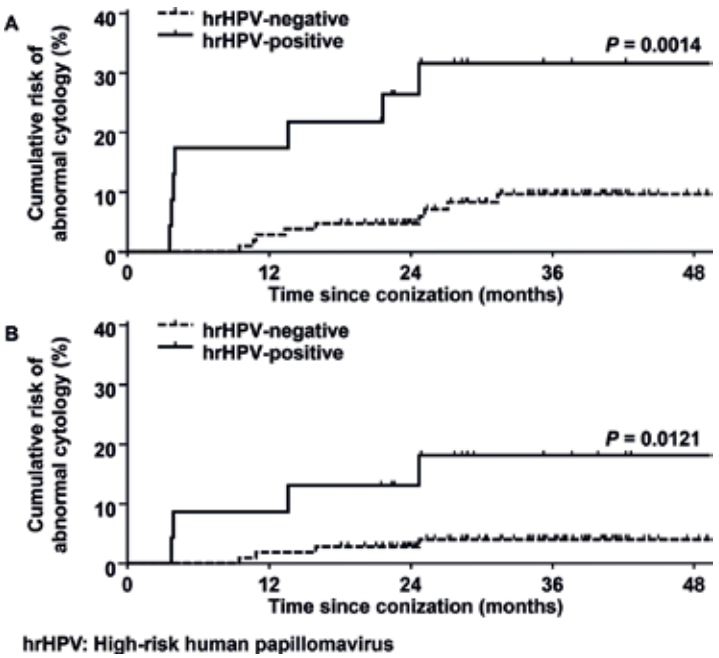

Figure 3. Postoperative infection with high risk (hr) HPV and risk of abnormal cytology. The cumulative risk curves for (a) atypical squamous cells of undetermined significance (ASC-US) or higher and (b) low-grade squamous intraepithelial lesion (LSIL) or higher show that the cumulative risks for recurrence of abnormal cytology and LSIL or higher were significantly increased in postoperative hrHPV-positive patients than in hrHPV-negative patients.

of cost-effectiveness and expansion of screening services [101]. Therefore, HPV-DNA testing by self-sampling has the potential to become the mainstream in cancer prevention.

CIN frequently regresses spontaneously within months or a few years [102, 103]. However, there is no biomarker to predict spontaneous regression of CIN. The standard treatment for CIN is still surgical resection such as conization; for a long time, there had been no other options for treatment. Although surgical excision is successful for CIN treatment most of the time, HPV infection cannot be completely eliminated. We reported that postsurgical hrHPV infection was a positive predictor of the recurrence of abnormal cytology (**Figure 3**). Furthermore, surgical procedure can lead to complications such as pregnancy problems, infertility, incontinence, and sexual dysfunction [104–106]. At the very least, overtreatment of women with fertility must be avoided. With the progression of CC screening, the importance of these problems has increased. In order to overcome this problem, development of a therapeutic vaccine as a new treatment option without surgery is urgently needed. The availability of low-cost therapeutic vaccines for patients with CIN or stage IA CC in the future will lead to a long-term reduction in medical costs [107].

6. Conclusions

Although elucidation of the mechanisms of HPV carcinogenesis and development of a prophylactic vaccine have made CC a preventable disease, eradication of CC is expected to take several decades. To decrease the mortality rate of CC, early detection by screening will remain important for a while. The clinical application of simple biomarkers to stratify HPV-positive women is important for maintenance of medical economy and avoidance of overtreatment of women in the reproductive age. To overcome cancer deaths due to CC, the development of inexpensive treatment options or therapeutic vaccines that can be readily used worldwide is necessary.

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Chapter 6

Locally Advanced Cervical Carcinoma Management

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Additional information is available at the end of the chapter

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Abstract

Cervical cancer is a public health burden to Low and Middle Income countries. Whereas strides are being made in the management of malignancies worldwide, resources limited settings are confronted with the paucity of basic awareness, health professionals, diagnosis and management modalities, all contributing to cervical cancer disease late presentation. Among available treatment modalities, the mainstay of treatment for locally advanced cervical cancer remains radiation therapy combined with chemotherapy. Radiation is delivered through external radiation and brachytherapy. The evidence leading to the decision making, the modern management modalities and the general side effects, will be reviewed here.

Keywords: cervical cancer, radiation, brachytherapy

1. Introduction

Cervical cancer represents the second most common malignancy and the third overall cause of cancer mortality in Low and Middle Income Countries (LMICs) [1]. Such countries contribute the majority of the cervical cancer burden worldwide.

The FIGO classification system, endorsed by the American Joint Commission on Cancer 2017, defines locally advanced cervical cancer, as a disease found between stages IB2 to IVA [2]. This subset of diseases is visible on clinical examination and usually predict worse outcome in terms of recurrence and survival rates when compared to early stage disease.

Several studies focusing on the different stages at initial presentation have shown consistently high rates of advanced disease in LMICs (**Table 1**).

Studies	Type	Country	Patients (N)	Stage (rate percentage) at presentation
Chirenje et al. [5]	CS[2]	Zimbabwe	196	Stages IIB - IVA: (80.3%)
Musa et al. [4]	R[1]	Nigeria	65	IA (1.5%) - IB (6.1%); IIA (20%); IIB (35.4%); IIIA (9.2%), IIIB (24.6%); IVA (3.1%)
Mlange et al. [6]	CS[2]	Tanzania	212	IA (1.4%), IB (28.2%), IIA (6.4%), IIB (20.4%), IIIA (15.8%), IIIB (17.3%), IVA (7.4%)
Sharma et al. [3]	R[1]	India	227	IA (3.9%), IB (8.7%), IIA (6.8%), IIB (32.5%), IIIA (6.8%), IIIB (30.7%), IVA (8.7%); IVB (1.9%)

[1]R = retrospective review.
[2]CS = cross-sectional study.

Table 1. Summary of rates of cervical carcinoma disease stage at presentation in LMICs.

Advanced stages present a considerable challenge to achieving adequate treatment. This is compounded by the absence of modern treatment modalities and technologies that are available in high-income countries (HIC).

LMICs face a double burden where patients for different reasons including health system factors presents at hospital with advanced diseases and at the same time there is a pronounced lack of infrastructure to take care of these patients. This leads to an overall poor survival rates.

2. Disease evaluation

Staging with full nodal evaluation remains a crucial aspect of the management of locally advanced cervical carcinoma.

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The pattern of spread of cervical cancer follows principles seen in numerous other solid malignancies namely, local extension, lymphatic spread and distant metastasis.

The disease usually spreads directly distally to the vagina, with extension along the parametria, the uterine ligaments (commonly utero-sacral) and the peri-rectal area.

Lymphatic spread occurs by echelon from the pelvic nodes, usually proximally toward the para-aortic nodes, and could substantially lead to left supraclavicular lymph node involvement in rare cases.

There is a high correlation between nodal status and disease outcomes. Contemporary studies have estimated close to 40% of survival at 5-years follow-up if evidence of para-aortic node involvement is established. Risk of nodal metastasis increases approximately by 15% for each stage, from FIGO stage I to III.

A Computed-Tomography (CT) guided biopsy is needed for nodal disease confirmation. For expert centers, an F-18 fluorodeoxyglucose-based positron emission tomography (PET) scan is necessary to establish nodal disease.

The patient needs also to be screened for competing risk factors. Importantly, patients should be evaluated for present or acquired (by local extension, Stage IIIB) kidney disease as the management of locally advanced cervical carcinoma incorporates the use of platinum-based chemotherapy as a radio-sensitizer. Corrections to the management protocols related to the renal disease stage have been suggested and carry promising grounds for further prospective studies.

Disease staging provides ground for prognosis, local control and survival prediction in the presence of adequate management modalities. Survival rates vary across studies and are inversely related to stage, with disease control and 5-year overall survival both ranging from 90% for stage IB to only close to 30% for stage IVA, in recent studies.

For aggregate analyses of locally advanced cervical carcinoma management outcome-based studies, newer treatment techniques are providing encouraging survival data.

3. Locally advanced cervical carcinoma management

The standard of care for advanced cervical carcinoma is Radiation Therapy alone (in case of palliation), or in combination with cisplatin-based chemotherapy.

Overall survival is usually a function of disease-free interval rates, highlighting the scarcity of salvage therapy options in case of recurrence.

With limited options for salvage in cases of recurrences, disease-free interval rates directly correlate to Overall Survival and comorbidities of the patients.

Generally, management of cervical carcinoma includes definitive surgery for selected cases, upfront radiation therapy and chemo-radiation. Surgery and radiation therapy amount to the

same effect yet with drastic differences in terms of debilitating toxicities, hence surgery alone is the treatment of choice for initial smaller lesions (<4 cm) with other treatment modalities offered as a salvage in case of recurrence.

4. Primary chemoradiation vs. surgery

As cited above, the widely used treatment scheme for locally advanced cervical carcinoma consists of upfront radiotherapy concurrently with a platinum-based chemotherapy.

Surgery has been proven to not be superior to chemo-radiation, but carries twice a risk of increased toxicity rates.

Adverse features arising post-surgery could be similar to other advanced diseases, including high grade disease, lympho-vascular space invasion (LVSI), positive lymph nodes, prompting the use of multiple modalities of treatment with subsequent considerable toxicities.

The largest comparison study to date by Landoni, compared 343 patients with early disease, contemporarily included in the locally advanced stage (IB - IIA) disease, to undergo an extensive surgery with pelvic lymph node dissection, with a possibility of Radiation Therapy boost in the presence residual disease. This arm was compared with upfront Radiation Therapy. The comparison yielded no differences in survival, but showed considerable difference in toxicity rates [7].

Chemo-radiation has been proven to offer a high survival benefit which is greatly influenced by disease stage. Additionally, concurrent chemo-radiation decreases the recurrence risk.

Radiation consists of external beam radiotherapy session and a stage-variable boosting dose achieved by brachytherapy. Details on patient simulation, field size and dose specification are found below.

5. Combined radiation and chemotherapy regimen

5.1. Chemotherapy regimens

Concurrent radiation with chemotherapy has come to age relatively recently, with cisplatin-based chemotherapy rising at the dawn of the twenty-first century.

Before this era, numerous institutional trials had been published with various chemotherapy regimens selections.

Among previously suggested regimens, hydroxyurea was used in the 1960s, perceived as being a radiosensitizer. Hydroxyurea induces a block on the G1-S phase of the cell cycle, hence enhancing cell kill by radiation; prevention of sub-lethal damage repair has also been proven.

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A Gynecological Oncology Group (GOG 56) study confirmed the benefit of added hydroxyurea to radiation therapy, with a higher Progression Free interval, though no significant survival benefit was found [8]. This study was controversial in its setting and the recommendations were not applied widely. Hydroxyurea involves a high risk of myelosuppression, and prospects of considering it as a viable combination therapy to radiation were abandoned overtime.

5-Fluorouracil has also been considered as an alternative chemotherapy regimen for combination therapy. However, the few published studies failed to prove local disease control and survival benefit with an added 5-Fluorouracil regimen [9].

Based on a five-study analysis, cisplatin added to radiation therapy was confirmed to have a superior survival when compared to radiation alone.

A large systematic review of 18 trials combining radiation and chemotherapy for locally advanced cervical carcinoma proved the survival benefit of adding chemotherapy. Platinum based chemotherapy was not seen to be significantly different from non-platinum based chemotherapy (HR: 0.84 vs. 0.76, $p = 0.48$). Platinum-based regimens were also found to have a non-significant increased toxicity trend. However, single agent platinum offered an important alternative with regards to local disease control, adherence to treatment and ease of administration.

Historically, the GOG 120 compared different chemo-radiation regimens, combined with a brachytherapy boost. The arms had a cisplatin alone, a hydroxyurea alone and a cisplatin/5-Fluorouracil/Hydroxyurea components. The arms containing cisplatin had improved survival and disease down-staging was achieved. Subsequent studies removed hydroxyurea and compared upfront radiation therapy with concurrent cisplatin (with or without 5-Fluorouracil) with radiation therapy, which established the standard of care of adding chemotherapy to radiation therapy, as it was shown to increase survival and decrease recurrence risks.

The rationale behind adding cisplatin to radiation is that it acts as a radiosensitizer, by preventing the Non-Homologous End Joining pathway, which is paramount in the Double Strand Breaks repair. Double Strand Breaks in DNA are induced by high energy radiation therapy.

Cisplatin is given on a weekly basis, a few hours before radiation therapy, and care needs to be taken for its administration. As a nephrotoxic agent, adequate hydration is to be ensured, before and after cisplatin infusion. Together with knowing prior the renal status of the patient, dosing can be altered to prevent toxicity. Carboplatin has been shown to provide a suitable alternative to cisplatin for patients who are not candidates to cisplatin infusion (**Table 2**).

Gemcitabine could also be a choice when cisplatin is contraindicated. However, in the absence of level I evidence, and with the increased toxicity risk associated with Gemcitabine, carboplatin remains the preferred choice in case of intolerance to cisplatin, and deranged renal function tests.

5.2. Radiation therapy

Radiation Therapy is provided by both External Beam Radiation (EBRT) and brachytherapy (BT) to increase local control.

Cisplatin - dosage and premedications	Details
Prior to Treatment Work-up	Complete Blood Count - with a low Absolute Neutrophil Count, consider adding Filgrastim (if available) prior to chemotherapy infusion
	Renal Function Tests - use the Glomerular Filtration Rate to determine fitness of the patient to receive cisplatin
	Serum Electrolytes (K^+, Na^+)
Dosage	40 mg/m^2 IV weekly
Pre-medications	Hydration – 2 l Normal Saline (0.9%) over 2 hours prior and after a cisplatin infusion
	Ondansetron 16 mg IV and Dexamethasone 16 mg IV, both mixed with 100 ml of Normal Saline (0.9%)

Table 2. Summary—cisplatin treatment planning and dosage.

Total doses above 45 Gy are preferred as they are proven to offer a survival advantage.

Radiation therapy is offered to post-operative patients confirmed to have adverse features (mainly Lympho-Vascular Space Invasion, positive pelvic nodes, involved parametria and positive surgical margins), with stages IA2, IB. It is also the definitive treatment, with concurrent chemotherapy for stage IIB- IVA.

The typical dose given by EBRT varies between 45 and 50 Gy depending on the stage and prior treatment. For patients treated with a prior hysterectomy, lower doses (typically below 45 Gy) are preferred to avoid radiation induced bowel toxicity; higher doses are safe to be delivered on an intact uterus and cervix.

Current RTOG and GOG protocols suggest total doses for cervical carcinoma treated with definitive radiation therapy to be around 80–90 Gy to a point defined within the paracervical triangle, namely the point A.

The point A has been varied over the years, and is defined as being at 2 cm above the external cervical os and 2 cm lateral to uterus midline. This corresponds to the paracervical triangle, where the uterine vessels cross the ureter, medial to the broad ligament.

Given the proximity of the cervix to the major pelvic organs and the femoral heads, the external beam radiation doses are limited to an overall dose of 50 Gy. Institutional practices vary, some preferring doses below 45 Gy before proceeding to brachytherapy, with options of lowering the field size to boost to gross residual and nodal disease to doses up to 50 Gy.

Addition of brachytherapy has shown high rates of cancer-specific and overall survival benefits when compared to external beam radiation therapy alone. The objective of brachytherapy addition is to reach the desired total dose of 80–90 Gy to the disease site while minimizing toxicity to organs at risk.

5.3. External beam radiation techniques

The distal most part of the disease needs to be marked with radiopaque gold seeds for disease localization prior to treatment imaging. In the absence of gold seeds, radiopaque materials

such as lead or steel-made wires can be used for disease localization. The same needs to be applied to the vagina and anus areas.

For a 4-field (antero-posterior, postero-anterior and lateral fields) treatment, the simulation is done in the supine position and CT or Fluoroscopic images taken. Patients are positioned with arms on the chest, knees and lower legs immobilized. Anterior and lateral tattoos are marked and aligned with lasers for lateral rotation prevention. Obese patients may benefit from prone belly boards, to avoid small bowel inclusion in the radiation volume.

Intra-venous contrast CT scans are taken to help highlight the pelvic vessels used as reference to delineate the pelvic nodes.

For centers using two-dimensional planning and fluoroscopic imaging, the same marking has to be done, with fluorescent markers, and tattoos where applicable.

The borders are:

- Superior: Lumbar spine level 4/5
- Lateral: 1.5–2 cm away from the pelvic brim
- Anterior: 1 cm anterior to pubic symphysis
- Posterior: Entire Sacrum to be included
- Inferior: Below the ischial tuberosity or the inferior obturator foramen if bony landmarks are used

For advanced disease involving the lower vagina (stage IIIA), include at least a margin of 3 cm away from the distal most part of the disease.

Extensive Radiation Therapy has been suggested in the presence of para-aortic lymph nodes, with the superior-most border being T12/L1, with kidney blocks [10].

Stage IIIA is associated with inguinal nodes, and the field needs to include the vaginal introitus as the inferior border; with a common iliac nodes disease presence, the superior border is to be raised up to L3/4.

Dosing should be up to 50 Gy delivered in 25 equal fractions, daily. This is usually given within 5 days a week for 5 weeks, allowing a 2-day rest between weeks of treatment.

Dose limiting organs are mainly the bladder, rectum, femoral heads, and with a lower instance, the small bowel and ovaries.

5.4. Brachytherapy

As per the American Brachytherapy Society guidelines, brachytherapy for cervical cancer needs to be applied for a disease not exceeding a size of 5 cm.

The preferred brachytherapy technique is the High Dose Rate Brachytherapy, delivering above 12 Gy/hour.

The point of interest for brachytherapy delivery is defined in the contemporary method as per the Manchester point A - 2 cm superior to the external cervical os and 2 cm lateral to the central uterine canal. The objective is to deliver a cumulative dose of 80–90 Gy.

Due to nodal disease associated with locally advanced cervical cancer, a Manchester point B is defined at 3 cm lateral to point A. With this system, bladder, vaginal and rectal points are also defined. Care needs to be taken to minimize the radiation dose to the bladder and rectum by anteriorly and posteriorly packing through the vagina and around the brachytherapy applicators.

Brachytherapy delivery is provided once weekly over a time interval of 3–6 weeks. Total radiotherapy treatment (EBRT and BT) should be completed within a time period of 7–8 weeks.

6. Complications

Acute complications commonly include local features, consisting with dry and moist skin desquamation, vaginitis, cystitis, and proctitis. Management of these complications varies between anti-inflammatory medications and anti-microbial drugs given for prophylaxis.

Brachytherapy side effects are mainly due to neighboring organ toxicity and include vaginitis, cystitis and uterine perforation.

Late complications include vaginal stenosis, recto-vaginal and vesico-vaginal fistula and intestinal perforation.

7. Conclusions

With increasing rates of advanced cervical cancer disease in Low and Middle Income countries, adherence to evidence-based literature for treatment is key. Radiation therapy combined with chemotherapy are the mainstay of management for locally advanced cervical carcinoma. The treatment should ideally not exceed eight (8) weeks after the baseline work-up and disease evaluation to maximize disease control.

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Cervical Cancer Prevention

Chapter 7

Immune Regulatory Network in Cervical Cancer Development: The Expanding Role of Innate Immunity Mechanisms

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Abstract

There is increasing evidence of a pivotal regulatory role of innate immune mechanisms in tumor-immune interplay. Among these diverse mechanisms, tumor-derived nucleic acids' sensing has recently emerged as one of the fundamental pathways linking innate and adaptive immunity, with DNA-sensor STING being the crucial member of this pathway. Another clear trend is understanding the striking diversity of innate and innate-like immune cell populations implicated in suppression or promotion of tumor growth. Papillomavirus-associated cervical cancer appears to represent a complex network of antiviral and antitumor innate immune mechanisms, whose regulation can be significantly influenced by developing neoplasia. In this chapter, we address new data on the problem of regulation of innate and acquired immunity in cervical cancer patients published in the past 2 years. To support the idea of multilevelness and diversity of changes in the innate arm of immunity, we also report our findings about (a) the expression of endogenous immune sensor STING in neoplastic tissue and peripheral blood lymphocytes, (b) altered frequencies of circulating natural killer and natural killer-like cell populations, as well as regulatory T lymphocytes from patients with precancerous or early cancerous lesions. Revisiting this problem may provide new insights into therapeutic options for cervical cancer.

Keywords: cervical neoplasia, innate immune system, antitumor immune response, innate-like lymphocytes, regulatory lymphocytes, immune suppression, DNA-sensing mechanisms

1. Introduction

Human papillomavirus (HPV)-associated cervical cancer is a type of oncopathology, one can consider as an example of a unique natural phenomenon of virus-related carcinogenesis, which realization is defined by dynamic interactions within a complex system "pathogen ('alien')-tumor ('altered-self')-host immunity." And while for the systems of "viral infection-immunity" and "tumor-immunity" interactions, the models well-describing molecular mechanisms supporting these interactions have been proposed, the situation when both pathological factors coexist seems to be much more complex. It is in these types of pathology that the dual (positive and negative) role of the immune system is most evident [1, 2], and it is for this reason that, obviously, despite a long history of studies, immunology of virus-related cancers still has a lot of blind-spots. The fact that clinical trials of immunotherapy methods to treat cervical cancer and other HPV-related cancers, which typically use unimodal approach, do not show the desired effect, particularly in advanced disease, underlines diverse multidirectional role of cellular, and molecular components of the immune system at different stages of disease development and points the need to study the combined multimodal approaches [3].

A large number of fundamental discoveries made recently in the area of oncoimmunology and immunology of infectious diseases have led to a substantial revision of the priorities in the studies of the antitumor immune response regulation mechanisms, including: (1) redefining the role for cellular components of the innate immune system, as well as the role for cells that represent a link between the innate and adaptive systems, in implementing an effective antitumor response; (2) understanding high phenotypic and functional heterogeneity (plasticity) of these components; (3) realization of the leading role of intrinsic (genetically encoded) mechanisms for stress-/damage-associated molecular pattern-dependent (neoantigen-independent) recognition and induction of immune response against transformed or virus-infected cells; (4) gaining insight into the expanding role of the immune checkpoint mechanisms (which normally have a protective, homeostatic function) tumors can adopt to resist antitumor immune response. It is clear that any attempts to activate (in clinical or experimental settings) specific T cell-mediated immunity, which is based on the T cell receptor (TCR) recognition of tumor-associated antigens (TAAs) presented by the major histocompatibility complex (MHC), to naught under the influence of immunosuppressive tumor micro- and macroenvironment. In this regard, current research has an explicit priority to study innate (genetically encoded) mechanisms of activation and suppression (i.e., immune regulation) of antitumor (and antiviral) response and cell subsets responsible for these mechanisms, as illustrated by thematic searching PubMed database for papers published in the last 2 years.

Among the innate mechanisms of immune recognition and immune regulation, the recognition of cell stress associated with the key hallmarks of carcinogenesis (such as uncontrolled cell mass accumulation, metabolic abnormalities, oxidative stress, and cell death program impairment) deserves special attention. These innate sensing mechanisms can be exploited not only in cells of the innate immune system itself, but also directly in neoplastic cells [4] and presumably even in adaptive immune cells of the system (see below). In general, they serve to detect mislocalized, normally non-immunogenic, molecules that can be regarded as damage-associated molecular patterns (DAMP), with the involvement of specific cell sensors that

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trigger downstream signaling to produce cytokines and other factors necessary for activation of effector functions of innate immune cells [4]. Among these signaling pathways, the innate response to extranuclear/cytosolic or extracellular DNA activated by various molecular DNA sensors (expressed virtually in all cell types) is an example of the most actively studied mechanisms, with the cGAS-STING molecular pair playing the main part. It is important to note that the mechanism of immune response to mislocalized/cytosolic DNA within the tumor site largely overlaps with the mechanism of recognition of viral infection (especially, in the case of DNA viruses such as HPV). However, even for such a common pathway of antitumor response induction, a dual (tumor-suppressing or tumor-promoting) role defined by the etiology or the stage of a disease has been reported.

More specialized populations of innate immune cells are also equipped with a large variety of receptors to detect mislocalized/ectopically expressed biomolecules in cancerous or virus-infected cells. Lymphoid cells (natural killer (NK) cells, NK-like T cells, and Tγδ lymphocytes), tumor-associated macrophages (TAMs, M1, and M2-polarized), tumor-associated neutrophils/myelocytes (TANs, N1, and N2-polarized), myeloid-derived suppressor cells (MDSCs), and other immature dendritic cells—all these cell populations (both tumor-infiltrating and circulating) are the main object of studies published recently. For most of them (including some innate-like T cell subpopulations), it has been established that they can significantly contribute to tumor progression, and at the same time, a crucial role in the elimination of malignant cells has been proved for innate-like lymphocytes (see below). The most difficult aspect of the functioning of these types of cells is their ability to produce the widest spectrum of cytokines that depends on the surrounding "context," thus defining their regulatory properties. In this sense, their activity should be considered in conjunction with the activity of regulatory/suppressor T and B cells (Treg, Breg) and different T helper subtypes, including pro-inflammatory Th17/Th22 cells, especially in light of the fact that the inflammation is appreciated as one of the most important tumor-promoting factors. Despite significant progress in the study of innate mechanisms of response to a developing tumor, which is implemented by the cell populations named above, many researchers point out that most of the information on this problem is obtained using laboratory mouse strains, which are certainly indispensable as experimental models, but this information cannot be simply extrapolated to the human body and thus requires a separate verification. This is especially important in case of virus-associated carcinogenesis because, due to high species-specificity of oncoviruses and their strong cell-type tropism, the range of *in vivo* models adequately reproducing the terms of the long-lasting, chronic infection, and gradual development of neoplasia in humans (which can take months and years), is limited [5].

HPV-associated cervical cancer as an object for studying the dynamics of "pathogen-tumor-immunity" interactions draws increasingly more attention due to the newly emerging findings demonstrating that during HPV-associated carcinogenesis, the immune system (and its innate components, in particular) acts as a double-edged sword and its role dramatically changes during the course of disease development [2]. Most HPV infections and low-grade lesions regress spontaneously in a short time; these cases are proposed to be considered as an "acute" infection [3], which is accompanied with the activation of inflammatory response superior in strength to a variety of mechanisms exploited by HPV to suppress inflammation and escape from immune recognition. However, in a number of cases, the infection turns into a persistent form, thereby

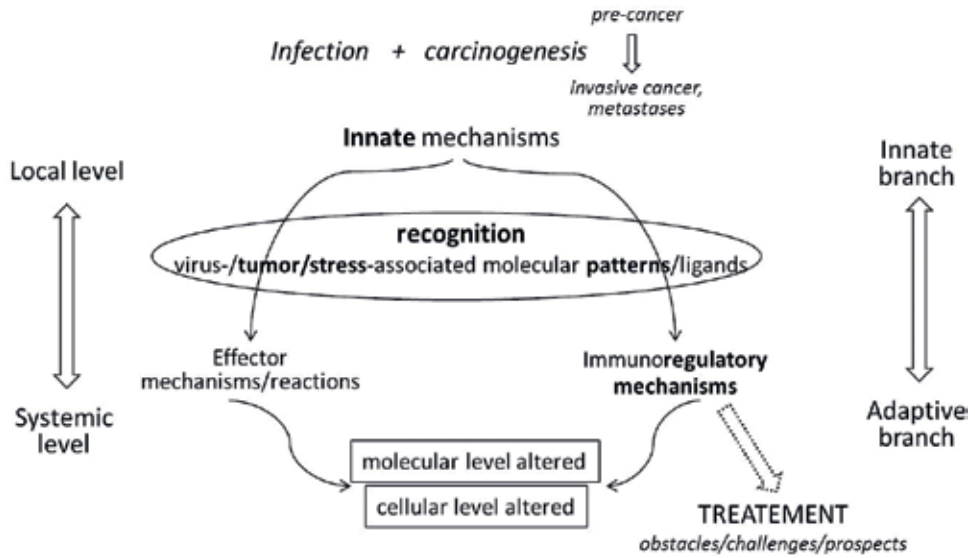

Figure 1. Scheme illustrating general relations between the key levels of immune response to cervical cancer that are addressed in the chapter.

increasing the risk of malignant transformation. In the later stages of carcinogenesis, in contrast to the stage of productive infection, HPV-transformed cells reprogram their environment in such a way that they gain the ability to recruit different populations of immune cells and to initiate chronic stromal inflammation, which contributes to further progression of precursor lesions into invasive cancer, facilitates tumor growth and metastastic spreading, and simultaneously promotes exhaustion of effector immune cells populations [2].

As a result of the fact that cervical cancer development is characterized by high genomic instability, the accumulated somatic mutations generate the enormous variety of neoantigens, which, together with the HPV-antigens, represent the potential targets for the T cell-mediated adaptive (TCR-restricted) response [6]. The range and immunogenicity (the ability to be presented to cytotoxic and helper T cells) of these antigens have been proved in high-throughput studies using integrated approaches to genome/transcriptome sequencing data analysis (see, for example, [7]). At the same time, the study by Qin et al. shows that increased mutation burden and neoantigen load correlates with HPV-dependent activation of master regulator genes that abrogate antitumor immune responses these neoantigens could cause by mobilizing immune regulatory, suppressive mechanisms. This again proves the rationale of studying the innate and innate-like lymphocytes, regulatory T/B lymphocytes, cells of myeloid lineage, as well as the mechanisms of antigen-independent innate immune response (including those involving DNA sensors) and the processes of immune regulation at different stages of cervical neoplasia development. In present chapter, the results of studies on these specific cell populations, mechanisms and processes published in last 2–3 years are described, with simultaneous discussion of our own experimental data on this problem, obtained from the patients with the diagnosis of pre- and microinvasive cervical cancer. Since a large number of constantly updated reviews are available on the issue of molecular strategies used by HPV to avoid immune response or other so-called cell restriction factors (see, for example, [8]), this question is not presented in the Chapter. In addition, we do

not discuss the preventive and therapeutic vaccines developed for cervical cancer, as one can find many specialized detailed articles devoted to this applied question (for example, [9–11]).

The issues which are accentuated in this Chapter are showed schematically in **Figure 1**. Those are: the relationships between local and systemic changes, cells of innate and adaptive arms of immunity, their regulatory and effector properties, their phenotypic and quantitative changes—at different stages of cervical cancer development. We give special attention to pre- and microinvasive cervical carcinoma when reporting our findings is due to the idea that these stages can be considered as tipping points in re-formatting of the host immune system.

2. Intrinsic molecular mechanisms bridging antiviral and antitumor immune responses in cervical cancer

2.1. The role of nucleic acid-sensing pattern-recognition receptors (PRRs) and related signaling pathways in controlling cervical cancer development: current concepts

To respond to ectopically localized nucleic acids of exogenous (infectious) or endogenous (tumor cell- or stressed cell-derived) origin, cells are "armed" with a set of nucleic acid-sensing pattern recognition receptors (PRRs). Members of this essential group of PRRs are expressed in cells of both immune (lymphoid/myeloid) and non-immune (for example, epithelial) origin and can recognize various forms of nucleic acids (single- and double-stranded DNA or RNA, DNA-RNA-heteroduplexes, CpG-islets, as well as specific chemical modifications or structures, typical for viral DNA/RNA, and messenger cyclic nucleotides) in different cellular compartments (cytosol, endosomes/phagosomes, and even in the nucleus). These include some representatives of Toll-like receptor family (TLRs: 3, 7, 8, 9), Absent in Melanoma 2 family, (AIM2, IFI16), RIG-I-like receptors (RLRs: RIG-I and MDA5), and other members of the DExD/H helicase family, as well as a "signaling pair" of cyclic GMP-AMP synthase (cGAS)—Stimulator of Interferon Genes (STING). In spite of the fact that these receptors/sensors activate different signaling pathways, they all eventually lead to the activation of transcription factors such as Interferon Regulatory Factors (IRFs) or Nuclear Factor kappa B (NF-kB), which are responsible for the production of type I interferons (IFN-I) or proinflammatory cytokines, respectively [12].

Among the listed molecular sensors, the STING protein is recognized as a signaling hub (**Figure 2A**): it can receive and redistribute signals coming from different upstream molecular partners, although the most well studied and, perhaps, most important for mammalian cells, is the cGAS-STING signaling axis [13]. Binding of cGAS with cytosolic DNA results in the synthesis of secondary messenger—cyclic dinucleotide cGAMP—a natural STING ligand; following interaction with cGAMP, STING (an endoplasmic reticulum membrane-resident protein) initiates assembly of a multiprotein complex (i.e., signaling platform) and, through activation of IRF3 transcription factor, triggers expression of a large number of genes, including IFN-I genes and IFN-stimulated genes (ISG). Moreover, the new data from high-throughput transcriptome analysis showed that depending on the cell type, STING can alter the expression of not only the immune response-associated genes, but also many other genes that govern crucial cellular processes (proliferation, apoptosis, and stress response) [14–16]. The existence of alternative pathways that lead to STING

activation (possibly ligand/agonist-independent) is also assumed, although the mechanisms have not yet been sufficiently described [17]. The key role of STING in antiviral innate immune defense has been confirmed by numerous studies, and it is not surprising, therefore, that different groups of viruses have evolved a variety of strategies to avoid/inhibit STING-dependent response, and oncoviruses are no exception: for most of tumor-associated mammalian viruses, STING, and other components of the STING-dependent signaling pathway were found to be specifically targeted by viral oncoproteins (in our previous paper, we summarized known mechanisms that are used by the oncoviruses, in particular, HPV, to evade STING-mediated recognition [18]).

The involvement of STING in regulation of the relationship between the tumor and the immune system (both innate and adaptive branches) mediated through the recognition of tumor DNA has been experimentally corroborated, although there are still many unresolved contradictions regarding its precise role in carcinogenesis: in different mouse tumor models, stimulation of expression, and/or activity of STING resulted in either restriction of tumor growth or tumor progression. What reasons could underlie these contradictions? On one hand, the STING-induced production of type I IFNs and activation of inflammatory reactions are obviously indispensable for the proper functioning of antigen-presenting cells (APC) and for further induction of adaptive antitumor response (discussed in [13, 19, 20]). On the other hand, the increased activity of STING leads to chronic inflammation within the locus of neoplasia which is a driving force of immunosuppression and tumorigenesis. In addition, there is still no clear understanding of exactly which cells within a tumor are responsible for STING-dependent recognition of tumor DNA. A previously proposed model, according to which it is phagocytizing cells (primarily dendritic cells and macrophages) that can engulf tumor DNA from dead/apoptotic tumor cells and activate the STING-signaling pathway, causes many doubts as it is not clear how endosomal/phagosomal DNA can reach cytosolic cGAS. Another model has been recently proposed, whereby the primary recognition of tumor DNA and synthesis of cGAMP occurs in tumor cells themselves because of the "leakage" of nuclear DNA into the cytosol (as a result of genomic instability, DNA damage, increased proliferation rates); cGAMP can diffuse to neighboring cells, including immune ones—presumably during the formation of immunological synapse—which are more efficient IFN-I producers and thus are able to promote recruitment of dendritic cells (DCs) and effector T cells [21]. APCs are widely recognized as such efficient producers, but other types of cells, for instance, lymphoid cells, can also be the candidates, considering that the level of STING mRNA/protein expression in lymphocytes was shown to be significantly higher than in macrophages [14, 16]. This model assumes that the initial stages of carcinogenesis are accompanied by an increased expression/activity of cGAS-STING, but as the tumor progresses, a disruption of cGAS-STING signaling—as a way to counterattack anti-tumor immunity—can occur. However, in virus-associated cancers, including cervical cancer, where STING activity can potentially be modulated by virus-derived and tumor-derived DNA, there may be the opposite sequence of events: in the initial phase of the establishment of a chronic infection, viral oncoproteins inhibit cGAS-STING pathway in infected cells (**Figure 2B**) and then, after undergoing malignant transformation, tumor cells gain the ability to support up-regulated state of cGAS-STING signaling in order to generate inflammatory immunosupressive microenvironment. Immunohistochemical study of HPV-infected cervical epithelium and low-grade cervical lesions indeed showed reduced expression of STING in relation to normal epithelium [22], but what changes are characteristic of high grade lesions and cervical cancer are as yet unknown.

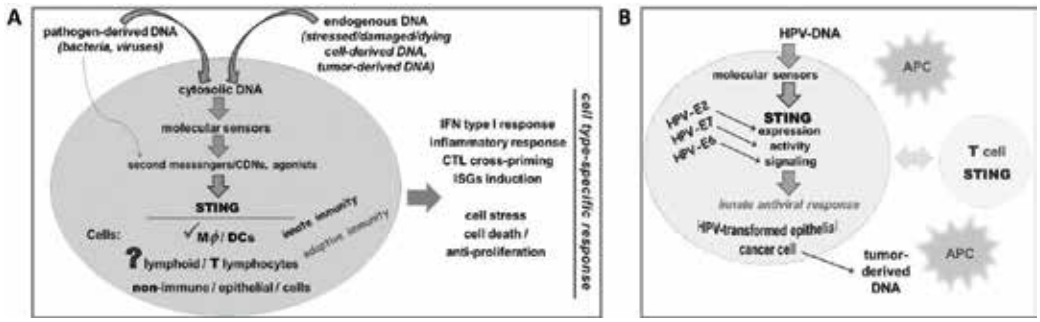

Figure 2. (A) Activating signals from various sources converge on STING to initiate cell type-specific innate response to cytosolic DNA. (B) In HPV-induced neoplastic lesions, STING can receive activating signals both from invading HPV-DNA and mislocalized self-DNA.

As mentioned before, STING-mediated signaling has been most thoroughly investigated in macrophages and dendritic cells while its role in other cell populations, specifically non-myeloid cells, is not fully understood. In this respect, recently published findings from *in vitro* and *in vivo* experiments (carried out using genetically engineered mice and STING ligands/agonists) demonstrating the functionality of canonical STING-dependent signaling in T cells [14–16] are of high importance. Surprisingly, besides activation of IFN-I response these experiments revealed T cell-specific ability of STING to modulate (inhibit) TCR-stimulated expansion and to induce cell death (through IRF3- and p53-dependent pathway), which is the fundamental difference from macrophages, in which stimulation of STING never leads to activation of death-associated genes [14–16]. The T cell-specific effect is extremely important for the prediction of therapeutic effect of STING agonists, which are currently undergoing extensive clinical trials as adjuvants in chemo-and immunotherapy of different types of cancer; however, in the case of cervical cancer the specificity of STING expression changes has not been investigated so far. At the same time, HPV-associated cervical cancer, in our opinion, can be used as a model object to study either cell type-specific or stage-specific involvement of STING in the innate/adaptive immune functioning at local and, most importantly, systemic level.

2.2. Altered patterns of STING expression indicate its putative role in cervical cancer

Based on the above facts, a study of the expression profile of STING (at mRNA/protein level) in tissue samples as well as in the major populations of peripheral blood T lymphocytes obtained from patients with preinvasive and microinvasive cervical cancer compared to healthy women (control group) has been started by our research team. We also took into account that: (1) increased expression of markers of apoptosis can be observed in circulating T lymphocytes in patients with early (pre-clinical) stages of cervical cancer [23]; (2) patients with early-stage cancer or precursor lesions display a variety of systemic alterations in the immune system including altered phenotype/activity and frequencies of different T cell populations, as evidenced by the large number of data (including those described below); (3) HPV-DNA (and possibly tumor DNA) circulates in the body and thus can be detected in various tissues and lymphoid organs long before the first detectable signs of metastases [24], whereby it potentially exerts a systemic effect on the activity of the STING.

2.2.1. STING protein levels in different subsets of circulating lymphocytes from early-stage cervical cancer patients

Intracellular STING level was measured in circulating CD4 and CD8 T cells, as well as in CD4CD25 subset (**Figure 3**) by flow cytometry using anti-human STING monoclonal antibody (MAb; clone 723505). Since the majority of lymphocyte population were stained positively for STING (which is in compliance with previously reported data showing that STING is robustly expressed in lymphoid tissue, specifically in T cells [14]), making the percentage values less informative, the level of STING protein was expressed as relative Mean Fluorescence Intensity value (ΔMFI) normalized to MFI of isotype control (IgG) with correction for autofluorescence of corresponding T cell subsets (Fluorescence Minus One, or FMO, control) (**Figure 3**).

As we did not find published works reporting on the level of STING in peripheral blood lymphocytes analyzed by means of immunofluorescence techniques, we first compared different commercially available kits for intracellular protein staining. The results of intracellular STING evaluation in peripheral blood T cells appeared to be sensitive to the permeabilizing ability of a fixation/permeabilization buffer set used, specifically: when kits designed for staining of intracellular proteins (such as cytokines) were applied, the level of anti-STING MAb binding did not differ from isotype control (**Figure 4A**); whereas the use of a reagent kit intended for intracellular detection of antigens such as nuclear transcription factors resulted in significant anti-STING MAb binding compared to isotype control (**Figure 4B**). This might be due to specific localization of STING and availability of its epitopes: homodimeric STING resides in the ER membrane and upon activation may form aggregates and translocate to Golgi and perinuclear space [25] (according to the manufacturer, the immunogen aa215-379 for the clone 723505 of anti-STING MAb corresponds to the C-terminal cytoplasmic domain of human STING).

In early-stage cervical cancer patients (with carcinoma *in situ* or microinvasive carcinoma), the level of STING protein showed a decreasing trend in both CD4 and CD8 T subsets compared to

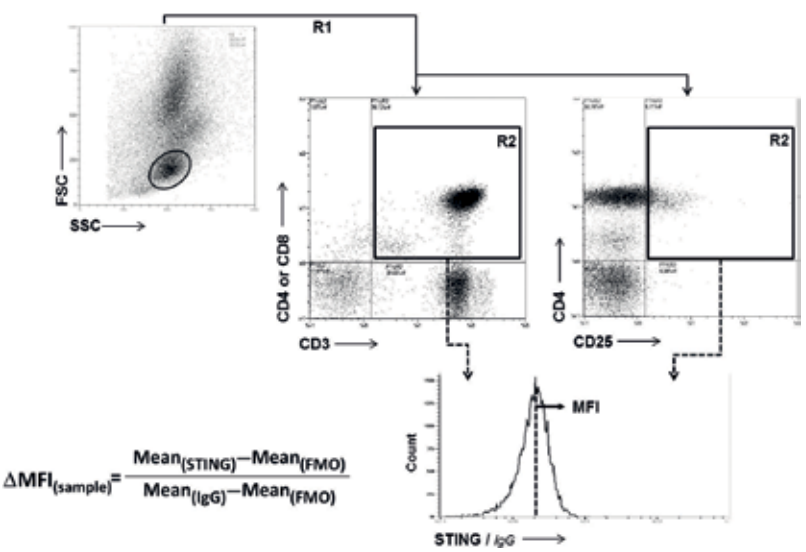

Figure 3. T cell gating and evaluation of STING protein level.

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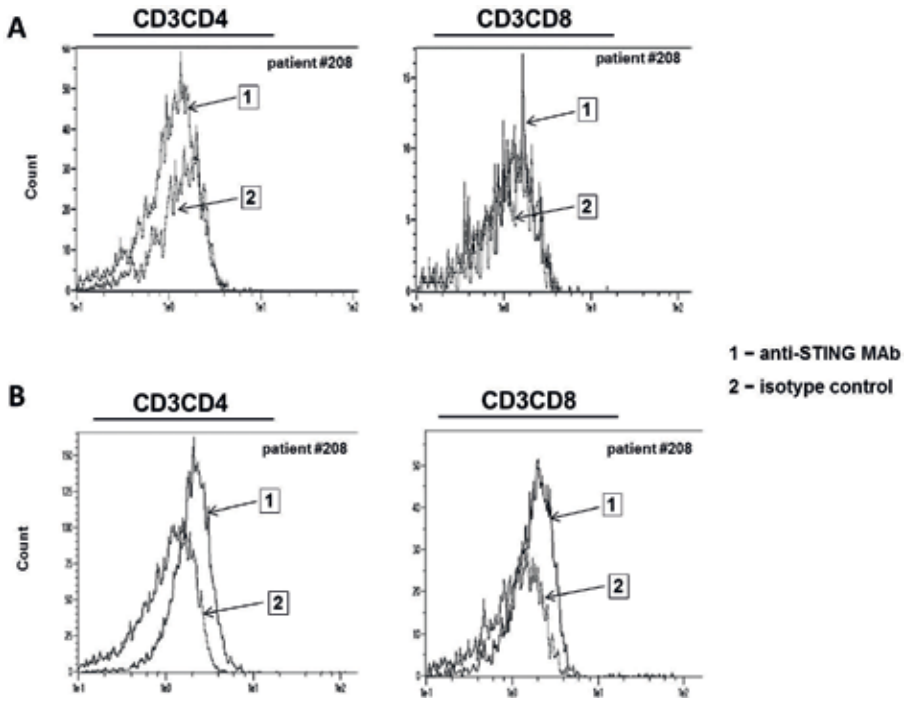

Figure 4. Representative examples of STING staining carried out using (A) a reagent buffer set for staining cytosolic proteins (e.g., cytokines), or (B) a reagent kit with stronger permeabilizing capacity for intracellular/nuclear protein staining.

healthy controls with this decrease being more pronounced in CD8 T lymphocytes (**Figure 5A**). No significant change was observed for CD4CD25 subpopulation. A notable increase in ΔMFI (CD4CD25)/ΔMFI (CD3CD8) ratio was revealed for circulating T cells from cancer patients (**Figure 5B**), implying that STING expression became more pronounced in CD4CD25 lymphocytes in relation to CD3CD8 subset. At the same time, the difference between STING levels in CD3CD4 and CD3CD8 cells from both controls and cancer patients was less significant; ΔMFI(CD3CD4)/ΔMFI (CD3CD8) ratios were close to 1 in all groups studied suggesting that the expression of STING is associated with both CD4 and CD8 T cell subsets. These results are, in a certain sense, in consistence with data reported previously by others for mouse models [14].

The percentage of STING-positive cells in total population of circulating lymphocytes from cervical cancer patients was on average lower than that in the control group, although this difference was not statistically significant ($p > 0.05$, U-test; **Figure 6**). When analyzing CD3 T cells, the same trend could be observed (while the total frequencies of T cells did not differ between patients and controls).

2.2.2. STING mRNA expression in peripheral blood mononuclear cells (PBMC) and neoplastic tissue samples

At the mRNA level, STING expression was analyzed in ficoll-isolated PBMC using semi-qPCR (RPLP0 and PGK1 genes were used as endogenous controls [26]): similar to flow cytometry results, in PBMC from patients with preinvasive/microinvasive cancer (stage 0-IA), STING-mRNA

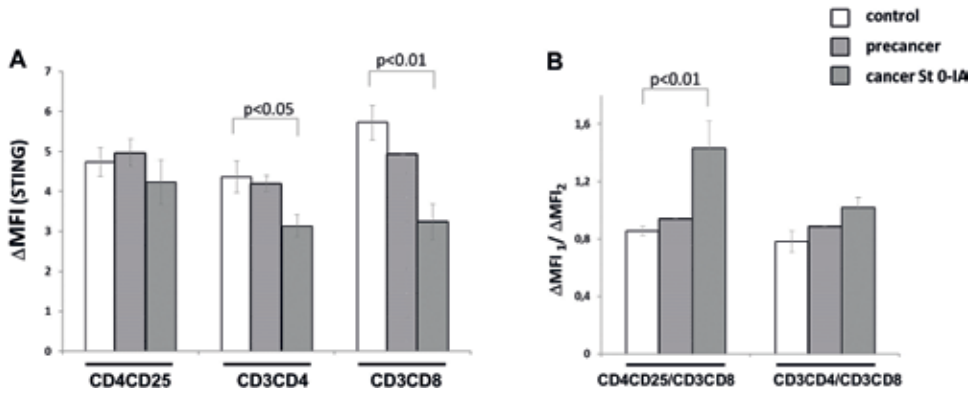

Figure 5. (A) The change of STING protein levels in major subsets of peripheral blood T cells from patients with precancerous cervical lesions or cancer (stage 0-IA, n = 20) relative to the control group of healthy donors (n = 15). (B) The ratio of the relative STING expression level in different T cell populations. Mean ± SEM values are displayed; p-value was assessed by U-test.

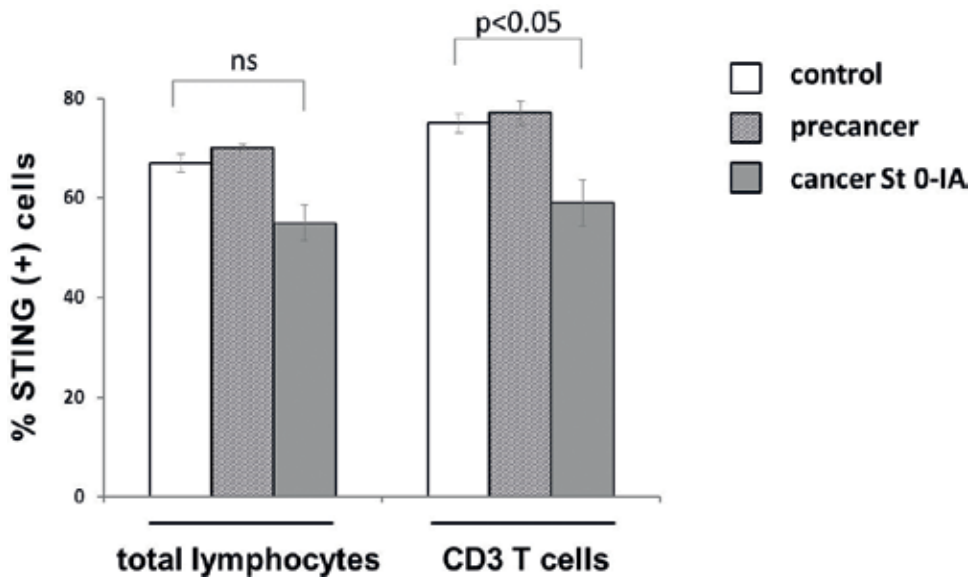

Figure 6. Percentage of peripheral blood lymphocytes stained positively for STING in patients with precancerous cervical lesions or cancer (stage 0-IA, n = 20) vs. healthy donors (control, n = 15). Mean ± SEM values are shown, ns—not significant.

level showed a slight decrease compared to the control group ($p > 0.05$, U-test; **Figure 7A**) suggesting the need for T cell (CD4/CD8) separation in further analysis. STING-mRNA expression was also assessed in samples of HPV-negative morphologically normal epithelium (control), HPV-positive precancerous lesions of the cervix, carcinoma in situ and microinvasive carcinoma (relative to four genes—EEF1A1, ACTB, GAPDH, and RPLP0—taken as endogenous controls due to their proved constitutive expression in cervical tissues [27]) (**Figure 7B**). In contrast to lymphocytes, a considerable (up to 50%) proportion of pathological samples

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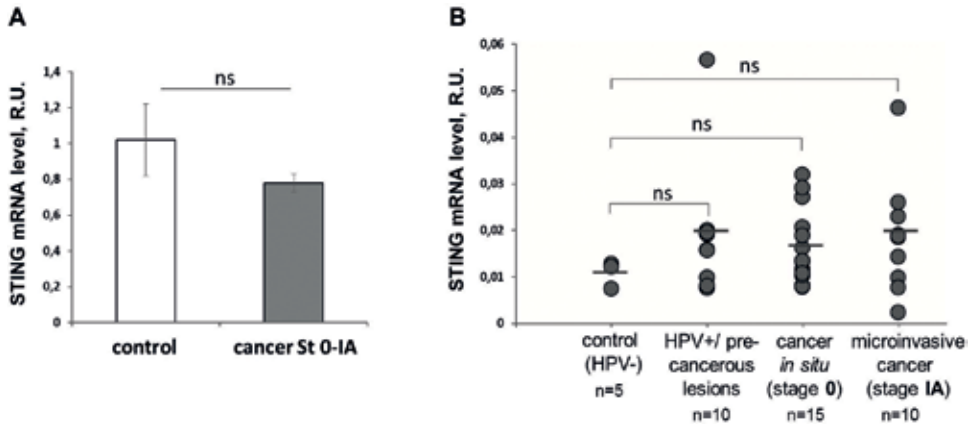

Figure 7. (A) The change of STING mRNA expression in PBMC isolated from cervical cancer patients compared to healthy women (controls); mean ± SEM values are shown. (B) The change of STING mRNA expression in cervical neoplastic lesions; group mean values are depicted as horizontal bars; ns—not significant.

in each patient group showed elevated STING expression as compared with normal non-infected epithelium (though the group mean values did not differ statistically). Up-regulation of STING at early stages of cervical carcinogenesis is consistent with some previous reports by other researchers and is, overall, in line with the conception of dichotomous role of STING-pathway in tumor development [28]. The data may also indicate STING's participation in as yet unexplored mechanisms promoting tumor development: for example, c-MYC proto-oncogene which overexpression is a hallmark of cervical cancer has been recently described as an essential transcription factor for the STING gene [29]).

Taking into account that cervical carcinogenesis can be associated with decreased proportion of STING-expressing T lymphocytes, as well as decreased level of STING protein in both T cell subsets (CD4 and CD8), one may assume the involvement of T cell STING in controlling papillomavirus infection and HPV-induced oncopathology. On the other hand, despite the lower level of STING observed in total CD3CD4 population of patients' peripheral blood lymphocytes, in CD25-positive subpopulation its expression was sustained at levels similar to the control: as CD25 is known to be a Treg marker, as well as a T-activation marker, this may be related to the processes of T cell activation/proliferation, interleukin (IL) 2-signaling, and T cell death. This assumption can be confirmed by recent findings [14, 15] demonstrating anti-proliferative or cell-death promoting activity of STING in TCR-stimulated T cells. Previously, we also showed up-regulation of apoptotic processes in circulating lymphocytes from early-stage cervical cancer patients [23], which was correlated with the expansion of CD25-positive cells (including FoxP3-expressing Treg) prompting further investigation of STING in T cells during virus-related carcinogenesis. Thus, the study of naturally occurred cervical neoplastic pathology that develops as a result of chronic viral infection suggests that STING being a key player in modulation of innate immune reactions may have an essential role in T cell functions. During the development of infection-related cancer, the importance of this specific role can be realized through redistribution of STING levels in different T cell subsets. Oppositely directed changes in STING expression observed in different compartments—blood T lymphocytes and neoplastic tissue—may illustrate the putative dual role of STING in virus-related

carcinogenesis, which, in turn, may represent an important point in prognosing therapeutic outcome of STING stimulation. While administration of STING agonists may occur beneficial, for example, for patients with T cell-derived cancer (or other lymphoproliferative disorders) due to promotion of apoptosis in malignant T cells [16], mobilization of STING activity in solid tumors may have an opposite effect due to increased apoptosis of T effectors.

Summarizing, it is worth noting that the abundance of STING in T cells may imply; on one hand, their engagement in the innate immune mechanisms (as was revealed by a study of Larkin and co-authors who observed induction of intact antiviral IFN-I response in mouse T cells upon stimulation with STING agonists [14]) or, on the other hand, the plausibility of noncanonical functions exerted by human STING in cells of the adaptive immune system, these issues to be further investigated in the norm and in various pathological states, including virus-induced cervical cancer. In conclusion, it is worth mentioning that such noncanonical activity of STING, specifically, ability to switch on the apoptotic pathway has been unraveled not only in T cells, but in murine B lymphocytes (normal and malignant) as well [30]. However, in another study, the expression of STING in human B cells could be detected only upon Epstein-Barr virus-mediated transformation, while normal B lymphocytes were unable to elicit IFN-I response upon treatment with STING agonists due to the absence of STING expression [31]. Regarding other types of lymphocytes, for instance, NK cells, there is limited or no information. According to our flow cytometry data, the level of STING protein in circulating natural killer cells from patients with cervical carcinoma in situ is notably lower than in CD3CD4 and CD3CD8 (ΔMFI for CD3negCD16pos population was 2.16±0.16), but nonetheless 35 ± 4% of NK cells appear to be STING-positive suggesting potential involvement of STING in NK cell functions.

3. Cellular component of innate immunity (natural killer lymphocytes, myeloid cell populations): its role in regulation of T cell-mediated antitumor/antiviral immunity

3.1. Regulatory functions of innate immune cells in relation to cervical cancer development: current knowledge

The regulatory role of myeloid cells (monocytes—dendritic cells and macrophages, and especially granulocytes) has been undervalued for a long time; however, recently emerged data have prompted reconsideration of significance of these cells, classically regarded as professional phagocytes or professional APC, in mediating regulatory/suppressor effects of tumor cells on T-effectors [32]. In addition, the systemic effect of local neoplastic lesions on deviations within these innate immune cell populations, which can become detectable even earlier than the distribution of tumor-infiltrating cell populations is changed, is becoming increasingly apparent [32]. In respect of these abnormalities, a number of fundamentally important data have been obtained for cervical cancer.

According to the model described by Smola et al., IL-6 secreted by HPV-transformed cells acts as a triggering factor that leads to multiple impairments in the key functions of myelomonocytic cells during the intraepithelial stage of cervical cancer development. Under the influence of IL-6 and chemokines, myelocytes are actively recruited into the site of neoplasia, where they can

differentiate into functionally impaired dendritic cells or M2-polarized macrophages to maintain pro-inflammatory environment. Despite they have mature phenotype, dendritic cells are not able to migrate to the lymph nodes to initiate adaptive response due to the lack of appropriate homing receptors; instead they accumulate within cervical cancer stroma and secrete protumorigenic and Th2-polarizing factors. Cervical cancer-infiltrating M2-macrophages not only fail to produce IFNs at levels required for T cell activation and proliferation, but also express ligands for the immune checkpoint molecules, for example PD-1L, thereby promoting cytotoxic T cell exhaustion [6, 32–34]. Interestingly, according to Swangphon et al., cervical cancer patients exhibit altered ratio of M1/M2-polarized (CD64+/CD163+) monocytes not only at the local level, but in systemic circulation as well; notably, circulating M1/M2 ratio was shown to be correlated with the number of stroma- or peritumoral area-infiltrating M2-macrophages (CD163+), and with severity of the disease [35]. Similarly, cervical cancer patients displayed increased numbers of circulating dendritic cells (CD11b+) expressing PD-1L [36]. Moreover, an increase in the number of tumor-promoting M2-macrophages/ monocytes has been found to occur not only locally, i.e., in the tumor site, or systemically, i.e., in circulation, but also in tumor-draining lymph nodes (TDLN) of cervical cancer patients implying that the number of PD-1L+ M2-macrophages and metastasis are interrelated; this association allows to suppose that metastasizing cancer cells have the ability to recruit CD14+ monocytes and drive their conversion into M2-macrophages further contributing to the expansion of highly suppressive Treg cells [34].

Progression of precursor lesions into cervical cancer is also accompanied by an increase in the number of infiltrating neutrophils (TANs) displaying suppressive phenotype. A negative correlation found between the amount of TANs and CD8 T cells in high-grade lesions (cervical intraepithelial neoplasia grade 3, CIN3) or cervical cancer samples suggests that TANs can potentially contribute to inhibition of T cell activity and thereby facilitate tumor growth [32]. This assumption was confirmed experimentally in *in vitro* cell system using co-cultures of SiHa-spheroids, *ex vivo*-stimulated T lymphocytes, and neutrophils, with the ratio of T cell/neutrophil numbers appeared to be the determining factor for the degree of suppression of T cell proliferation, their expression of activation markers, secretion of IFNγ, and cytotoxic activity against SiHa cells [32]. At the systemic level—in the peripheral blood of cervical cancer patients—higher frequency of immature low density neutrophils has been also revealed, with elevated serum levels of granulocyte colony stimulating factor (G-CSF) discovered not only in cervical cancer patients, but also in women with precursor lesions (CIN2-3). Furthermore, patients diagnosed with cervical cancer are characterized by a systemic increase in the frequency of the tolerogenic monocyte-derived dendritic cells (MoDCs), the differentiation of which is modulated by G-CSF: MoDCs that were differentiated from monocytes taken from patients with CIN3 or cervical cancer and showing higher serum level of G-CSF were able to significantly more intensively inhibit proliferation of T cells from healthy donors and to promote Treg differentiation in the *ex vivo* system [32]. The effect of cervical neoplastic lesions on the process of MoDCs differentiation (expression of maturation markers, the profile of secreted cytokines) has been also demonstrated in a study by Lopes et al. [37]. Altogether, these data once again prove that early neoplastic lesions can be accompanied by systemic deviations in innate immunity, which in turn can influence redistribution of innate and adaptive cell populations and their interactions with each other within the tumor locus. The entirety of systemic and local immune changes is also an important point to consider when developing antitumor therapies based on adoptive DC transfer, because it is obviously these changes that determine the absence of the desired therapeutic effect (such developments

aimed at overcoming the suppressive impact on DC are conducted using preclinical murine models of cervical cancer, see, for example, [38, 39]). In addition, a study performed by van Meir et al. showed that myeloid cells from cervical cancer patients can systematically respond to radiotherapy (RT): during the course of RT and 3–9 weeks after its completion (regardless the administration of cisplatin), increased frequencies of circulating CD3(-)CD19(-)HLA-DR(+) monocytes as well as CD3(-)CD19(-)HLA-DR(-) MDSCs were detected in parallel with the loss of T cell reactivity and stimulatory capacity of APC in *ex vivo* testing [40].

Unlike neutrophils and suppressor populations of myeloid cells, whose contribution to the progression of solid tumors has only recently come under intense investigation, the functions of natural killer cells have always been considered in the context of cancer immunosurveillance. However, in spite of the fact that for this group of innate lymphoid cells, a detailed spectrum of receptors allowing for recognition of transformed cells has been described and a vast diversity of mechanisms for their cytotoxic action has been established, attempts to use them in anticancer therapy occurred to be unsuccessful—the reasons for this situation are reviewed in [41], and among these reasons are the underappreciated regulatory properties of NK cells implementing via production of a wide range of cytokines, the specificity of which is largely determined by the surrounding molecular context. Nevertheless, recently there has been considerable revival of interest in NK cells brought about by the invention of chimeric antigen receptors (CAR) technology that made possible creation of engineered CAR-NKs with "improved" properties (e.g., increased migrating and proliferating ability, up-regulated expression of activating receptors) for their subsequent adoptive transfer into a cancer patient. Another promising concept seems to be the use of Cord-Blood NK cells that can retain a highly activated phenotype and whose expansion capacity substantially exceeds that of peripheral blood NK cells (successful implementation of this approach in the preclinical model system using cervical cancer cell lines has been recently reported in [42]).

Cervical cancer cells' ability to withstand NK cell-mediated response is clearly confirmed by the observation that the prevalence of NK cells in CD45(+)-infiltrating leukocytes is greatly reduced with the progression of intraepithelial neoplasia to invasive cancer [32]. In addition to the known mechanisms recruited by cervical cancer cells to escape from NK-mediated recognition (including down-regulation of activating NK-cell receptor ligands MICA/B, ULBPs, or aberrant expression of non-classical HLA-G [43]), inhibition of NK cell activity can be driven by intra-tumoral Tregs, as was confirmed in ex vivo experiments with Tregs and NK cells isolated from primary tumors of cervical cancer patients [44]. Whether these negative processes have any influence on circulating NK cells during the development of cervical cancer remains a poorly studied question.

Despite the high phenotypic heterogeneity of NK cells, they can be divided into two subsets depending on the level of expression of CD56 marker: CD56bright and CD56dim. These two populations differ not only phenotypically and functionally—they are differently represented in the systemic circulation and tissues [45]. CD56dim population comprises the vast majority (80–95%) of peripheral blood NK cells and is characterized by high expression of markers of mature phenotype (including CD16/FcγRIIIa required for activation of antibody-dependent cytotoxicity, perforin and granzyme B cytotoxic proteins); traditionally, this population is associated with anti-tumor response. Unlike CD56dim, CD56bright NK cells represent the minor population in peripheral blood, while in the secondary lymphoid organs and other tissues CD56bright cells account for the majority of peripheral NKs. In addition, they are characterized by the absence or low expression of CD16 (CD16dim/neg) and low cytotoxic activity, so their role in direct

killing of tumor cells is less clear; on the other hand, CD56bright NK cells are known for their high cytokine and chemokine production capacity (including IFNγ, TNFα, GM-CSF, IL-10, IL-13, CCL3, and CCL4), and immunomodulation of activity of other innate or adaptive immune cells is therefore believed to be a key feature of CD56bright NK cell subset.

Presently, increasing attention is being paid to CD56bright NK cells as new facts are emerging suggesting that there is no strict functional dichotomy between the so called regulatory CD56bright and cytolytic CD56dim subsets, and that CD56bright cells are capable of acquiring cytotoxicity upon appropriate stimulation with specific combinations of cytokines [45]. Indeed, it has been recently shown that priming of CD56bright NK cells with IL-15 is accompanied by a burst of cytotoxic activity against tumor cells; however, this has only been confirmed so far for hematological malignancies [46]. Upon treatment with different stimuli, CD56bright NK cells exhibit ability of suppressing proliferation of autologous CD4 T cells via both cytotoxic and immunoregulatory mechanisms, e.g., by secreting the immunosuppressive molecule adenosine (these mechanisms are reviewed in detail in [47]). For some types of solid cancers, in particular lung cancer and breast cancer, the proportion of CD56bright cells in a total amount of tumor-infiltrating NK cells was found to be significantly higher than in the corresponding normal tissues, however, they express low perforin and rather play an immunoregulatory role, but not cytotoxic [48].

3.2. Analysis of NK cell subpopulations in peripheral blood lymphocytes of early-stage cervical cancer patients

Taking into consideration, the proposed model that describes the ability of CD56bright NK cells to circulate among tissues, lymphoid organs, and peripheral blood [45, 48], it can be assumed that altered frequencies of these cells in the blood of cancer patients are highly relevant to immune regulation at the tumor locus. Quantitative assessment of circulating CD56bright NK cell population has been performed for head and neck cancer [49], prostate [50], and breast cancer [51, 52]); we also recently reported our findings concerning circulating NK subsets in women with CIN3 (including carcinoma in situ) and microinvasive carcinoma (stage IA1) of the cervix [23].

Based on the intensity of CD16/CD56 staining, we could distinguish four main subsets of circulating NK cells within CD3-negative lymphocytes (gates P1–P4, **Figure 8**). As expected, we found no significant difference in the frequency of cells within CD16brightCD56dim gate (which encompasses the major pool of circulating cytotoxic NKs), as well as within CD16dim/negCD56dim and CD16brightCD56neg gates (comprising less abundant populations with poorly established functions) between patients and controls. As opposed to these subsets, a decrease in the frequency of CD16dim/negCD56bright NK cells and, accordingly, higher CD56dim/CD56bright ratio were observed in cervical cancer patients relative to the control group. We hypothesized this specific alteration reflects a systemic shift in the balance between effector and regulatory NK subsets that occur early in invasive cervical cancer development. One can also speculate this change, along with those described above for M2/M1, neutrophils and MoDC subsets, is part of a complex cervical cancer-related immunoregulatory network. As it is well known that activation of NK cells occurs locally, in our attempt to interpret the obtained data we therefore use the idea that circulating CD56bright NKregs are recruited to the lymphoid tissue (regional lymph nodes) and the primary tumor site, where they are thought to serve as precursors for cytotoxic/effector CD56dim NK cells [45]. This assumption encourages further investigation into the regulatory role of NK cells in cervical cancer progression.

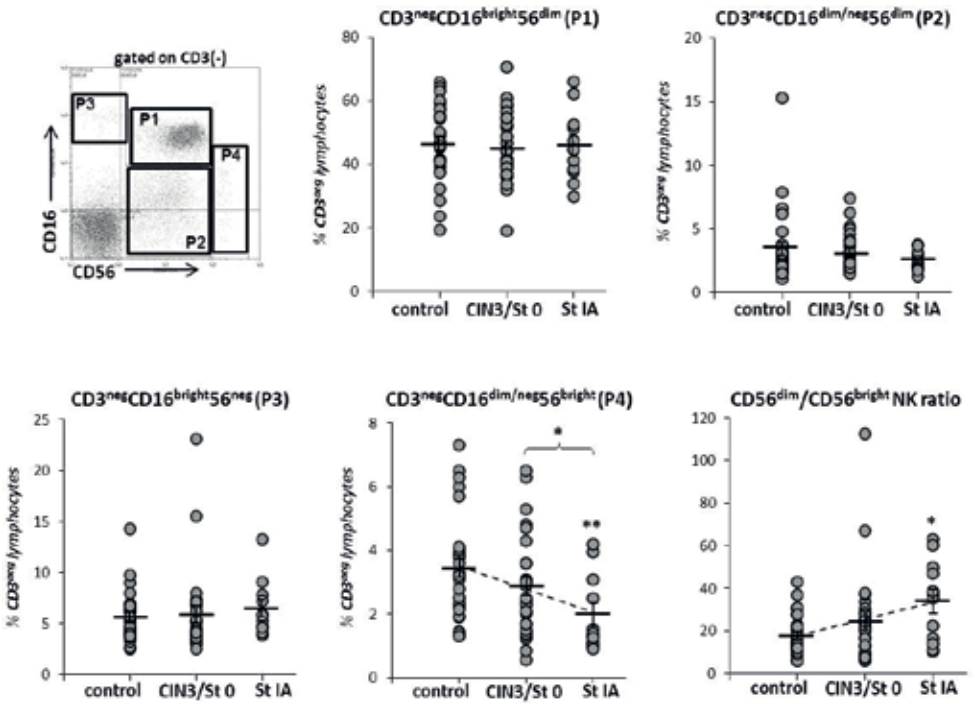

Figure 8. Percentage of peripheral blood CD56bright NK cells and CD56dim/CD56bright ratio within circulating NK cell population in patients (n = 30 for CIN3/stage 0, n = 15 for stage IA) vs. healthy controls (n = 30) as measured by flow cytometry. Lymphocytes were gated for CD3-negativity (a diagram on the left) and a population of interest was defined according to CD16/CD56 membrane expression levels (gates P1–P4). Here and below, individual values are shown as dots; bars correspond to the mean ± SEM values; statistically significant difference between the patient group and the control group are marked with asterisk: *p < 0.05, **p < 0.01 (U-test).

4. Innate-like T lymphocytes: pivotal players in the tumor: immunity interplay

4.1. The emerging role of NK-like T cells (NKT) and γδT lymphocytes in cervical cancer progression

Since recently, a heterogeneous group of innate-like T lymphocytes linking the two branches of immunity, innate and adaptive, is being increasingly acknowledged as a valuable source of novel opportunities for antitumor therapies development. This was facilitated by increasing realization of the pivotal role of innate-like T cells in tumor immune surveillance and their unique ability to recognize cancerous and virus-infected cells in a highly specific, though MHC-unrestricted, manner. Similar to innate immune cells, they are equipped with a rich set of germline-encoded receptors conferring them ability to undergo rapid activation upon interaction with ectopically expressed or stress-associated molecules on the surface of target cells. In addition, like conventional αβT cells, innate-like T lymphocytes (NKT and γδT) express T cell receptors (TCR), although their repertoire differs from αβT cells. The range of antigens the innate-like lymphocytes' TCRs are able to recognize is defined by their structural properties (for example, lipid antigens in the case of NKT cells

or phosphoantigens in the case of γδT cells) and therefore is thought to be restricted and universal; at the same time, these antigens are present within a plenty of natural ligands, which is doubtless advantageous from a therapeutic standpoint. Quick activation in response to antigenic exposure followed by intense production of a broad range of cytokines is another valuable characteristic of innate-like lymphocytes they have in common with typical innate lymphocytes; it is known, for instance, that even in the absence of stimulation NKT cells permanently stay in pre-activated state. That is why innate-like lymphocytes supposedly perform "guarding" functions by being the first to respond efficiently to pathological changes (infection, transformation) and stimulate further activation of dendritic cells and adaptive response. However, in spite of their apparent beneficial properties, there are several features that greatly impede potential manipulations of innate-like lymphocytes, among them are: high structural and functional population heterogeneity, low abundance, heterogeneous distribution of different subpopulations in tissue and blood compartments, ability to provoke chronic inflammation and to secrete not only Th1-cytokines, but Th2 as well. If structural heterogeneity of innate-like lymphocytes is defined by their receptor repertoire, their functional heterogeneity is believed to be driven by polarizing factors coming from the environment. Importantly, conclusions about existing functional subtypes of innate-like lymphocytes were made based mostly on the results of in vitro stimulation [53–55].

Like conventional T lymphocytes, NKT cells express αβTCR, but can undergo activation only on interaction with lipid antigens presented by CD1b (a nonpolymorphic MHC-I-like molecule). In spite of such a relatively narrow specificity, NKT cells however exhibit an important feature—ability for TCR-independent activation upon stimulation with proinflammatory cytokines IL-12, IL-18, IL-25, and IL-23. According to the structure and binding specificity of TCRs, two NKT subsets can be distinguished: NKT-I, or iNKT—invariant NKT cells (with α-galactosylceramide being a prototypic ligand), and NKT-II cells—variant NKT having less restricted specificity. NKT-II cells are thought to be the most prevalent NKT subset in humans (in contrast to, for example, mice, where NKT-I cells are known to be more abundant), although their identification and characterization is still a challenging task due to the lack of distinctive NKT-II markers or agonists specifically targeting their receptors. In general, following the results of *in vivo* modeling of various cancers, NKT-I cells have been associated with the protective antitumor response, while NKT-II have been implicated in immunosuppression/immunoregulation and tumor promotion. The mechanisms of antitumor activity of NKT-I cells consist in their ability for both direct tumor lysis and generation of copious amounts of IFNγ (along with other Th1 cytokines) required for recruitment and activation/full maturation of APC, CD8 cytotoxic T lymphocytes, and NK cells. Immunosuppressive effect of NKT-II cells is thought to be due to their ability to produce high levels of IL-4 and IL-13 that shift immune response towards Th2 type. Nevertheless, this functional dichotomy is at present actively debated, and there is growing conviction that it is not so firmly associated with NKT-I or -II subset; rather, it is determined by the context (for example, tissue location) or microenvironment where activation of NKT cells occurs [53]. (Due to limited space, in our characteristic of NKT cells and γδT cells, here and below we refer to several recently published comprehensive reviews that contain links to original papers).

In spite of the relatively low abundance of NKT cells, there is constantly growing body of evidence showing this cell population undergoes quantitative and phenotypic changes (both in peripheral blood and within the tumor locus) in patients with different types of cancer, however there is only scares information available for cervical cancer. It has been found that

HPV can escape from NKT cell-mediated CD1d-restricted recognition of infected keratinocytes and low-grade cervical neoplastic lesions via HPV-E5 dependent inhibition of CD1d expression (while normal keratinocytes express high levels of CD1d molecule) [56]. Despite this evasion mechanism, CIN2-3 lesions were shown to be associated with increased numbers of infiltrating iNKT, with these numbers being higher for HPV-positive lesions than for HPV-negative [57]. Elevated frequency of circulating NKT have been revealed in peripheral blood of women with CIN1 and HPV infection, compared to the control group or HPV-positive women without signs of neoplastic abnormalities [58]. It can be inferred from these findings that the population of NKT cells may undergo early changes upon persistent HPV infection and progressing neoplasia, although there is no data available for more advanced stages of the disease.

γδT lymphocytes differ from both conventional T cells and NKT cells in their TCR chains composition and ability to recognize phosphoantigens, while many other features characteristic of NKT cells are shared by γδT as well, specifically: rapid activation, direct cytotoxicity against infected or transformed cells, reliance on natural killer receptors that enable fast (MHC-independent) response to stress-related ligands expressed on the surface of cancer cells, strong regulatory properties and ability to modulate activity of other immune cells via production of a wide range of cytokines. Further, similar to NKT cells, γδT also demonstrate functional heterogeneity (polarization) with regard to antitumor response, with this heterogeneity partially overlapping with the structural features of γδTCR, but nevertheless being mostly driven by differential environmental stimulation, as was mentioned for NKT cells. In humans, Vδ2 T cells were found to be the most frequent subpopulation of peripheral blood γδT cells (70%); Vδ1 T cells constitute the remaining 30% of γδT in circulation, although they represent the dominant γδT subset in epithelial and some other tissues. Antitumor activity of Vδ2 T cells is attributed to not only their ability to directly recognize (via congenital receptors) and kill tumor cells, but also their ability to effectively cross-present antigens to CD8 αβT effectors and NKT cells, as well as facilitate DC maturation and co-stimulate cytolytic activity of NK cells. Pro-tumor role of Vδ1 T cells can be explained by their IL-17-producing ability; at the same time, however, these cells show unique specificity for B7-H6 molecule expressed exclusively on tumor cells, being able thereby to exert antitumor effect. Immunoregulatory (suppressive) function of γδT cells in antitumor immunity is thought to be mediated by IL-10 and TGF-β, or adenosine secreted by tumor-infiltrating γδT cells [54, 55].

The impact of γδT cells on pathogenesis of cervical cancer is largely unexplored. Gosmann and co-authors analyzed total population of CD45+IL-17+ cells infiltrating CIN2-3 lesions and found them to be represented by not only CD3CD4 T helpers (Th17), but also by γδT cells, although the percentage of γδT cells was significantly lower than that of Th17 [59]; this observation may indicate their putative involvement in the promotion of proinflammatory suppressive microenvironment as CIN progresses to invasive cancer. The cytotoxic activity of γδT cells isolated from PBMC against cervical cancer cell lines (HeLa, SiHa, and CaSki) pre-treated with bisphosphonate pamidronate was also confirmed in [60]. A vast amount of clinical data on the role of γδT cells in viral infections, as well as their correlation with cancer prognosis allows speculation on γδT cell involvement in pathogenesis of virus-associated cervical cancer. Whether this cell population experiences any changes at different stages of cervical cancer, and if so, in which tissue compartments or depending on which clinical-pathological parameters—remains an open question.

Taken together, innate and innate-like (NK, NKT, and γδT) lymphocytes proved to have non-redundant functions in antitumor immune response, which makes them attractive objects

for the development of adoptive cell transfer therapy combined with immune checkpoints blockade or neutralization of other immune-suppressive factors [55, 61]. At the same time, the results of preclinical studies and attempts to translate them to clinical settings explicitly point to our insufficient knowledge of the role of innate-like lymphocytes and the mechanisms, whereby they contribute to cancer progression [53, 55].

4.2. Analysis of CD3+CD56+ population and its CD3bright subset in PBMC from early-stage cervical cancer patients

CD56 is a natural killer prototypic marker, but, apart from NK cells, its expression is shared by T lymphocytes (NKT and γδT) and is commonly considered as a marker of an activated state, NK-like cytotoxicity and IFNγproduction [62]. Accordingly, CD3+CD56+ population comprises of both NKT and γδT lymphocytes, but within this population, a CD3bright subset can be observed [63]. Studies on phenotyping of CD3bright subpopulation have identified it as γδT lymphocytes [63, 64]. Furthermore, Paget and co-authors [64] established mouse CD3bright γδT cells were identical to Vδ1 sub-lineage (Vγ6/Vδ1+ TCR) and possessed high IL-17-producing capacity; the high CD3 expression (CD3bright phenotype) could hence be considered as a surrogate marker of γδT identity. In humans, the population of circulating CD3brightCD56+ cells has been analyzed in patients with chronic hepatitis B infection: it has been shown that, despite increased numbers, their activity and phenotype are substantially impaired, this impairment includes down-modulation of IFNγ and LAMP1 expression (i.e., markers of antiviral and killing activity) and, conversely, up-regulation of NKG2A [65].

Given, the described data on the ability of CD3bright T cells to respond to inflammation and chronic infection observed either in model animals or in humans, in the norm or under pathological conditions, we decided to examine whether changes of the frequency of this cell population could be detected in the circulation of patients at early stages of cervical cancer progression (**Figure 9**). Using clone UCHT1 of anti-human CD3 MAb, we were able to clearly identify subpopulation of CD3bright lymphocytes in peripheral blood of CUN3/cervical cancer patients and the controls. In spite of the relatively wide range of individual values in all studied groups, a trend towards a decreased number of circulating CD3brightCD56+ cells ($p > 0.05$) was observed for women with microinvasive carcinoma (gate P1, **Figure 9**); at the same time, no difference in the frequency of cells falling within gate P2 and the total frequency of CD3+CD56+ (including CD3+CD16+/-CD56+) cells was revealed (data not shown). Then, within the CD3bright population, we also analyzed the expression of CD16, a marker of antibody-mediated cytotoxicity, but did not find any significant difference between the controls and the patients groups (data not shown). According to Lambert et al., CD3bright T cells (i.e., γδT) do not express CD4, but express low levels of CD8 [63]. We compared the frequencies of CD3brightCD8low cells (gate P3) between the study groups, but again did not observe any difference. Therefore, in contrast to regulatory NK cells, the combination of CD3/CD16/CD56 markers is not sufficient to show if there are any significant changes occurring within the population of circulating innate-like T cells in early-stage cervical cancer. Although these results cannot be compared with the results reported by Pita-Lopez et al., who used the same combination of CD markers to analyze blood samples from women with low-grade lesions (CIN1), nevertheless, observations made for CD3brightCD56 cells, along with published data mentioned above underline the need for continuing investigation into innate-like lymphocytes at various stages of cervical cancer development and progression with the use of lineage-specific (e.g., anti-TCR) antibodies.

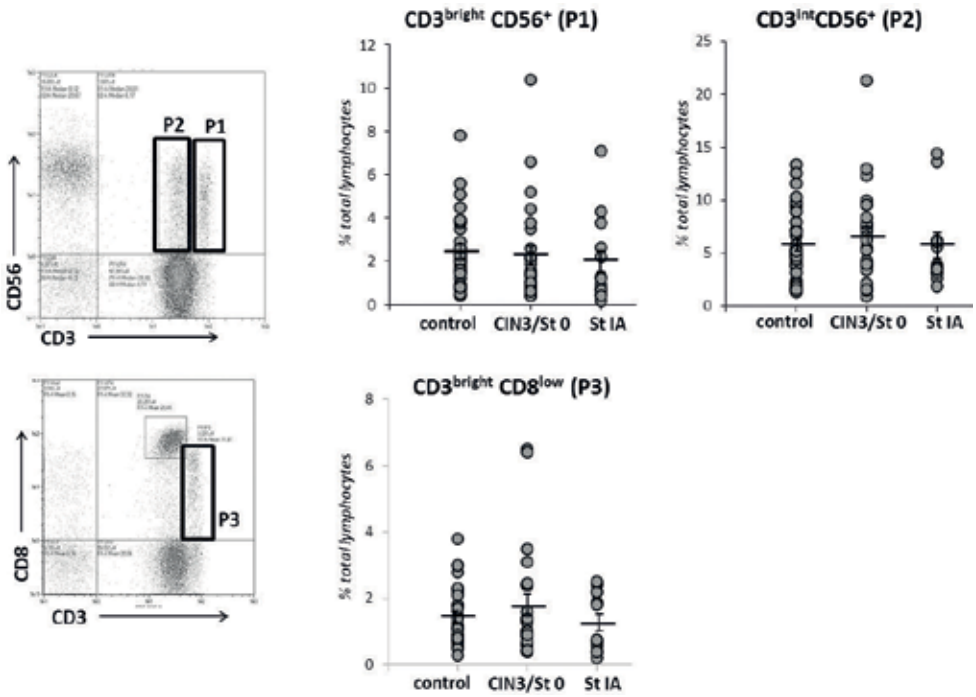

Figure 9. Percentage of peripheral blood T cells with NK-like phenotype in patients with CIN3 or microinvasive carcinoma (St IA) and healthy controls. Lymphocyte populations of interest were defined according to CD3/CD56 expression levels (gates P1-P3).

5. Regulatory T and B cells and immune checkpoint molecules in cervical cancer: at the crossroads of immune suppression mechanisms

Mobilization of intrinsic immune checkpoint mechanisms by a tumor to restrict antitumor immune response is one of the topical issues currently discussed; the phenomenon of effector cell exhaustion and the expression of checkpoint markers have been described for various innate/acquired immune cell populations, including innate-like cells. Regarding cervical cancer, one can observe an avalanche of new data emerged in recent 2 years on the expression of immune checkpoint markers, first of all PD-1/PD-1L, a hallmark of T cell exhaustion caused by chronic antigenic stimulation [66], as well as other members of B7 and CD28 protein families (e.g., B7-H3 [67] and B7-H4 [68]). For example, patients with CIN or cervical cancer show increased expression of PD-1 both in infiltrating lymphocytes and macrophages (TAMs) [69, 70], as well as in circulating CD4 and CD8 T cells [36], and, furthermore, in the sentinel lymph nodes [71]. Cervical neoplastic cells are considered as the primary source of PD-1 Ligand (PD-1L) [69, 70], with HPV16-E7 oncoprotein proved to be the driving force for elevated PD-L1 expression [72] and copy number gains of PD-L1 gene being one of the putative underlying reasons [73, 74]. Tumor-infiltrating and stromal M2 macrophages are another such source [34]. Finally, there is one more important source of PD-L1 among adaptive immune cells represented by regulatory T cells (Treg); a

correlation between PD-L1 expression and FoxP3+Treg was reported by Ma et al. [75]. Moreover, CD4CD25 Tregs are able to upregulate PD-1 expression in patients with CIN/ cervical carcinoma [36], which, however, does not result in Treg exhaustion, but, conversely, favors upregulation of their immunosuppressive activity. Lastly, it has been reported that regional lymph nodes from stage IB1 cervical cancer patients, along with elevated PD-1, have increased expression of FoxP3 Treg-marker, which may shed light on the establishment of pre-metastatic niches [71], as well as on systemic expansion of suppressive mechanisms Tregs are engaged in.

Several studies have previously reported on increased frequency of circulating CD4 Tregs at initial stages of cervical cancer development [76–78]. Furthermore, we have recently confirmed systemic expansion of Tregs within not only CD4 cell subset, but within CD8 subset as well, at as early as preinvasive and microinvasive cancer (**Figure 10**); we have also revealed correlations between the number of circulating Tregs and the T cell expression of markers of apoptosis, whose induction is supposed to be one of the mechanisms mediating exhaustion of T effector pool during cervical cancer progression [23]. In parallel, the search of new mechanisms providing conditions for Treg expansion during the course of cervical cancer progression is continued: for example, it has been recently found that cervical cancer cells, as well as mesenchymal stromal cells isolated from cervical tumor tissue can upregulate CD73 ectonucleotidase to generate high amounts of adenosine, a potent inducer of Treg differentiation and recruitment [79, 80].

Apart from regulatory T cells, the potential involvement of regulatory B lymphocytes (Breg) in cervical cancer promotion should not be ignored. This can be supported by the results obtained

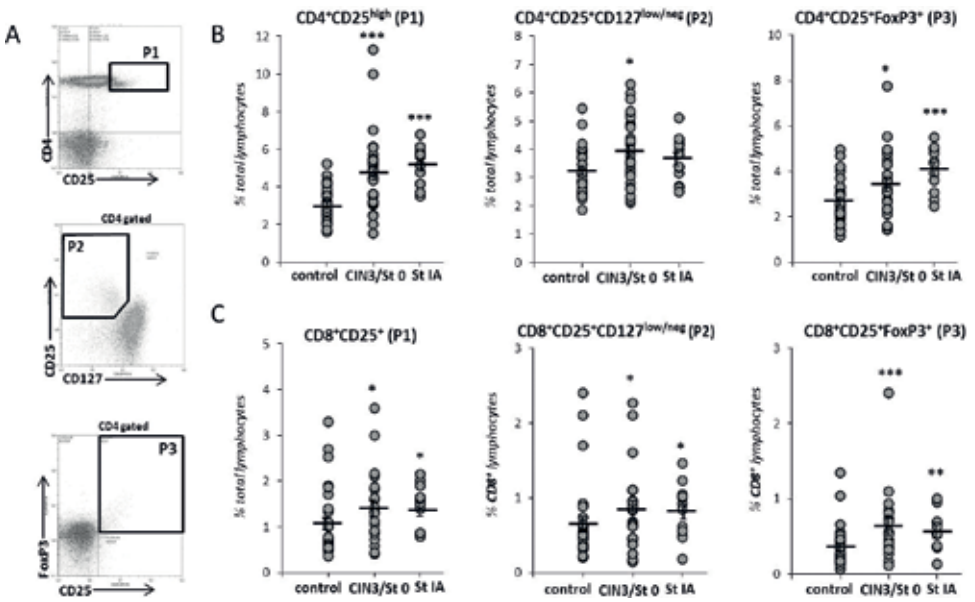

Figure 10. The frequencies of peripheral blood Treg lymphocytes in patients with CIN3 or microinvasive carcinoma (St IA) and healthy controls. (A) CD4 Tregs were gated according to the level of CD25, CD127, and FoxP3 expression; gating of CD8 Tregs was performed in a similar way. (B) The change in the frequency of circulating CD4 regulatory cells in patients compared to healthy donors. (C) The change in the frequency of circulating CD8 regulatory cells in patients compared to healthy donors: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (U-test).

by Tang et al. who used mouse model of HPV-related cancer to demonstrate that Bregs accumulate in tumor-draining lymph nodes, have altered phenotype (specifically, altered expression of cell surface markers, such as MHC II, PD-L1, and CD39), exhibit high regulatory potency, thus fostering tumor growth [81]. In humans, many types of solid tumors were found to be accompanied with increased numbers of both tumor-infiltrating and circulating Bregs capable of producing suppressor cytokines (e.g., IL-10) and immune checkpoint ligands, thus impairing T cell function (see reviews [82, 83]), suggesting this issue to be investigated for cervical cancer patients.

6. Conclusion

Further ways to develop approaches for the treatment of HPV-associated malignancies, including cervical cancer, belong to the area of combined therapies, where particular attention is to be paid to restoration the effectiveness of innate mechanisms of immune response, including those trigged by PRRs (e.g., STING). PRR agonists are expected to serve potent adjuvant function; another promising area is the use of agonists to stimulate NK, NKT, and γδT receptors. Despite substantial progress, there is clear understanding that stimulation of innate immune cells "per se" is senseless without concomitant inhibition of immunosuppressive factors (such as inhibitory molecules of immune checkpoint or other Treg-associated factors). Therefore, as illustrated by recent findings summarized in the chapter, there is obvious need for continuing comprehensive characterization of functional diversity of innate immune cells that organize cervical cancer immune regulatory network, exploration of noncanonical functions of innate immunity mediators, identification of precise resources of immune suppression and assessments of local and systemic changes in immune parameters.

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Conflict of interest

Authors have no conflict of interest to declare.

Abbreviations

APC Antigen-presenting cell

Breg Regulatory B cells

CAR Chimeric antigen receptor

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CD	Cluster of differentiation
cGAMP	Cyclic GMP-AMP
cGAS	cGAMP synthase
CIN	Cervical intraepithelial neoplasia
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
G-CSF	Granulocyte-colony stimulating factor
HPV	Human papillomavirus
IFN-I	Type I interferon
IL	Interleukin
IRF	Interferon regulatory factor
MAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cells
NK	Natural killer cells
NKT	Natural killer-like cells
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood monocyte cells
PRR	Pattern recognition receptor
SEM	Standard error of the mean
STING	Stimulator of Interferon Genes
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T cell receptor
TDLN	Tumor-draining lymph nodes
TGFβ	Transforming growth factor beta

Th	Helper T cell
TLR	Toll-like receptor
Treg	Regulatory T cell

A. Appendices and nomenclature

A.1. Experimental procedures

Patients and specimens: Samples of peripheral blood and epithelial tissue were obtained from 55 patients who were diagnosed with CIN2-3 (including cancer *in situ*) or microinvasive carcinoma (FIGO stage IA1) and underwent surgery in Oncological Dispensary of the Republic of Karelia. CIN and cervical cancer diagnosis was based on comprehensive physical examination, extended colposcopy findings, cytology, and histopathology tests, in full compliance with the approved standards for the diagnosis and treatment of patients with gynecological malignancies. All women engaged in this this study were informed and gave voluntary written consent. The research was approved by the Committee on Medical Ethics of Petrozavodsk State University and the Ministry of Healthcare and Social Development of the Republic of Karelia, and was done in accordance with the Declaration of Helsinki and good clinical practice guidelines. All women from patient group were positive for oncogenic HPV types (with the prevalence of HPV16 > 80%). Thirty healthy non-pregnant women without cervical abnormalities and HPV-infection at the time of blood sampling served as normal controls. Venous blood was collected immediately before the surgery or any other treatment and immediately processed for multicolor flow cytometry. Tissue samples were submerged in RNA stabilizing reagent right after excision and stored at −80.

Flow cytometry: The following fluorophore-conjugated monoclonal antibodies were used: CD3-APC (Clone: UCHT1), CD4-FITC (Clone: MT310), CD8-FITC (Clone: DK25), CD16-FITC (Clone: DJ130c), CD56-RPE (Clone: C5.9) (Dako, Austria), CD25-APC (Clone: 4E3), CD45-VioBlue (Clone: 5B1), CD127-RPE (Clone: MB15-18C9), FoxP3-RPE (Clone: 3G3) (Miltenyi Biotec, Germany), STING/TMEM173-RPE (Clone: 723505, R&D Systems, USA). For blocking of non-specific antibody binding, FcR Blocking Reagent (Miltenyi Biotec) was used. For intracellular detection, cells were fixed and permeabilized using "FoxP3 Staining Buffer Set" (Miltenyi Biotec). Cells were acquired on a MACSQuant Analyzer flow cytometer (Miltenyi Biotec) and analyzed using MACSQuantify software.

Real-time PCR: Total RNA was extracted from tissue samples or ficoll-isolated PBMC with Trizol Reagent (Invitrogen). cDNA was synthesized from DNAse I-treated RNA (1 mg RNA per 1 reaction volume) using ProtoScript II (New England BioLabs, UK) or RevertAid First Strand cDNA Synthesis Kit (Fermentas, ThermoScientific, USA). Amplification was performed in StepOnePlus thermal cycler (Applied Biosystems, USA) using qPCRmix-HS-SYBR+HighROX reaction mix (Evrogen, Russia).

Statistical analysis: Data analysis was performed using R software. Mann-Whitney U-test was used to evaluate the differences between the patient and the control groups; the difference was considered to be statistically significant at $p < 0.05$.

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This book entitled *Cervical Cancer - Screening, Treatment and Prevention Universal Protocols for Ultimate Control* is the fourth successful endeavor of the Editor with InTech publisher. The four books serve as four pillars in cervical cancer control, globally. This book is unique and sensational. Public health "topics" are assuming a status of "phenomenon," through deliberations and research. Screening is riddled with global limitations of availability, affordability, acceptability, and accessibility. The treatment of precancers has questionable efficiency. Prevention is costly with the inclusion of HPV vaccine. This book helps to find the solutions. The authors, editor, and InTech publisher wish the readers a pleasant and purposeful reading. This book is a "readers' feast," "receivers' choice," and "respondents' delight." Enjoy and treasure the international facts and flavors.

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