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Current Perspectives in Human Papillomavirus

Edited by Shailendra K. Saxena

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Infectious Diseases

Volume 2

Prof. Shailendra K. Saxena is vice dean and professor at King George's Medical University, Lucknow, India. His research interests focus on understanding the molecular mechanisms of the host defense during human viral infections and developing new predictive, preventive, and therapeutic strategies using Japanese encephalitis virus, human immunodeficiency virus, and oncogenic viruses as a model, via stem cell and cell culture technologies. His research work has been published in various high-impact factor journals (*Science*, *PNAS*, and *Nature Medicine*) with high citations. He has received many awards and honors in India and abroad, including various Young Scientist Awards, the BBSRC India Partnering Award, and Fellows, and he has been named the "Global Leader in Science" by *The Scientist* magazine (USA) and "International Opinion Leader/Expert" involved in the vaccination for Japanese encephalitis by IPIC (UK).

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Scope of the Series

The series will give a most comprehensive overview of recent trends in various infectious diseases (as per the most recent Baltimore classification), as well as general concepts of infections, immunopathology, diagnosis, treatment, epidemiology and etiology to current clinical recommendations in management of infectious diseases, highlighting the ongoing issues, recent advances, with future directions in diagnostic approaches and therapeutic strategies. This book series will focus on various aspects and properties of infectious diseases whose deep understanding is very important for safeguarding human race from more loss of resources and economies due to pathogens.

Contents

Preface

Globally, human papillomavirus (HPV) is one the most sexually transmitted infectious agents. About 5% of all cancers are attributed to HPV with enormous cases of cervical and other sites of cancers. Global epidemiological and virological studies may help to understand the origin, evolution, carcinogenesis, and immunopathogenesis of HPV, which divulge the opportunities to effectively treat and prevent the global disease burden of HPV. The most prevalent HPV types (16 and 18) are responsible for 70% of cervical cancers, including precancerous cervical lesions. Identification of precancerous lesions during the screening of HPV cases allows us to effectively treat disease progression and combats at least 80% of cervical cancer cases in developed countries. However, the limited access of preventive measures in developing countries often results in poor early detection and treatment. In addition, the scarcity of advanced levels of treatment during later stages of infection is limited, which results in a higher morbidity profile.

Current Perspectives in Human Papillomavirus covers a collection of articles by brilliant researchers who have devoted their time to combat HPV. This book gives a comprehensive overview of recent advances in HPV, as well as general concepts of molecular biology of HPV infections, epidemiology, immunopathology, prevention, and current clinical recommendations in the management of HPV. It examines novel insights into future directions in the prevention and therapeutic strategies that control HPV infection in developed and developing countries. The book also focuses on various aspects and properties of HPV, whose deep understanding is very important for safeguarding the human race from further loss of resources and economies due to pathogens. This book aims to target the interdisciplinary experts of medicines, including researchers, gynecologists, pathologists, microbiologists, oncologists, healthcare physicians, epidemiologists, policy makers, and funding agencies. This book will be a part of the book series "Infectious Diseases," series editor Shailendra K. Saxena.

I anticipate that this work will augment attention in this field of research and prospective readers will irrefutably find it useful for their clinical usage, management, and further exploration. I would like to thank our contributors, colleagues, family, and friends who gave us a lot of encouragement and support during the production of this book.

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Section 1

Introduction

Chapter 1

Recent Advances in Human Papillomavirus Infection and Management

Shailendra K. Saxena, Swatantra Kumar, Madhu Mati Goel, Apjit Kaur and Madan LB Bhatt

Abstract

Human papillomavirus (HPV) accounts for approximately 4.5% of all cancers which differs at the level of economic development and geographical regions. The life cycle of the HPV is completely dependent on the epithelium differentiation without the involvement of cell death and systemic viremia. Carcinogenesis is the consequence of viral gene expression, dysregulated cell proliferation, and genomic instability. Keratinocytes are the target cell for HPV which act as the physical and immunological barrier. In cervical carcinogenesis, the enhanced level of Th17 infiltration has been observed which increases with the disease progression and is coupled with CCL20 expression in the stromal mesenchymal compartment. IL-6 and M-CSF are known as "switch factors" which are imperative for pro-tumorigenic response in monocytes. Screening of cervical cancer includes three major procedures: cytology, nucleic acid test, and co-testing. For evaluating anal lesions, high-resolution anoscopy is performed which is similar to colposcopy. Prophylactic vaccination is the primary preventive measure to control the HrHPV infection and reduce the burden of HPV-related cancer. The precancerous stage of HPV infection includes excision, ablation, and immunotherapy. Radiotherapy is the acceptable primary treatment for the early stage of anogenital cancer, whereas for the advanced-stage metastatic cancer, palliative therapy is the only option.

Keywords: HPV, infection, management, prevention

1. Introduction

Human papillomavirus (HPV) attributes to approximately 4.5% of all cancers which differs at the level of economic development and geographical regions [1]. *Papillomaviridae* family comprises of more than 200 types of HPV which are classified into five genera: *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus*, and *Nupapillomavirus* [2]. All the genera are responsible for the various types of HPV-associated cancers. Furthermore, on the basis of oncogenicity, the mucosal type (alpha) is grouped into two subtypes which are low risk (LR), HPV 6 and HPV 11, which are known to cause benign genital warts and high-risk (HR) cervical cancer [3]. HPV 6 and HPV 11 are also known to cause respiratory papillomatosis predominantly in children [4]. According to IARC classification, high-risk HPV (HrHPV) is of 14 types: HPV 16, HPV 18, HPV 31, HPV 33,

HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 66, and HPV 68 [5]. High-risk papillomavirus is responsible for causing cancer related to the cervix, vulva, vagina, anus, penis, and oropharynx [6]. HPV 16 and HPV 18 represent approximately 60% for adenocarcinoma of the endocervix (ADC-CX), and both are equally associated with 15% of adenosquamous carcinoma (ASC) of the endocervix [7]. Among the HrHPV, HPV 16 is the most efficient in evading the host immune response and responsible for persistent infection in the oropharynx, an imperative step in development of malignant lesion [8]. The mucosal HPV is transmitted by sexual contacts, and the transformation zone in females is characterized as the area adjacent to the border of the ecto- and endocervix which are the preferential sites of primary infection [9]. Approximately 80% of the sexually active women get HPV infection where most of them are asymptomatic with the immune system-mediated clearance of infection within 6–12 months [10]. With the impairment of immune function, the infection becomes symptomatic in a small number of individuals. Persistent infection of HPV and the development of cervical intraepithelial neoplasia (CIN) after the period of latency may lead to the regression or progression toward the development of invasive carcinoma [11]. The prevalence of HPV infection is higher in immunocompromised individuals such as HIV [12] and organ transplant recipients (OTRs) [13] suggesting the role of T cell in the clearance of HPV. Other factors including smoking, parity, sexual habits, genetic factors, oral-contraceptives have shown to increase the risk of progression of HPV-driven diseases [14].

Beta genus of HPV principally accounts for progression of nonmelanoma skin cancer (NMSC). The association of beta HPV types with skin cancer can be explained as the prevalence of epidermodysplasia verruciformis (EV). Patients diagnosed with EV are highly susceptible for HPV beta-type infection which results in flat warts and pityriasis versicolor-like lesions [15]. With the advancement detection methods, beta HPV types have been shown to be abundantly present in the skin of the asymptomatic HPV-infected individuals. So far, 43 HPV of beta genus have been isolated. Furthermore, beta HPV types have been speculated as HPV facilitates the accumulation of ultraviolet-mediated DNA damage [16]. In order to prevent the disease, the neutralizing antibody (nAb) has been shown to provide protection. However, immunization with attenuated or killed pathogen is imperative.

2. Global HPV epidemiology

The global burden of HPV-driven cervical cancer accounts for approximately 630,000 cases annually [1] where five other HPV-associated sites of cancer are responsible for further 113,400 cases [17]. In spite of the availability of several preventive strategies, HPV subtypes cause worldwide morbidity and mortality particularly in the less developed countries (**Figure 1**) [18]. Asia contributes to the immense majority of cervical cancer cases of 285,000 with 144,000 deaths followed by Africa where 99,000 cases with 60,000 deaths and America with 83,000 newer cases with 36,000 deaths. Asia, China, and India contribute to 62,000 and 123,000 cases along with 30,000 and 67,000 deaths, respectively [19]. Data from the meta-analysis and systemic reviews are suggesting that the vast majority of the infection is asymptomatic and transient. The incidence of HPV infection particularly cervical cancer is higher in sub-Saharan Africa (SSA) [20]. The worldwide distribution of age-specific HPV infection also varies with the predominance in young women of age less than 25 years in Europe and America [21]. However, in Africa and Asia no significant decline with the age has been observed. 3.2% of HPV 16 infected women shows normal cytology [22]. Cervical precancerous lesion is an imperative factor for HPV prevalence where HPV is detected in 52.5%

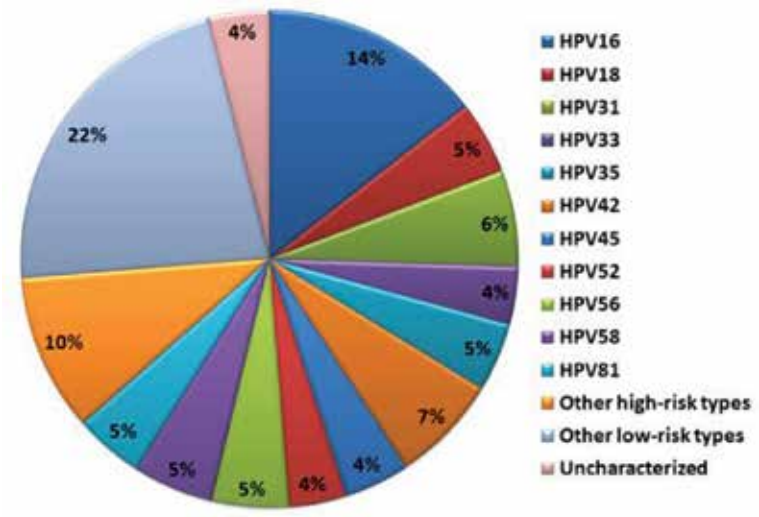

Figure 1.
Worldwide prevalence of HPV (n = 1938) [adapted from ref. 18].

of ascus lesion, 74.8% of low-grade cervical lesions, and 88.9% of high-grade cervical lesions. HPV 16 has been detected in 19.3% of low- and 45.1% of high-grade cervical lesions [23]. Furthermore, anal HPV infection is the most frequent HPV infection of other anogenital areas. The global burden of anogenital cancers caused by HPV is very high where 88% of anal and <50% of cases attribute to the lower genital tract [24]. About 30,000 cases are reported in men with the 56.56% attributed to the anus and 43.33% to penile origin. In the case of women, 38,500 cases are reported with 46.75% cases attributed to the anus, 22% to the vulva, and 31.16% from the vaginal origin [1]. Furthermore, the predominance of anal HPV infection that has been observed in the men sex with men (MSM) is 58.8% or HIV-infected individuals. The oral HPV infection significantly differs by gender and with higher incidence in men [19]. The worldwide prevalence of HPV-associated head and neck cancer is 8.15%, that is, 37,200 cases, where 77.95% of cases belong to the oropharynx, 11.82% of cases correspond to the oral cavity, and 10.21% of cases correspond to the larynx [1].

3. HPV genome and replication

Human papillomavirus is a histone-bound double-stranded DNA virus of ~8 kb with either eight or nine open reading frames (ORFs) on the same DNA strand. HPV genome can be subdivided into three regions: coding region comprises of early genes such as E1, E2, E4, E5, E6, and E7; region encoding major (L1) and minor (L2) capsid proteins; and a noncoding region present between ORFs L1 and E6 known as long control region (LCR), involving in viral DNA replication and transcriptional regulation [25]. HPV genome is highly conserved; however, exclusive features are represented by different genera. As compared with the mucosal HPV types, beta HPV genome is relatively shorter due to reduced size of LCR (400 bp) which ranges from 7.4–7.7 kb, whereas the E2 ORF is relatively larger in beta HPV types (**Figure 2**) [26]. Apart from this, most of the beta HPV types lack E5 gene with the exception of HPV 14 [27]. An additional early protein, E8Ê2C, is expressed exclusively by some of the HrHPV types as HPV 16, HPV 18, and HPV 31 that repress the expression of viral oncoproteins E6 and E7 following proliferation [28].

Recently, three gamma HPV types (101, 103, and 108) have been isolated from the cervical specimens which lack the E6 ORF [29].

Epithelial abrasion is the typical site of HPV infection where the squamocolumnar junction (SCJ) of the cervix is susceptible to HPV transformation [9]. The life cycle of the HPV is completely dependent on the epithelium differentiation without the involvement of cell death and systemic viremia. Heparan sulfate proteoglycans (HPSGs) are exposed by the epithelial abrasion which is the binding site for L1 and defined as the extracellular event of HPV replication [30]. Upon binding the conformational changes in the capsid expose the N terminus of L2 which gets cleaved by extracellular furin, a prerequisite for the virus internalization [31]. Virus uptake is mediated by L1 and endosomal vesicles, following transport of endosomal virus to the nucleus along with the retromer. L1 is degraded, whereas L2 forms the complex with viral genome following the escape from endosome to the trans-Golgi networks [32]. L2 entry into the nucleus is dependent on the cell cycle progression and transpired upon transient nuclear membrane breakdown [33]. The L2 genome complex interacts with ND-10, a promyelocytic leukemia nuclear bodies following initiation of early viral transcripts [34]. The host DNA replication machinery system is utilized by the viral early proteins E1 and E2 to establish 50–100 episomal copies per cells [35]. Dependence on host DNA replication system is the reason for the slow rate of virus evolution due to proofreading mechanism. The productive phase of HPV life cycle exhibits a unique spatial and temporal regulation and differentiation of keratinocyte in squamous epithelium. Upon leaving the basal membrane, HPV enters into the productive phase of life cycle which can be characterized as high copy number (1000 copies per cell) of HPV genome along with

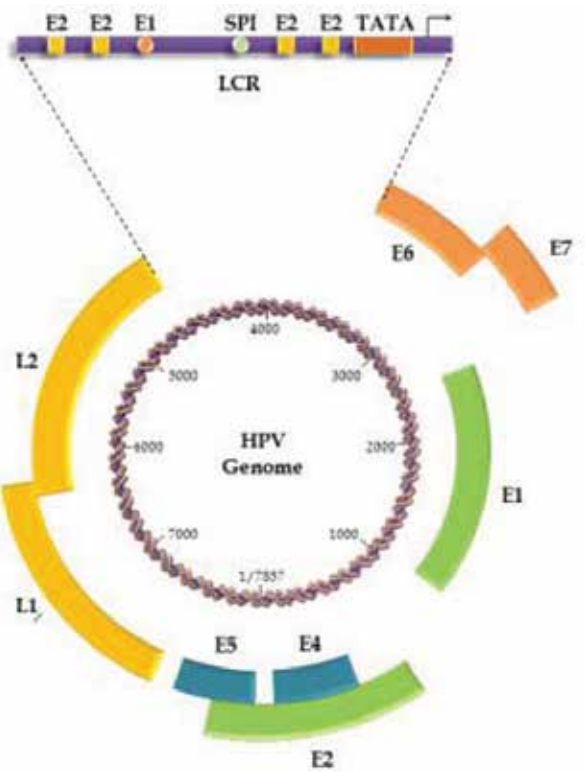

Figure 2.
HPV genome.

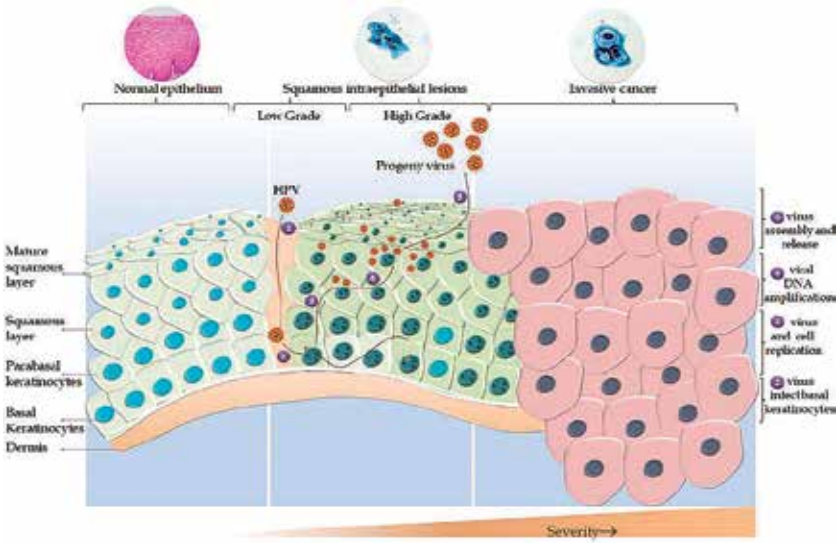

Figure 3.
HPV life cycle.

the late viral capsid expression for assembly transpired only in the upper terminally differentiated epithelial layer (**Figure 3**) [36].

Several of the host factors also play an imperative role in HPV replication where HPV drives the keratinocytes into S phase of the cell cycle mediated by E5, E6, and E7 viral oncoproteins [37]. E5 increases the expression of epidermal growth factor receptor (EGFR) which promotes the progression toward G1 phase [38]. E6 mediates proteasomal degradation upon binding with p53, a PDZ protein to promote de-differentiation of cells and other pro-apoptotic factors to promote the cell survival [39]. Moreover, MYC expression and telomerase get activated by E6 protein [40]. E7 protein is known to bind with multiple targets specifically RB to overcome the restriction points [41]. Primary keratinocytes get immortalized due to ectopic expression of E6 and E7 which also induces the genomic instability [42]. Carcinogenesis is the consequence of viral gene expression, dysregulated cell proliferation, and genomic instability; however, the progression of cancer is not beneficial to the virus.

4. Immunopathogenesis during HPV infection

The skin and mucosal surfaces act as the first line of defense during infection; however, cutaneous and mucosal HPV are known to diminish the recognition process mediated by TLR9 [43]. Mucosal HPV is known to inhibit the IFN signaling and expression which is mediated by both E6 and E7. HPV E6 protein interacts with IFN regulatory factor 3 (IRF3) [44], whereas E7 interferes with pro-apoptotic factor IRF1 and antiviral [45]. Keratinocytes are the target cell for HPV which act as the physical and immunological barrier. The activation of inflammasome in keratinocytes by other DNA viruses results in acute inflammatory responses; however, diminished inflammatory response has been observed in mucosal HPV infection. Mucosal HPV oncoproteins are responsible for abrogated posttranslational modification and secretion of IL-1β via targeting p300/CBP-associated factors/NF-κB pathway [46]. Upon infection the epithelial cells release chemotactic factors for recruiting, differentiation, and activation of Langerhans cells which are the professional antigen-presenting cells (APCs). HPV infection interferes with homeostasis of Langerhans cells in the

epidermal compartment. Low levels of chemokines (CCL20 and CCL2) are mediated by HPV oncoproteins which result in the reduced level of APC recruitment toward the epithelium [47]. HPV infection during the progression of an invasive form of cancer results in expansion of epithelial stem cell compartments.

The increased level of stromal infiltration with the immune cells is associated with the increasing dysplasia. Secretion of chemoattractants is upregulated by HPV-transformed cells, specifically the CCL2 production by monocytes which attracts myelomonocytic cells following maintenance of inflammatory microenvironment via CCR-2 dependent pathway. Remarkably, CCL-2 secretion results in higher production of MMP-9 via intracellular $Ca2^{+}$ signaling by monocytes [48]. MMP-9 has been shown to be involved in the progression toward malignancy where monocytes become infiltrating into high-grade cervical lesions. In cervical carcinogenesis, the enhanced level of Th17 infiltration has been observed which increases with the disease progression and is coupled with CCL20 expression in the stromal mesenchymal compartment [49]. Most importantly, IL-6 and monocyte colony-stimulating factors (M-CSF) are known as "switch factors" which are imperative for pro-tumorigenic response in monocytes and are highly upregulated during the late stage of human cervical cancer which results in activation of JAK/STAT3 signaling pathway in monocytes [46]. In cervical cancer, inhibition of NF-κB in CD83+ dendritic cells is the consequence of the diminished CCR7 expression that leads to interrupted dendritic cell migration to the lymph node-homing chemokine. Antigen transport to the secondary lymphoid tissue by stromal dendritic cells gets impaired due to diminished CCR7 expression. Cervical cancer cell-derived IL-6 immobilizes the dendritic cells in the tumor stroma via suppression of CCR7 resulting in local MMP-9 production [50]. Furthermore, the low level of IFN-γ production by M2-polarized macrophages causes a reduced level of T-cell proliferation in cervical cancer stroma [51]. The programmed cell death ligand-1 (PD-1) is expressed by most of the CD8+ T cells in cervical cancer, and the suppressed cytotoxic cell response is probably due to M2-macrophages [52].

5. Diagnosis for HPV

Screening of cervical cancer includes three major procedures: cytology (microscopic evaluations of sample acquired from cervical regions), nucleic acid test (detection of HrHPV DNA or RNA), and co-testing (combination of microscopy and nucleic acid test) [53]. In the clinical settings, some of the routine tests for HPV detection are biopsy, DNA-based test, Pap smear, colposcopy, and acetic acid test. Colposcopy is the clinical examination of the cervix, vagina, and vulva upon application of acetic acid solution which is known as visual inspection with acetic acid (VIA) and is mostly coupled with biopsy of regions suspected of neoplasia [54]. Colposcopy findings are evaluated and represented on the basis of acetowhite lesion, mosaic pattern, punctuation, and surface contour [55]. Due to the higher chance of false-positive results, colposcopy screening is recommended for various conditions such as HIV-infected individuals, dyskaryosis, borderline nuclear change in endocervical cells, and cervical cytology positive for malignant [56].

For evaluating anal lesions, high-resolution anoscopy is performed which is similar to colposcopy. However, anoscopy is more complex since it requires manipulation to visualize the entire SCJ along with lower accuracy. High-resolution anoscopy is performed as a first-line test for HPV surveillance in MSM population to follow up anal cytological findings [57]. Histopathological evaluations are considered as the reference standards for deciding the treatment in precancerous or cancerous stages of HPV [58]. CIN3 is considered as the precancerous stage of the HPV infection and can be treated, whereas CIN1 is the morphological representation of HPV infection

and cannot be treated [59]. The CIN2 lesions are considered as severe appearing HPV infection rather than a precancerous one. Several of the biomarkers have been developed to screen CIN2 form of HPV infection. p16 staining can be used to discriminate among the p16-positive CIN2 with CIN3 and p16-negative with CIN1 [60]. DNA-based test utilizes the principle of direct probe hybridization such as dot blot and Southern blot which are associated with disadvantages as low sensitivity and requirement of large amount of DNA sample. Currently, hybrid capture HPV DNA test 2 (HC2) and PCR-based test have been approved by the FDA [61]. HC2 test can be used to detect as low as 1 pg. of HPV DNA/ml and its sensitivity is comparable with PCR. HC2 can detect both low-risk (6, 11, 42, 43, 44) and high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) HPV. PCR-based diagnosis of HPV is more accurate and robust as compared to the Pap smear test which is a screening test for HPV detection.

6. Prevention and treatment

Prophylactic vaccination is the primary preventive measure to control the HrHPV infection and reduce the burden of HPV-related cancer [62]. Currently available vaccine for HPV comprises viruslike particles (VLPs) which is the major HPV coat protein L1. VLPs are noninfectious in nature which lacks DNA and have similar geometry to the native virus [63]. Currently, three prophylactic VLP vaccines have been licensed, namely, as the bivalent Cervarix, the nonavalent Gardasil, and the quadrivalent Gardasil [64]. Cervarix is based on the HPV 16 and HPV 18 antigens and proprietary adjuvants for enhancement of immunogenicity [65]. While Gardasil provides protection against wide range of HPV such as HPV 6, HPV 11, HPV 18, HPV 31, HPV 33, HPV 45, HPV 52, and HPV 52 [66]. All the vaccines are intended to provide complete protection against HPV if administered before the exposure and have been shown to be highly efficacious without any adverse effects. More than 64 countries have introduced the HPV vaccination program primarily targeting girls of age 9–13 and boys of higher age group [67]. In both males and females, systemic immunization with HPV vaccine results in higher amount of antibody generation as 1–1000 times higher than the natural infection.

The management of HPV infection depends on the type and severity of the HPV infection. For the precancerous stage of HPV infection, it includes treatment strategies as excision, ablation, and immunotherapy. The gold standard for the management of HPV treatment is various excisional procedures of the transformation zone where the extent of excision depends on the lesion size [68]. Ablation procedures are less invasive and include cold coagulation, laser therapy, and cryotherapy with the disadvantage of procurement of tissue sample for confirmatory diagnosis. The immunotherapy has shown to be effective but is still present in the clinical trial stages [69]. Anogenital cancers are treated by radical local excision and by regional lymphadenectomy during the spread of infection to the lymphatic system [70]. During cervical cancer radical hysterectomy which is the removal of the whole uterus including the upper vagina and supporting ligaments along with excision of paracervical soft tissue is performed. Surgical intervention not only defines the involvement of lymph nodes but also suggests the treatment alternatives as radiotherapy and chemoradiotherapy.

Radiotherapy is the acceptable primary treatment for the early stage of anogenital cancer, whereas for the advanced-stage metastatic cancer, palliative therapy is the only option. In the recurrent cervical cancer, the antiangiogenic treatment such as bevacizumab along with cisplatin and paclitaxel can prolong the survival [71]. In case of invasive form of cervical cancer, treatment is based on surgery, brachytherapy, external beam radiation, and

cisplatin-based chemotherapy [72]. In case of oropharyngeal squamous cell cancer, cisplatin-based chemotherapy has been the primary therapeutic approach [73]. Radiotherapy or surgical intervention is the choice of treatment during the primary early stage of oropharyngeal cancer.

7. Conclusions

Globally, over 4.6% of all the cancer cases are attributed to HPV each year where vast majority of the cases are of cervical cancer. HPV 16 and HPV 18 are predominantly responsible for progression toward cancer development. The greatest burden of HPV infection comes in the form of cervical cancer and can be prevented. The burden of cervical cancer in the form of high toll number can be reduced via vaccination, screening programs, and implementation of the early treatment along with palliation. Vaccination is the ultimate preventive strategy for all the forms of HPV-associated cancers, and the evidences are suggesting the safety of the strategy.

8. Future perspectives

From the last 36 years, the research in the area of HPV focuses on the prevention strategies and led to the development of vaccine and screening programs. However, new treatment for HPV infection and HPV-related cancer is still under investigation. Immunotherapy for the systemic treatment of HPV infection is the most promising area research worldwide, but none of them have been FDA approved. In order to reduce the burden of HPV-associated cancer, global implementation of vaccination and risk-based screening programs must be followed. The use of HPV-FASTER should be compelled to exemplify the current need of vaccination and screening among the higher prevalence of HPV in young generation. Currently, preventive vaccines are being developed targeting a wide range of HPV subtypes which might reduce and eventually eliminate the need of cervical cancer screening.

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Section 2

Epidemiology and Pathogenesis of Human Papillomavirus

Chapter 2

Human Papillomavirus and Cervical Cancer

Kehinde Sharafadeen Okunade

Abstract

Cervical cancer is by far the most common HPV-related disease. About 99.7% of cervical cancer are caused by persistent genital high-risk human papillomavirus (HPV) infection. Worldwide, cervical cancer is one of the most common cancer in women with an estimated 528,000 new cases reported in 2012. Most HPV infections clear spontaneously but persistent infection with the oncogenic or high-risk types may cause cancer of the oropharynx and anogenital regions. The virus usually infects the mucocutaneous epithelium and produces viral particles in matured epithelial cells and then causes a disruption in normal cell-cycle control and the promotion of uncontrolled cell division leading to the accumulation of genetic damage. There are currently two effective prophylactic vaccines against HPV infection in many developed countries and these comprise of HPV types 16 and 18, and HPV types 6, 11, 16 and 18 virus-like particles. HPV testing in the secondary prevention of cervical cancer is clinically valuable in triaging low-grade cytological abnormalities and is also more sensitive than cytology as a primary screening. If these prevention strategies can be implemented in both in developed and developing countries, many thousands of lives could be saved.

Keywords: cervical cancer, high-risk HPV, HPV vaccines, screening, triaging

1. Introduction

Human papillomavirus (HPV) is the commonest viral infection of the reproductive tract and is one of the most common causes of sexually transmitted infection worldwide [1]. Even though it is sexually transmitted, HPV transmission does not require penetrative sexual intercourse. Skin-to-skin genital contact is a well-established mode of transmission. Over 70% of sexually active women and men will be infected at some point in their lives and some may even be infected on more than one occasion [2]. The peak period for acquiring HPV infection is shortly after becoming sexually active. The infection usually clears up spontaneously within a few months after the acquisition with about 90% clearing within 2 years. There are over 200 HPV types recognized based on DNA sequence data showing genomic differences, and many of these are harmless. HPV can infect basal epithelial cells of the mucocutaneous membrane, and it is associated with a variety of clinical conditions that range from innocuous lesions to cancer. Most of the infections are benign, causing lesions such as cutaneous warts on the hands, feet and anogenital regions. Warts are areas of hypertrophied skin filled with keratin and are mainly a cosmetic nuisance; generally, they resolve spontaneously within 1–5 years. Only a

small proportion of infections with certain types of HPV can persist and progress to cancer such as oropharyngeal, cervical, vulvar, vaginal and penile cancer [1].

Cervical cancer is by far the most common HPV-related disease [1]. Nearly all cases of cervical cancer are due to persistent or chronic HPV infection. Cervical cancer is the fourth most common cancer in women worldwide and it accounts for an estimated 528,000 new cases [2]. About 85% of the global burden occurs in the less developed regions, where it accounts for almost 12% of all female malignancies. In 2012, an estimated 266,000 deaths were attributed to cervical cancer, accounting for 7.5% of all female cancer deaths with almost 90% these deaths occurring in the less developed regions [2]. In these developing countries, cervical cancer may constitute up to 25% of all female cancer cases [3] and is only preceded by breast cancer as the most common cause of cancer deaths in women worldwide [4].

2. Basic virology of HPV

HPV is a member of the Papovaviridae family. It is a relatively small, non-enveloped virus of about 55 nm diameter. It has an icosahedral capsid with 72 capsomers and these contain at least two capsid proteins, L1 and L2. Each capsomer is a pentamer of the major capsid protein, L1 [5]. Each virion capsid contains about 12 copies of the minor capsid protein, L2 [6]. The HPV genome consists of a single molecule of double-stranded, circular DNA [7] with all Open Reading Frame (ORF) protein-coding sequences confined to one strand. There are three functional regions in the genome (**Figure 1**) [8]: The first is a "non-coding upstream regulatory region" also referred to as the long control region (LCR), or the upper regulatory region (URR). This region contains the highest degree of variation in the viral genome and contains the p97 core promoter along with enhancer and silencer sequences that control ORFs transcription in the regulation of DNA replication [9]. The second is called the "early region (E)" and it consists of ORFs E1, E2, E4, E5, E6, and E7, which are involved in viral replication and tumorigenesis. The third is referred to as the "late region (L)" and this encodes the L1 and L2 ORFs for the viral capsid. The E6, E7, and L1 ORFs of a new or unknown HPV type should be 90% or less homologous to the corresponding sequences of known HPV types [10].

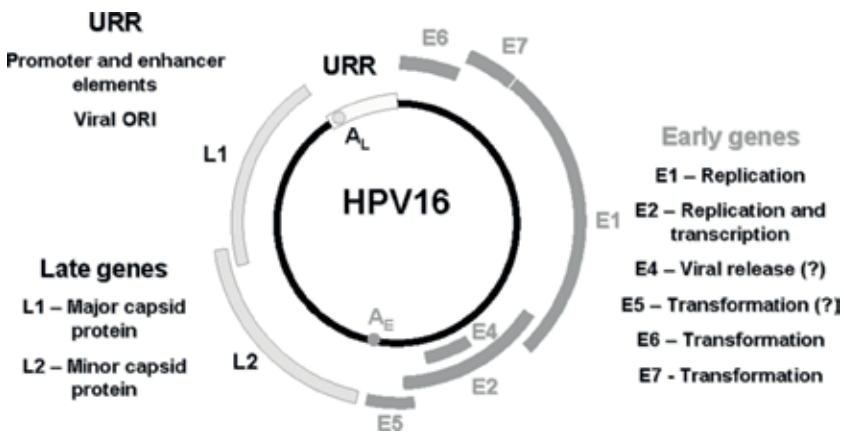

Figure 1.
Genome organization of HPV [9].

3. Epidemiology of genital HPV infection

The worldwide prevalence of high-risk HPV infection is 10.4% [11] and it can be as high as 36.5% in some developing countries [12, 13]. Several epidemiologic studies have clearly shown that the risk of contracting genital high-risk HPV infection and cervical cancer is influenced by sexual activity [14, 15]. An individual is at increased risk of having HPV infection if he or she has had multiple sexual partners at any time or if he or she has a partner who has had multiple sexual partners. Having sexual activity at an early age as well as having a history of other sexually transmitted infections, genital warts, or cervical or penile cancer in an individual or sexual partner may also increase the risk of becoming infected with HPV. In addition to sexual activity, age is an important determinant of the risk of HPV infection [16, 17]. The infection is most common among sexually active young women between the age of 18 and 30 years with a sharp decline in prevalence after the age of 30 years. Although, cervical cancer is more common in older women of 35 years and above, thus suggesting that the infection occurs at a younger age with a slow progression to cancer at an older age. Persistence of HPV infection is commoner with the high-risk or oncogenic types and this plays an important role in the development of invasive cancer of the cervix [1]. Cervical cancer arises at the transformation zone, which is the region between the squamous epithelium of the ectocervix and the columnar epithelium of the endocervix, where continuous metaplastic changes occur. The period of greatest metaplastic activity coincides with the greatest risk of HPV infection and this occurs at puberty and the first pregnancy and subsequently declines slowly after the occurrence of menopause.

4. Link between genital HPV infections and cervical cancer

In the past 3–4 decades, the natural history of cervical cancer has been well studied, and persistent infection of the cervix with certain types of HPV has been reported as a necessary causative factor for its occurrence [18]. The link between HPV and cervical squamous cell carcinoma has become well established since the early 1980. The magnitude of the association between HPV and squamous cell carcinoma of the cervix is higher than that for the association between smoking and lung cancer [19]. About 30 HPV types that are transmitted through sexual contact and infect primarily the cervix, vagina, vulva, penis, and anus have been identified. At least one of these HPV types has been implicated in 99.7% of cases of squamous cell carcinoma of the cervix [18]. HPV is a family of closely related viruses with each designated as a type based on their nucleic acid sequencing and then numbered in the order of discovery. More than 200 HPV types are known to exist [1, 20] with 15 types associated with cervical cancer. Genital HPV types can be grouped as high-risk (oncogenic) and low-risk (non-oncogenic) HPV types based on this association with cervical cancer and its precursor lesions. Low-risk or non-oncogenic HPV types include types 6, 11, 42, 43, and 44 while the high-risk or oncogenic HPV types include types 16, 18, 31, 33, 34, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 [18]. Low-risk subtypes are also occasionally found in cervical carcinomas. The virus usually infects the mucocutaneous epithelium and produces viral particles in matured epithelial cells and then causes a disruption in normal cell-cycle control and the promotion of uncontrolled cell division leading to the accumulation of genetic damage [20]. Adenocarcinomas of the cervix are also less commonly related to HPV infection and is age dependent [21]. Almost 90% of adenocarcinoma of the cervix in women younger than 40 years of age are related to HPV infection, whereas it was observed in only 43% of adenocarcinomas in those aged 60 years and older. Most

HPV-induced cervical changes are transient with 90% regressing spontaneously within 12–36 months [22–26]. However, various other factors such as the individual's genetic predisposition, genetic variation within different HPV type, coinfection with more than one type of HPV, frequency of reinfection, hormone levels, and immune response may alter an individual's ability to clear the infection. Therefore, detection of high-risk HPV is necessary but may not be enough for the development of cervical cancer. Whether a woman will develop cervical cancer depends on several factors that act in conjunction with oncogenic HPV types in a process that leads to cervical cancer. These factors or modifiers of HPV activities include:

4.1 Suppressed primary immune response

Immune response to HPV infection is cell mediated and thus conditions that impair cell-mediated responses such as renal transplantation or HIV disease increase the risk of acquisition and progression of HPV [10, 27, 28]. Studies have consistently shown higher prevalence of HPV infection and cervical cancer precursors in HIV infected women [29–31].

4.2 Long-term use of oral contraceptives

This is a significant risk factor for high-grade cervical disease according to some studies [16, 32]. This is because the upstream regulatory region of high-risk HPV contains sequences which are similar to the responsive elements of glucocorticoid that can be induced by steroid hormones such as progesterone which is the active component of oral contraceptives and dexamethasone.

4.3 Cigarette smoking

The suppression of local immune response induced by smoking and the mutagenic activity of tobacco components have been demonstrated in cervical cells and this may contribute to HPV persistence or to malignant changes in the cervix [33–35]. It appears that smoking is the most important risk factor independent of HPV infection for high grade cervical disease [16]. Smoking shows little or no relationship to low grade cervical disease [1].

4.4 Increasing parity

Having an increasing number of full-term pregnancies is a significant independent risk factor for persistent HPV infection and cervical cancer [36, 37]. The possible mechanisms proposed for this are the increased hormone levels and impaired immune response of pregnancies [38]. In multiparous women, the transformation zone remains longer on the ectocervix and this facilitates its direct exposure to the virus and other potential cofactors [39]. However, the most plausible mechanism is the local tissue damage occurring during vaginal childbirth or cellular oxidative stress with the increased likelihood of DNA damage and HPV integration [40, 41].

5. Prevention of HPV-associated cervical cancer

The natural history of cervical cancer offers unique opportunities for prevention of the disease [42]. Conventionally, Pap smear and liquid-based cytology, combined with treatment of cervical pre-cancerous lesions and early-stage cancer, has been successful in preventing up to 80% of invasive cervical cancer cases in

the developed world [43, 44]. Cervical cancer screening involves testing for HPV infection and cervical cancer precursor lesions among women who have no symptoms. When screening detects cervical pre-cancerous lesions, treatment can easily be instituted, and cancer avoided. Screening can also detect early stage cervical cancer at a time when treatment has a high potential for cure. Currently, primary approaches to HPV prevention include both risk reduction and development of vaccines again HPV. The risk of contracting HPV may be decreased with the use of latex condoms and spermicides. However, these are not totally reliable, since HPV infection may be transmitted through contact with other parts of the body, such as the external genitalia, or anus, that are not protected by a condom [1].

5.1 HPV testing

This is a laboratory test in which cells from the cervix are tested for DNA from certain types of HPV that are known to cause cervical cancer. This may be done alone (primary HPV screening) or in combination with cervical cytology (hybrid HPV screening). These 2 screening strategies are meant to minimize unnecessary follow-up visits and invasive procedures without compromising the detection of disease.

5.1.1 Hybrid screening

This test is usually done using the sample of cells removed during a Pap smear test or Liquid Based Cytology (LBC). It is done if the results of a Pap smear test show certain abnormal cervical cells (reflex testing). When both the HPV test and Pap test are done using cells from the sample removed during a Pap test, it is called a Pap Smear/HPV co-testing. Large-scale studies to evaluate management options for women with abnormal Pap smear results have been conducted and these studies indicate the potential utility of HPV DNA testing in the management of women with Pap smear results of Atypical Squamous Cells of Undetermined significance (ASCUS) [45–47]. Based on the results of these studies, screening strategy options that include testing for high-risk HPV DNA as an adjunct to cytology have been developed to triage and monitor ASCUS patients. These improvements in cytologic screening through LBC as well as the introduction of HPV DNA testing greatly facilitate the identification of women at risk for cervical cancer. There are three recommended options in the management of women with ASCUS [47] and these include:

5.1.1.1 Repeat cervical cytology

In this approach, ASCUS patients would undergo cytology at 4 to 6-month intervals until two negative results are obtained after which the patient can be returned to routine cytologic screening. If any repeat cytology shows ASCUS or greater, referral to colposcopy is recommended.

5.1.1.2 Immediate colposcopy

If this is used, women with biopsy-confirmed CIN are treated as per standard protocol for management of cervical intraepithelial neoplasia (CIN) using excision or coagulation techniques. If biopsy is negative for CIN, patients will undergo repeat cytology at 12 months. In postmenopausal women who have ASCUS and clinical or cytologic evidence of atrophy, a 6-week course of intravaginal estrogen is recommended if there are no contraindications to estrogen use. A repeat cytology is performed after completion of the estrogen regimen and if this is negative, the test

is repeated in 4 to 6 months. If the repeat test shows ASCUS or greater, the patient is referred to colposcopy. Immunosuppressed women with ASCUS should be referred directly to colposcopy.

5.1.1.3 HPV DNA testing

This the most preferred approach especially if liquid-based cytology (LBC) is used or if specimens are co-collected for HPV DNA testing. If HPV DNA testing is negative for high-risk HPV types, the patient undergoes repeat cytology testing at 12 months. However, direct referral to colposcopy is recommended for women who test positive for any of the high-risk HPV types. If biopsy confirms CIN, patients are treated per standard protocol for management of CIN. If biopsy does not confirm CIN, then cytology should be repeated at 6 and 12 months with referral back to colposcopy if results show ASCUS or greater or repeat HPV DNA testing at 12 months with referral back to colposcopy if high-risk HPV types are detected.

5.1.2 Primary HPV screening

HPV DNA testing alone without a Pap smear test may also be used for screening in women aged 25 years and older [48, 49]. It is as effective as a hybrid screening strategy that uses cytology in women aged 25–29 years and co-testing in those at 30 years or older [49]. However, HPV primary screening requires less screening frequency (every 5 years). This involves direct referral to colposcopy for women who test positive for HPV types 16/18 and cytology for those who test positive for any of the other high-risk HPV types [50]. The International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) have endorsed HPV testing as the primary screening method for cervical cancer. Several developed countries are now changing to HPV primary screening [50–52].

5.2 HPV vaccination

One of the major prevention strategies for cervical cancer is vaccination against HPV infection among adolescents prior to their first sexual exposure [15]. HPV vaccines are composed of virus-like particles (VLPs), which contains the major and minor HPV capsid antigens but lacking viral DNA. The vaccines are produced by expressing the L1 or L1 and L2 ORFs in eukaryotic cells. These proteins then self-assemble into VLPs which are highly immunogenic. There is no cross-protection among the HPV types due to the high level of antigenic specificity of HPV capsid antigens and thus protection against each HPV type requires vaccination with VLPs of that type. Optimal vaccines would contain a cocktail of VLPs of the most common high-risk HPV subtypes. There are currently 2 commonly used vaccines (Bivalent and Quadrivalent) which protect against both HPV 16 and 18, which are known to cause at least 70% of cervical cancers. In addition, the quadrivalent vaccine also protects against HPV types 6 and 11 which cause anogenital warts. Both vaccines are more effective if administered prior to exposure to HPV and thus, it is preferable to administer them before first sexual activity. The WHO recommends vaccination for girls aged 9–13 years as this is the most cost-effective public health measure against cervical cancer [1, 53, 54]. Some countries have started to vaccinate boys as the vaccination prevents genital cancers in males as well as females, and the quadrivalent vaccine also prevents genital warts in males and females. These vaccines may provide some cross-protection against other less common HPV types which cause invasive cervical cancer. At present, vaccination against HPV is not recommended as a replacement for cervical cancer screening and in countries where

the vaccine is introduced, cervical screenings still need to be developed or further strengthened [53]. However, in most developing countries, there is still a generally low level of awareness of the existence and availability of these HPV vaccines [55] compared to the developed countries with well-organized cervical cancer screening and HPV vaccination programs. Recently, a nonavalent vaccine against HPV types 6, 11, 16, 18, 31, 33, 45, 52 and 58, which has shown a better impact compared to the bivalent and quadrivalent vaccine, has been approved by the US Food and Drug Administration (FDA) and is now commercially available [56].

Other recommended preventive interventions against HPV infections that are appropriate for both boys and girls are education about safe sexual practices including delayed onset of sexual activity; promotion and provision of condoms for those already engaged in sexual activity; male circumcision; and warnings about tobacco smoking.

5.3 Future perspectives

5.3.1 Measurement of HPV oncoprotein levels

Measuring the levels of HPV E6/E7 oncoproteins is now a potential biomarker for high-risk HPV infection and this may have a role in the future screening of women for high-risk HPV especially type 16 which accounts for more than 50% of all cervical cancer cases [57–59]. The E6/E7 oncoproteins are overexpressed after HPV invasion into the host cervical cells in the form of HPV DNA or viral integration into the host's genome and are closely related to the development of cervical cancers [60]. In a recent pilot study, HPV16 E6/E7 oncoprotein test has a satisfactory diagnostic value for cervical cancer screening and demonstrated a better sensitivity than cytological test and a better specificity than HPV DNA testing [61].

5.3.2 Therapeutic HPV vaccines

There are currently no approved therapeutic vaccines against HPV in humans. However, there are many recent studies that have generated promising vaccine candidates tested in clinical trials [62–64]. Despite the success of these vaccine candidates, there still remains the concern that conventional expression methods when fully developed might result in very expensive products [65, 66] that will be inaccessible to the resource-constraint countries who have the highest incidences of cervical cancer.

6. Executive summary/conclusions

Molecular and epidemiologic studies have solidified the association between high-risk strains of genital HPV and squamous cell carcinoma of the cervix. The incidence of cervical cancer and its associated mortality have declined in recent years, largely due to the widespread implementation of screening programs. Screening for cervical cancer remains an important public health and economic concern throughout the world. Large-scale studies to evaluate management options for women with abnormal Pap smear results have been conducted and these studies highlighted the potential utilization of HPV DNA testing in the management of women with ASCUS Pap smear results. From these studies, screening strategies that include testing for high-risk HPV DNA as an adjunct to cytology have been developed for the triage and surveillance of women with ASCUS. Several other studies, such as the ATHENA study [45], have also examined and confirmed the role of HPV

DNA testing as a primary screening for cervical precursor lesions. In addition to the changes in screening strategies, HPV 16 testing through measurement of HPV E6/E7 oncoprotein levels and effective therapeutic HPV vaccines that have the potential to contribute significantly to the control and prevention of cervical cancer are also currently being developed for future use.

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Chapter 3

HPV Infection and Vulvar Cancer

Nicolae Bacalbasa, Irina Balescu, Ioan Suciu, Simona Dima and Nicolae Suciu

Abstract

Although the strong association between human papilloma virus (HPV) and cervical cancer has been widely demonstrated, it seems that uterine cervix cancer is not the only gynecologic malignancy induced by this pathogenic agent. It has been shown that HPV infection plays a central role in the development of vulvar cancer too, HPV 16 and 18 being the most frequently reported genotypes that might induce this kind of lesions. This aspect presents a particular importation, patients diagnosed with HPV-related vulvar cancer reporting a more favorable trend in regard with the long-term outcome. The current chapter aims to describe the pathogenesis as well as the therapeutic options and the long-term outcomes of patients in which association between HPV and vulvar cancer can be assessed.

Keywords: HPV, infection, squamous cell carcinoma, vulvar cancer, preneoplastic disease

1. Introduction

Discovering human papilloma virus (HPV) represented a crucial step in understanding and preventing the apparition of cervical cancer in women worldwide, the most frequently incriminated carcinogenic subtypes including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 [1]. However, it has been demonstrated that HPV infection plays a central role in the development of other malignancies such as vulvar, vaginal or anal cancer in women and anal or penile cancer in men [2].

2. The role of HPV in the development of premalignant vulvar lesions

Due to the fact that the incidence of vulvar squamous cell carcinoma has reported a continuous increase in the last decades, attention was focused on determining the pathogenesis of this lesion as well as on improving the therapeutic options [3, 4].

The main premalignant vulvar lesion consists of vulvar intraepithelial neoplasia (VIN), with an increasing incidence in the last decades; moreover, it seems that the age at diagnosis of this pathological feature has been consistently dropping in the last period of time, especially due to the relative increase of HPV infections [5, 6]. However, it seems that there are two different pathways leading to the apparition of this premalignant lesion; the first one is mainly related to type 16 HPV infection, while the second one is rather related to the presence of a nonneoplastic chronic inflammatory condition, lichen sclerosus [7, 8].

Squamous cell carcinomas represent more than 90% of all vulvar cancer and are associated with several histopathological subtypes such as keratinized, basaloid warty or verrucous lesions; moreover, it seems that basaloid and warty lesions are more commonly seen in younger women, being usually associated with HPV DNA positivity. Contrarily, keratinized lesions that usually develop from chronic dermatoses such as lichen sclerosus are not associated with HPV infection and develop in older patients [9]. In cases associated with lichen sclerosus, the premalignant disease is usually referred as differentiated VIN (dVIN) [10]. A third category has been also proposed, comprising the VIN lesions, which could not be classified in either of the two above-mentioned classes. This category is referred as VIN, unclassified type [11].

In order to study the clinicopathological characteristics of lichen-related vulvar carcinomas, Regauer et al. conducted a study on 38 patients diagnosed with this pathology [12]. Among these cases, 32 patients presented solitary lesions, while the remaining 6 patients presented multifocal lesions, all cases being HPV and p16 negative. As for the stage of disease, inguinal metastases were present in 42% of cases at the time of presentation. When it comes to the performed therapy, radical surgery (consisting of radical resection with negative margins) was performed in 36 cases, while the remaining 2 cases were submitted to radiochemotherapy. However, 14 of the 36 surgically treated patients developed recurrent disease on the residual mucosa, 68% of them being diagnosed within the first year. Moreover, 14 of the 38 patients died of the disease. In this way, the study came to demonstrate the strong association between the presence of lichen, the absence of HPV and a relatively poorer outcome of this class of patients [12].

In HPV-related lesions, it seems that the immune system of the host encounters a failure in order to produce an effective response to HPV; the longer time the HPV infection persists, the longer certain oncoproteins such as E6 or E7 will interfere with the cyclic cellular mechanisms, inducing cellular escape from the apoptotic process, and therefore malignant transformation [13, 14].

As for the VIN grading system, the first classification was proposed in 1986 by the International Society for the Study of Vulvo-Vaginal Diseases and included the division of VIN in three grades [15]; two decades, later the same organization decided to change the grading system. Lesions that had been previously considered as VIN 1 have been regarded since that moment as warts or HPV infections, while VIN 2 and 3 have been generally referred as VIN [7, 15].

More recently, in order to provide a more specific classification, the Lower Anogenital Squamous Terminology Committee graded all the HPV-related tumors of the anogenital tract into two categories depending on the degree of differentiation: the first category, LSIL (low-grade squamous intraepithelial lesions) refers to lesions presenting a lower grade of pathological transformation, while the second type of lesions HSIL (high-grade squamous intraepithelial lesions) refers to lesions with a higher grade of pathological transformation [16].

When it comes to the HPV-related VIN development, it seems that HPV DNA integration into the host cell genome plays a crucial role [17, 18]. An interesting study conducted on this theme was published by Peter Hillemanns and Xiuli Wang in Gynecologic Oncology in 2006 [19]. The study included 30 patients diagnosed with VIN at the University of Munich-Grosshaderm, Germany; among the 30 patients, HPV DNA was detected in 25 women, the main identified subtypes including HPV 16 and HPV-18. The presence of HPV-16 or HPV-18 DNA was reported in eight cases, all of them being diagnosed with multicentric lesions of VIN, one of them also associating areas of vulvar carcinoma. Therefore, the authors concluded that the integration of HPV-DNA in the hosts presents a central role when it comes to the progression of the vulvar lesions to advanced or multifocal VIN lesions or even vulvar carcinomas [19].

3. Epidemiology of HPV-related vulvar neoplasms

A recent study regarding the epidemiology of HPV-related vulvar neoplasms originates from the National Cancer Institute, Bethesda MD, United States of America, and was conducted by Brinton et al. [20]. The study was conducted on 201,469 women, 370 of them being diagnosed with vulvar neoplasms including 198 cases with grade 3 vulvar intraepithelial neoplasms. The mean age at the time of conducting the study was 61.8 years; among these cases, most patients were white, married, with a high level of education and parous. Moreover, a significant number of cases had been previously submitted to hysterectomy or reported a chronic usage of oral contraceptives or menopausal hormones, while more than one quarter of patients were obese. After a mean follow-up interval of 13.8 years, 170 cases developed vulvar cancer (the mean age at diagnosis being 71 years), while 198 cases developed grade 3 vulvar intraepithelial neoplasia (at a mean age of 67.5 years). Demographic risk factors included nonwhite women, as well as the marital status (divorced or separated women reporting a significantly higher risk of development of the disease); however, the educational status did not influence the risk of the development of any kind of vulvar lesions. As for the parity status, the risk of vulvar lesions was significantly decreased among women who had delivered. Moreover, patients with a previous history of hysterectomy reported a trend to a higher risk of vulvar neoplasm development, although this fact was not statistically significant. However, this difference was not found among cases submitted to a prior oophorectomy. When it comes to the relationship between the body mass index and the risk of developing vulvar lesions, obese patients (defined through a body mass index higher than 30 kg/m^2) had a significantly higher risk of developing invasive vulvar cancer; however, this relationship could not be established for patients with vulvar intraepithelial neoplasms. As for other lifestyle factors, diabetes or alcohol consumption did not influence the risk of vulvar neoplasm development, but smoking constituted in a significant risk factor. In the meantime, administration of hormonal therapies or oral contraceptives was only associated with the risk of development of intraepithelial lesions and not with the apparition of vulvar invasive cancer. Moreover, smoking was a risk factor especially among patients with HPV-related diseases [20].

4. The influence of HPV infection on the overall prognostic in patients diagnosed with vulvar squamous cell carcinoma

Although vulvar squamous cell carcinoma is not a common malignancy, its estimated incidence ranging between 3 and 5% of all gynecological malignancies, it seems that HPV infection plays a central role in its development; therefore, it is estimated that up to 70% of cases diagnosed with this malignancy present in fact HPV-related lesions [21, 22]. However, the rate of correlation between HPV infection and vulvar squamous cell carcinoma widely varies between different studies depending on the detection method and the included tumoral histopathological subtypes [23, 24].

Due to the fact that other related HPV infection malignancies (such as head or neck tumors) are associated with a significantly better outcome when compared to HPV-negative lesions, attention was focused on determining whether a similar relationship could be established between this type of infection and the overall survival in vulvar cancer patients. The improved outcome of patients diagnosed with HPV-related head or neck tumors seems to be explained especially through a better response to radiochemotherapy [23].

However, scarce data have been reported so far. An interesting study conducted on this theme was published by Alonso et al. in Gynecologic Oncology Journal in 2011 [23]. The study included 98 patients diagnosed with vulvar squamous cell carcinoma between 1995 and 2009 in which the authors studied the presence of HPV DNA; among these cases, 19 patients were diagnosed with HPV-associated infection, HPV-16 being the most prevalently detected subtype. Therefore, HPV-16 subtype was reported in 14 cases, one of these cases presenting in the meantime HPV 56 co-infection; HPV-33 was found in two patients, whereas HPV-31, 51 and 52 were each reported in 1 patient. When it comes to the clinical characteristics for the two subgroups, patients presenting with HPV-related tumors were significantly younger when compared to those in whom the presence of the infection could not be demonstrated (68 years versus 78 years, $p = 0.005$). As for the other clinical factors that had been studied (such as FIGO stage at diagnostic, the median dimension of the tumor, association of ulceration, invasion depth and lymph node metastases) as well as for the type of performed therapeutic strategy (resection or radiochemotherapy), there was no significant difference between patients with HPV-related tumors when compared to those in whom HPV infection had not been observed [23].

The most relevant studies that focused on the correlation between HPV status and clinicopathological findings of patients with vulvar cancer are summarized in **Table 1**.

When it comes to the long-term outcomes, both disease-free and overall survival were significantly influenced by the FIGO stage at diagnosis, while the association of HPV infection showed no significant influence. Moreover, no significant difference was reported between the association of radiotherapy, HPV infection and overall survival; however, cases in which radiotherapy was associated reported a higher morbidity rate. In univariate analysis, the most important factors associating with the risk of disease progression and mortality were represented by the age over 78 years, FIGO stages III–IV, tumor size larger than 20 mm, ulceration, invasion depth and the presence of lymph node metastases; however, in multivariate

Name, year	No of cases (total)	HPV-related cases	Non–HPV-related cases	Factors significantly associated with HPV infection
Hinten, 2018 [25]	318	55	263	Patients' age, smoking status, immune status, history of lichen sclerosus, diameter of the tumor, lympho-vascular space invasion, FIGO stage, risk of recurrence
Yap, 2018 [26]	40	14	26	Lower risk of recurrence *- No correlation between HPV status and age, TNM classification or type of treatment, disease-free survival interval, overall survival*
Brinton, 2017 [20]	370	—	—	Smoking, age, obesity
Rasmussen, 2018 [27]	1638	541	1097	Higher overall survival rate in HPV-related lesions
Monk, 1995 [28]	55	33	22	Patients' age, smoking status, histopathological subtype *- No correlation between HPV status and FIGO stage, grade of the tumor, type of therapy*

Table 1.
Correlation between HPV status and clinicopathological findings in patients with vulvar cancer.

analysis, only the association of lymph node metastases was still significantly associated with the mortality risk. When it comes to the influence of HPV infection, a significant association could not be seen even after adjusting for age. These data come to suggest that a supplementary mechanism might be involved in HPV-related vulvar neoplasms when compared to head and neck HPV-related neoplasms [23].

However, these results were not sustained by more recent studies conducted on the theme of the prognostic significance of HPV infection in patients diagnosed with vulvar squamous cell carcinoma and submitted to radiotherapy. In the article published by Lee in the same journal in 2016, contradictory results were found [29]. The study included 57 patients diagnosed with this pathological entity between 1985 and 2011 in Brigham and Women's Hospital and Dana Farber Cancer Institute who were treated with postoperative radiotherapy with radical intent or as part of the salvage setting. In all cases the presence of the following genotypes was studied: 6, 11, 16, 18, 26, 31, 33, 35, 40, 45, 51, 52, 56 and 59; similar to Alonso's study, HPV-16 genotype was the most commonly encountered subtype. When it comes to the long-term outcomes, patients with p16-positive tumors reported a significantly better five-year progression-free and overall survival rates. Moreover, in univariate analysis, older age at diagnosis as well as higher FIGO stage and development of recurrent disease were associated with increased risk of progressive disease and mortality-related disease; however, association of chemotherapy did not significantly impact on the overall survival. When a multivariate analysis was performed, the presence of p16 staining was associated with higher progression-free survival rates as well as with lower rates of recurrence [29]. The reported results of this study were similar to those regarding head and neck HPV-induced malignancies, the presence of HPV infection being associated with a better response to radiotherapy.

A recent study that was conducted by Hinten et al. that will be published in 2018 in Gynecologic Oncology Journal demonstrated that in fact HPV-positive and negative vulvar cancer represent in fact two different pathologic entities with different localization and different prognosis. The study was conducted between March 1988 and January 2015 and included 318 patients. Among these cases, HPV-related disease was reported in 55 cases, while the remaining 263 had non–HPV-related vulvar neoplastic lesions [25]. The authors demonstrated that HPV-related lesions were more often localized on the perineum when compared to non-HPV lesions. When it comes to the long-term outcomes, the authors demonstrated that patients with HPV-induced lesions reported a better outcome in terms of both disease-free survival and overall survival when compared to non-HPV lesions; therefore, the 5-year and total disease-free survival were 76 versus 46%, and 28 versus 13% in HPV-related lesions versus non–HPV-related lesions. In the meantime, the 5-year and total overall survival rates were 85 versus 57% in HPV-related lesions, and only 16% in non-HPV lesions. Another important prognostic factor that significantly influenced survival was the site of the lesions; therefore, even among patients with HPV-related lesions, cases presenting with perineal development reported a significantly better prognosis when compared to nonperineal HPV-induced lesions; the difference remained significant in terms of both disease-free and overall survival. Moreover, among patients presenting with perineal vulvar cancer, HPV-induced malignancies reported a more favorable outcome when compared to non–HPV-induced lesions. In the meantime, disease-free survival was also significantly influenced by FIGO stage and diameter of the tumor, while the overall survival was significantly influenced by age at primary treatment, stage at diagnosis, tumor diameter and relapse as well as by the perineal localization of the lesions. Moreover, the 10-year survival rate was significantly influenced by age at the time of initial treatment, FIGO stage at diagnosis, tumoral diameter, p16 expression and perineal localization of the lesions. In terms of histopathological characteristics,

non–HPV-related lesions presented a larger diameter and were associated with a deeper invasion, more frequent metastases at the level of the lymphatic nodes and, therefore, a more frequent association of adjuvant radiotherapy. This different outcome could be explained by a more aggressive biological behavior of non–HPV-related lesions as well as by the older age at diagnosis, elderly patients feeling most often ashamed to address to the gynecologist for such lesions [25]. All these data enabled the authors to conclude that most probably HPV and non–HPV-related lesions are in fact two different entities with different pathogenesis and different outcomes. Another possible explanation is related to the p53 status, non-HPV lesions being most commonly associated with a higher level of p53, and, in consequence, with a more aggressive biology of the tumor [30].

Another recent study that focused on determining the prognostic significance of human papilloma virus and p16 expression in patients with vulvar squamous cell carcinoma submitted to radiotherapy was conducted by Yap et al. and has been recently published in Clinical Oncology Journal [26].

5. Factors influencing relapse in patients with premalignant or malignant diseases

Starting from the observation that patients with similar stages of disease who receive similar treatment strategies had a very different evolution, the researchers tried to identify the potential factors that influenced this evolution.

5.1 The influence of DNMT expression in development of recurrent vulvar cancer

DNMT (DNA methyltransferases), the enzyme that dictates and maintains DNA methylation patterns through the genome, seems to have significant differences in terms of expression in patients with vulvar carcinomas. Among the general name of DNMT, there are in fact three enzymes with various influences on the methylation process. DNMT1 is directly involved in the methylation process in normal cells; however, it seems that it plays a certain role in tumorigenesis too. DNMT3A and DNMT3B represent two other enzymes that present low expression levels in adult cells; however, these molecules seem to be overexpressed in several epithelial tumors [31, 32]. Moreover, their expression is associated with poor prognosis in patients with such epithelial tumors. A recent study conducted on this theme was published in Gynecologic Oncology Journal in 2016 and included patients treated for vulvar squamous cell carcinomas at the Pan Birmingham Gynecological Cancer Center between 2001 and 2008 [33]. The authors demonstrated the overexpression of DNMT1 in 83% of cases and of DNMT3A in 44% of cases, while the overexpression of DNMT3B was present in 42% of patients. After determining these parameters, the authors studied their influence on the risk of recurrence. DNMT3A was associated with a 4.5 fold increased risk of developing recurrent vulvar cancer; in multivariate analysis, the overexpression of this enzyme was also significantly correlated with a higher risk of local recurrence. Moreover, the authors tested the patients with overexpression of DNMT3A for CDKN2A, an indicator of HPV-induced dysplasia, and demonstrated that among patients with negative staining for CDKN2A, the overexpression of DNMT3A was significantly higher. Similar to DNMT3A, a higher level of DNMT3B was significantly associated with the risk of recurrence; however, the levels of this enzyme could not be correlated with CDKN2A expression. As for DNMT1 levels, there was no significant

correlation between this parameter and the risk of vulvar cancer recurrence; similar to DNMT3B, no significant correlation could be found between DNMT1 levels and CDKN2A expression [33].

5.2 The influence of pretreatment subtype of HPV on the risk of relapse in patients with VIN

Another interesting topic in regard to the influence of HPV on the overall prognostic in patients with premalignant or malignant lesions is related to the effect of various viral subtypes on the long-term outcomes of these patients. A recent study conducted in Milan by Bogani et al. and published in 2017 in the European Journal of Obstetrics and Gynecology and Reproductive Biology included 64 patients diagnosed with high-grade VIN [34]. Among these cases, 41 patients had a previous history of HPV infection, the most commonly incriminated subtypes being HPV 16, 18, 31 and 33. As for the performed procedures, most often it consisted of LASER ablation, excision or diathermocoagulation. After a mean follow-up of 56.7 months, 10 patients were diagnosed with VIN2+ persistence or relapse, the mean disease-free survival being 51.7 months; the authors demonstrated that a pretreatment infection with HPV 31 or HPV 33 subtype was associated with an increased risk of developing recurrent or persistent disease. Moreover, patients submitted to surgical excision followed by LASER ablation experienced a lower rate of relapse when compared with other types of therapies. These facts were explained through two mechanisms: the first one is related to the fact that HPV16, as well as HPV 31 and 33, was associated with multifocal lesions, while multifocal lesions usually associate with a higher risk of persistent/recurrent disease; the second mechanism is probably related to the fact that HPV 31- and HPV33-induced lesions usually associate with a more rapid pattern of growth [34].

When it comes to the influence of HPV infection on the risk of recurrence of VIN, a recent study published by Satmary et al. in Gynecologic Oncology Journal in 2018 comes to demonstrate a significant relationship between these two entities [35]. The study included 784 patients with histopathological diagnostic of vulvar intraepithelial neoplasia which were treated with curative intent; however, 26,3% of cases developed recurrent intraepithelial neoplasia while 2,2% of these cases developed vulvar cancer. Among these cases, 25.9% of patients were 40 years of age or less, 23.9% were aged between 41 and 50 years, 24.6% were aged between 51 and 60 years, while the remaining 25.6% of patients were aged over 60 years. As for the immunity status, immunosuppression was reported in 189 cases and was caused by immune suppressant therapies (such as prednisone or methylprednisolone) in all but two patients (who were known to have HIV infection). When it comes to the performed therapy, it consisted of local excision in 54.8% of cases, laser therapy in 19.3% of cases and topical medication as single therapy or in association with excision or with laser in the remaining patients; in 17% of cases, data regarding therapy were not reported. However, cases in which the initial therapeutic option was not known were excluded from further study regarding recurrent disease. Among the 650 patients who benefited from any kind of treatment recurrence occurred in 171 cases, after a median disease-free survival interval of 16.9 months, while the median follow-up period was 89 months. Moreover, it seems that 75% of cases recurred within 43.1 months. When analyzed according to the age at the time of diagnosis, recurrence rate was significantly higher among patients over the age of 50 ($p = 0.0031$) when compared to younger patients. In univariate analysis, a significant association was also found between the risk of recurrence and the immunity system of the

patient, association of cervical intraepithelial neoplasia and increased BMI; in multivariate analysis, only age over 50 years, immunity status and association of cervical intraepithelial neoplasia were significantly associated with the risk of recurrence. When it comes to the influence of the type of treatment, in multivariate analysis, a trend toward a higher rate of recurrence was reported in cases submitted to nonexcisional therapies. As for the cases in which progression to vulvar cancer was encountered, the median time to progression to malignancy was 36.2 months. When studying only the patients who developed recurrences, the authors demonstrated that the relapse was significantly associated with increased age (patients over 50 years of age reporting a higher risk of recurrence), immunosuppression, positive resection margins and adjacent areas of lichen sclerosus or HPV infection [35].

5.3 The influence of TP53 gene on the risk of recurrence of vulvar cancer

Another factor that seems to influence the evolution of patients with vulvar cancer is represented by TP53 expression. Moreover, it has been widely demonstrated that antitumor agents activating the TP53 tumor suppression gene can be safely used as adjuvant therapy for cases exhibiting this gene. When it comes to the association between HPV-related infection and TP53 expression, in cases diagnosed with head and neck malignancies, disruptive TP53 mutations were exclusively seen in HPV-negative tumors; moreover, these lesions were associated with poorer outcomes when compared with HPV-positive lesions [36].

6. The potential preventive role of HPV vaccination against HPV-induced vulvar cancer

In order to diminish the risk of development of HPV-related malignancies, the quadrivalent and the two-valent HPV vaccines were approved to be used in both males and females in the European Union since 2006 and 2007, respectively [37, 38]. The main viral subtypes that are controlled by these two vaccines are HPV-16 and HPV-18, while the quadrivalent vaccine also contains proteins derived from HPV6 and 11 [37]. Studies have demonstrated that using HPV vaccines in HPV-naïve persons protects against both benign and malignant conditions such as condylomas, perineal and anal neoplasia in men as well as cervical cancer in women [38]. Therefore, routine HPV vaccination has been recommended in Europe in 12-year-old girls since 2009, in order to decrease the risk of development of such pathologies [39]. Due to the fact that there is a strong relationship between HPV infection and certain cases of vulvar cancer, a decreased incidence of this pathology is to be expected in the next decades, once the HPV vaccination has been widely implemented [40].

In order to maximize the protective effect of vaccination against HPV-related diseases, a nine-valent second generation of HPV vaccine was proposed; this nine-valent HPV vaccine is expected to offer protection against the seven high-risk HPV subtypes (HPV 16/18/31/33/45/52/58) as well as against low-risk subtypes such as HPV 6/11. In this way, it is expected to provide a significant degree of protection against the main nine subtypes of HPV, which are responsible for up to 90% of all genital warts. The nine-valent vaccine proved to be effective in order to prevent 97% of all high-grade premalignant lesions of cervix, vulva and vagina. In the study conducted by Hartwig et al. and published in 2015, which included all HPV-related malignancies reported in Europe in the year 2013, the authors demonstrated the efficacy of the second-generation HPV vaccine [2].

7. Future perspectives

Once the benefits of vaccination in terms of prevention of HPV-related malignancies are widely demonstrated, another problem is reported, the one of the vaccine's costs. It seems that, especially in the developing countries, where the incidence of HPV-related malignancies is higher, the accessibility to HPV vaccines is lower due to its price. Therefore, one of the future perspectives in regard to HPV-related diseases is lowering the price of the vaccine and increasing in this way the general accessibility to these products. Moreover, if we take into consideration the fact that a significant number of women self-refer to the gynecologist when neoplastic disease is already present, it seems that an important future perspective should refer to the development of a vaccine that could also have a therapeutic role [41].

8. Conclusion

HPV infection seems to play a central role in developing premalignant or malignant vulvar lesions. Patients diagnosed with HPV-related lesions tend to have a younger age at diagnosis, especially due to the association with the presence of this virus. However, HPV-induced vulvar neoplastic lesions seem to have a better outcome in terms of both disease-free survival and overall survival when compared with non–HPV-related lesions. When it comes to the prevention of these lesions, it seems that anti-HPV vaccination might play a role; however, more studies are still needed in order to clearly state this aspect.

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Chapter 4

Human Papillomavirus Infection and Lung Cancer

Andrés F. Cardona, Alejandro Ruiz-Patiño, Luisa Ricaurte, Leonardo Rojas, Zyanya Lucia Zatarain-Barrón, Oscar Arrieta and Rafael Rosell

Abstract

Lung cancer continues to be the most common neoplasia and represents the leading cause of cancer-related death in the world. Nonetheless, contrary to expected projections, the decrease in incidence expected by decrease in tobacco exposure has been partially halted due to an increasing amount of lung cancer cases in nonsmokers, particularly in female patients. This led to the development of new hypotheses in terms of lung cancer etiology, including the involvement of oncogenic viruses such as the human papillomavirus (HPV). HPV role in the pathophysiology of lung cancer, including adenocarcinoma and squamous cell carcinoma, is currently under research. Exposure to HPV, and the resulting infection, can occur in several possible ways, including sexual transmission and airborne fomites. Main pathogenic occurrences include alterations in inhibition of p53 and retinoblastoma. This chapter presents the current evidence as to the role of HPV in the development of lung cancer, methods to establish HPV infection, and also explores the role of predisposing factors, as well as immunological and inflammatory factors in nonsmokers. Additionally, the role of other molecular factors, such as EGFR, interleukins 6 and 10, and others, is discussed. Finally, future perspectives in this new paradigm of lung cancer in nonsmokers are broadly reviewed.

Keywords: HPV, lung cancer, inflamation, immunogenicity, viral DNA

1. Introduction

Lung cancer is the most common cancer in the world. In 2012, 1.8 million new cases were diagnosed and 1.6 million people died as a consequence of this disease [1]. It is one of the top 10 leading causes of death worldwide [2]. About 90% of lung cancer cases in men and 75% in women in the United States and Europe are caused by tobacco smoke [3, 4]. An important proportion of lung cancer cases presents in non-smokers, as shown in several reports. In the Caucasian population, the rate of non-small cell lung cancer (NSCLC) in non-smokers is 10% for men and 20% for women, while for Asian populations the rate reached 30–40% [5, 6]. In the United States, the overall lung cancer incidence rates and mortality have been declining for the past two decades, and the reduction in both of these parameters has been more prominent in men than in women, a trend that likely reflects the decrease in smoking rates in the

male population [7]. Interestingly, in developed countries, lung cancer incidence has been gradually increasing for non-smokers [8–10]. In Asian countries, the situation is similar; lung cancer incidence and mortality have been increasing despite the implementation of successful anti-smoking campaigns [11, 12].

Among the histologic subtypes of NSCLC, squamous cell lung cancer (SCC) is more common in men (44% cases in men vs. 25% in women) and adenocarcinoma (ADC) is more common in women (28% cases in men and 42% in women). SCC and small cell lung cancers (SCLC) are more closely associated with smoking, in contrast to ADC that is most commonly found in non-smokers [13]. In fact, the calculated histological distribution of lung cancer among smokers and non-smokers in 17 different studies has shown that 53% of the cases in smokers and 19% in non-smokers are SCC while 62% in non-smokers and 18% in smokers are ADC [14]. Furthermore, ADC in non-smokers appears to have a less complex histology with a higher presence of targetable driver mutations, particularly *EGFR*, *Her2* as well as *ALK* and *ROS* translocations [15, 16].

The differences in epidemiology, genetic profile, and survival outcomes of lung cancer in non-smokers have made it clear that this malignancy is a separate entity from lung cancer in smokers [17]. Over the last decades, the investigation of the preventable risk factors associated with lung cancer in non-smokers has gained much attention. Of interest, human papillomavirus (HPV) has been reported in

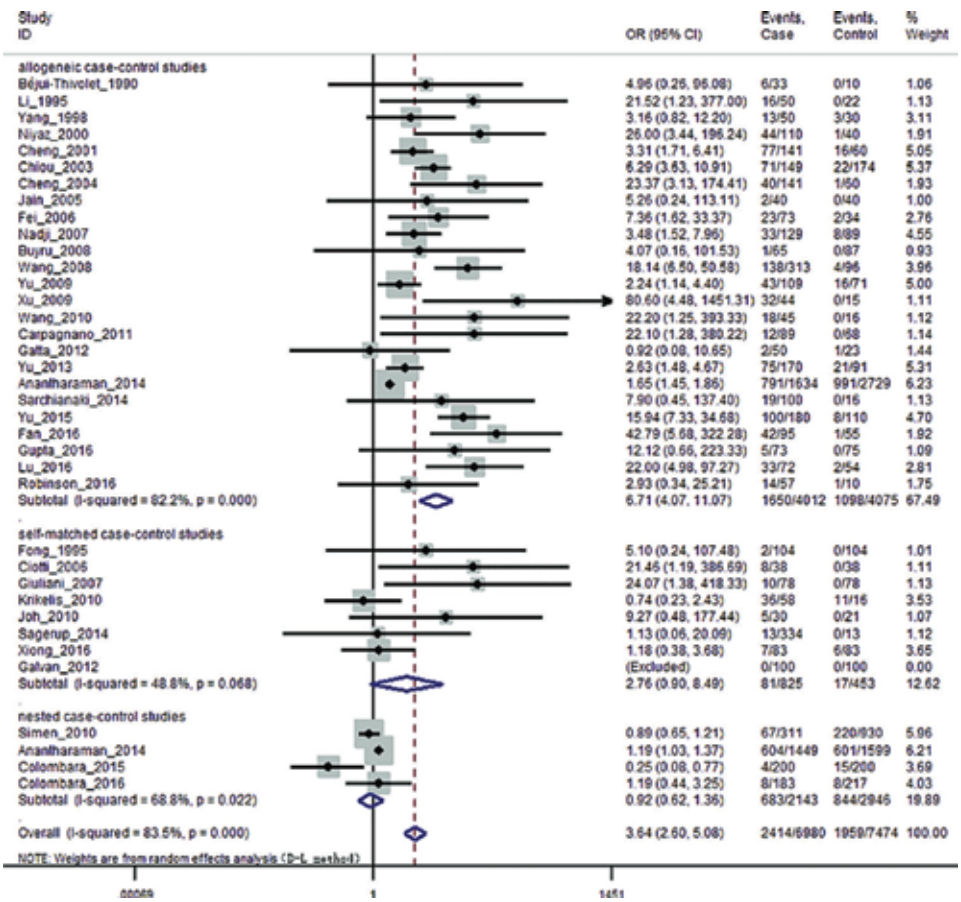

Figure 1.
Forest plot of random effects model stratified by study design. Individual study OR and grouped ORs sub (reproduced with authorization from Ref. [24].

lung cancer tissues from Western and Eastern countries, being types 18 and 16 the most common pathogenic species [18–21]. Likewise, recent studies have demonstrated a significantly increased risk of acquiring lung cancer in non-smokers who are exposed to HPV infection (OR 5.32; 95% CI 1.75–16.17) [22, 23]. **Figure 1** depicts a meta-analysis of recent studies evaluating the association between HPV infection and lung cancer.

Importantly, the public health impact of these recent findings has been recently explored, since vaccination against HPV could represent an efficacious measure to prevent lung cancer. Additionally, the timely detection of HPV in the respiratory tract could warrant a method for early diagnosis of lung cancer, particularly in the non-smoker population [25, 26].

2. Immune and inflammation markers in lung cancer among never smokers

Multiple studies have shown an increased incidence of non-smoker lung cancer in females [3, 9, 10, 27–30]. After analyzing a cohort of 975 patients in Singapore, Yano et al. identified that non-smoker lung cancer patients presented at a younger age and with an earlier stage at diagnosis than their smoker counterparts [10]. The most important risk factors for lung cancer in non-smokers are second-hand smoke, indoor air pollution, occupational exposures, genetic susceptibility, family history, estrogen levels, HPV infection, and pre-existing respiratory diseases [15].

Respiratory diseases elicit a deleterious chronic inflammatory response in lung tissue, which in turn causes an increased rate of cell division leading to an augmented risk of DNA damage [31]. Additionally, inflammation stimulates anti-apoptotic signal activation and angiogenesis further promoting tumorigenesis [32, 33]. Previous studies have also suggested an important role for lung diseases in the development of lung cancer, even when not associated with tobacco use. In 2012, Brenner et al. carried out an extensive analysis from 17 studies and identified that a history of emphysema, pneumonia, and tuberculosis elevated the risk for lung cancer development among non-smokers [34]. Likewise, other case-control studies across various populations have concluded that a history of respiratory diseases, including chronic obstructive pulmonary disease (COPD), asthma, pneumonia, and tuberculosis, would increase the risk of developing lung cancer [35–40]. A systematic review demonstrated that pre-existing tuberculosis increased lung cancer risk in non-smokers (RR 1.78, 95% CI 1.42–2.23); interestingly, the increased risk was only associated with ADC histology, while SCC and SCLC showed no association [41].

Due to the association between respiratory diseases and lung cancer among non-smokers, inflammatory pathways and markers have been the focus of much interest in recent years and hence have been widely studied. A case-control study nested within three prospective cohort studies carried out in Australia and Sweden identified a higher lung cancer risk for participants who had elevated concentrations of interleukin (IL)-6 and IL-8. These associations were stronger for former smokers (smoking cessation at least 10 years before study was performed) than for current smokers, both for IL-6 and IL-8; however, these associations were not observed in never-smoker patients [42]. Other inflammatory markers have been found to be significantly associated with lung cancer risk. In a previous case-control study within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening trial (PLCO), 11 markers of importance were identified. Interestingly, nine of these markers were significantly associated with lung cancer among non-smokers, which

included the epithelial neutrophil-activating peptide 78 (ENA-78/CXCL5) and IL-7, and also associated with lung cancer overall, and others not associated with lung cancer overall, which included human granulocyte chemotactic protein-2 (GCP2/CXCL6), granulocyte colony stimulating factor (G-CSF), IL-6, macrophage inflammatory protein 1B, 2 and 4 (MIP-1B/CCL4, MCP-2/CCL8, MCP-4/CCL13), and stromal cell derived factor-1 (SDF-1 A-B/CXCL12) [43]. Two years later, the PLCO trial was nested to its replication in a case-control study. Both of the nested case-control trials demonstrated that circulating levels of C-reactive protein (CRP), serum amyloid A (SAA), soluble tumor necrosis factor receptor 2 (sTNFRII), and monokine induced by gamma interferon (CXCL9/MIG) were associated with lung cancer risk [43, 44]. These associations were limited to smokers, and the study was considered to be underpowered to evaluate associations among non-smokers [44]. Finally, a nested case-control study carried out within the Shanghai Women's Health Study evaluated 61 inflammatory markers among non-smoker Chinese women. Nine markers were statistically significantly associated with lung cancer: soluble IL-6 receptor (sIL6R) and chemokine ligand 2/monocyte chemotactic protein 2 (CCL2/MCP-1) were associated with an increased risk of lung cancer, while IL-21, chemokine (C-X3-C motif) ligand 1/fractalkine (CX3CL1/fractalkine), soluble vascular endothelial growth factor receptor 2 (sVEGFR2) and sTNFRII and CRP were associated with a decreased risk. Interestingly sIL-6R was associated with an increased lung cancer risk even 7.5 years prior to diagnosis. The results of this study further support our current knowledge in terms of the role of inflammation and immune response on the development of lung cancer among the female non-smoker population.

3. HPV transmission in lung cancer

Since the respiratory tract is composed of two different epitheliums, it contains various squamous columnar junctions (SCJ). In the bronchi, the SCJ may occur naturally or most commonly as squamous metaplasia (SQM) secondary to cigarette smoking [45]. The SQM process initiates with the activation and posterior hyperproliferation of the SQM quiescent basal cells present in the pseudostratified epithelium. As a consequence, the epithelial cells begin to show a squamous cell differentiation, given by the expression of squamous epithelial cytokeratins and cell adhesion molecule SQM1. Finally, cells express involucrin, a protein which indicates that the cells are in a terminal differentiation stage [46–52]. The biochemical and metabolic changes that SQM induces make the bronchial epithelium susceptible to HPV infection. Hence, the multiple foci of SQM in the bronchi are analogous to the transformation zone of the uterine cervix, as both work as the point of entry for HPV [53, 54].

To date, there are three hypotheses regarding HPV infection and transmission into the pulmonary tissue [24, 55]. The first one states that the HPV infection occurs in the reproductive system (male or female) and then hematogenously transmitted to the lung tissue. A study reports that approximately 80% of female HPV-positive lung cancer patients also have cervical intraepithelial neoplasia [56]. Peripheral blood cells (B cells, dendritic cells, NK cells, and neutrophils) on healthy men have been shown to be infected by high- and low-risk HPV [57]. Furthermore, Bodaghi et al. identified HPV types 16 and 18 on peripheral blood from healthy transfusion donors [58]. Since the lung is a highly vascularized tissue, this makes it susceptible to capture the virus and eventually develop the tumor. More evidence to support this hypothesis is the high prevalence of HPV DNA in peripheral blood samples

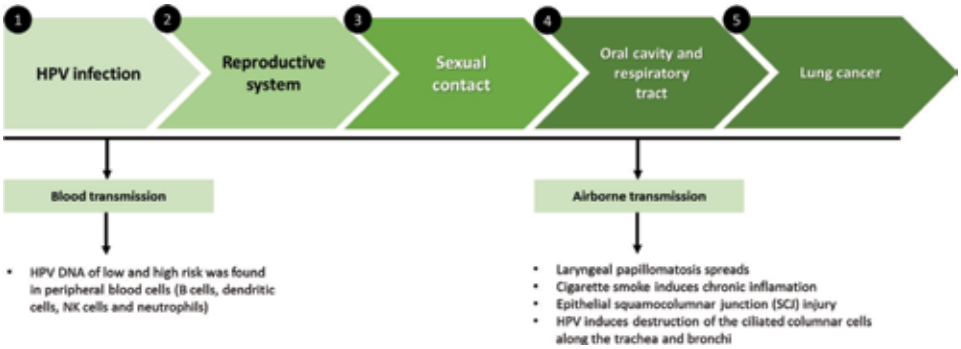

Figure 2.
Possible transmission pathways of HPV infection.

seen in NSCLC patients [25]. These findings have led some authors to suggest that these peripheral blood cells can be a viral reservoir for the infection of other organs and even contribute to viral spread in a sexual contact-independent manner [55]. Another hypothesis refers to oral-genital HPV infection that causes transmission through the throat into the lung. HPV infection may be propagated either through oral-oral contact or genital to oral contact. A survey that followed 222 men and their female patterns showed an infection rate in men of 7.2% with the majority of their female partners having either cervical or oral HPV infection. And finally, a third hypothesis is that HPV may be transmitted as an airborne disease. Carpagnano et al. reported the presence of HPV DNA in exhaled breath condensate of lung cancer patients, hence proposing that HPV transmission can occur through inhalation [59]. These hypotheses are summarized in **Figure 2**.

4. Possible molecular mechanisms of HPV-induced lung carcinogenesis

HPV initially interacts with cellular receptors and infects the basal lamina, transferring its viral genomes to the nucleus [60]. These events are followed by an initial phase of genome amplification and afterwards by a steady maintenance of the viral episome at low copy number [61, 62], particularly around 200 copies per cell, based on the study of episomal cell lines derived from cervical lesions [63]. The replication process requires the hijacking of the of the Retinoblastoma family of proteins (RB, p108 and p130), which regulate cell proliferation, as well as the inhibition of p53, which disrupts apoptosis [64]. The carcinogenic potential comes from the presence of the genes E6 and E7 in the HPV genome, especially in serotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. The E6 protein is composed of roughly 150 amino acids and has the ability to interact with many targets due to its structure containing two zinc fingers built by two pairs of CXX motifs [65]. The main target of this protein is p53. After binding with E3 ubiquitin ligase UBE3A/E6AP (E6-associated protein), the E6/E6AP complex marks p53 for proteasome-dependent degradation. E6 is also involved in other steps of oncogenesis including guarantying survival of the infected cells and offering evasion of the infected cells from the immune system. This protein has in turn the ability to stimulate interleukin-10 (IL10) expression, a cytokine responsible for immunomodulation and anti-inflammatory effects. Among its effects, IL10 has the ability to induce autocrine and paracrine immunologic tolerance due to activation of T helper 2 and T regulatory lymphocytes, inhibition of pro-inflammatory cytokines, and downregulation of the MHC classes I and II, arresting DC maturation

and downregulating intercellular adhesion molecules and co-stimulating mediators. Additionally, IL10 has been shown to promote early E6 and E7 expression, consolidating a vicious cycle [66]. Other interleukins such as IL6 have been shown to be both a stimulator and an inhibitor of cell proliferation, depending on the cell line exposed. Through a complex process in which E6 causes STAT3 activation and IL6 expression, the cancer-associated fibroblast cells, present in the tumor microenvironment, suffer from IL6-induced and p16-mediated senescence. This phenomenon of adjacent cell dysfunction has been shown to promote neoplastic growth due to paracrine stimulation, chronic inflammation, and loss of oncolytic countermeasures [67]. Furthermore, IL6 has the ability to induce antiapoptotic Mcl-1 expression in HPV-infected lung cancer cells [68]. Mcl-1, member of the BCL-2 gene family is responsible, together with the Bcl-2 protein, for the apoptotic response of the mitochondria. Mcl-1 inhibits apoptosis by capturing BH3 and thus inhibiting Bax/Bcl-xl translocation, crucial steps in propagating an apoptotic signal in the cell [69]. Similarly, Bcl-2 offers a comparable Bax translocation inhibition, thus offering a strong antiapoptotic signal [70]. All in all, E6-activated and Il6-mediated BCL2 family modulation seems to be responsible for contributing to apoptosis inhibition and immortalization of HPV-infected cells. Other mechanisms of E6-induced immortalization include the expression of cIAP2, which is considered to be a very potent antiapoptotic factor in these cells by being the upstream inhibitor of caspase 3 activity. This hypothesis was validated in a study of induction of apoptosis in HeLa cells transfected with E6 and E7 proteins by knockdown of this molecule. E6 in turn causes the binding of p52 to NF-κB leading to an upregulation in the expression of cIAP [71, 72]. Additionally, this molecule lays as a downstream step in EGFR signaling, and its expression has also been strongly correlated with the presence of *EGFR* mutations [73]. One possible hypothesis to explain this phenomenon relates to the E6 inhibition of p53. Inactivation of this protein in turn leads to the loss of function in the MMR pathway, especially in MLH1 and MSH2, thus causing an increase in reactive oxygen species, which also strongly correlates with exon 19 *EGFR* mutations in lung cancer [74]. Finally, matrix metalloproteinases (MMP) seem to be also influenced by E6 interactions. These enzymes are responsible for degradation of the extracellular matrix, process required for cell migration and development of metastasis. MT1-MMP, MMP-2, and MMP-9 are the main members of this family that were upregulated by the expression of this oncogene [75], possibly by the induction of microRNAs [76].

E7, on the other hand, is responsible for binding and degrading pRb, p105, p107, and p130, especially in the upper epithelial layers. Additionally, E7 causes genome instability by deregulating the centrosome cycle [60]. Additionally, occurs the induction of the aryl hydrocarbon receptor signaling, a transcription factor involved in proliferation, differentiation, and apoptosis. Members of this family and inhibitors of cyclin-dependent kinases p16 and p21 have been proven to bind to E7 increasing pRb phosphorylation and promoting furthermore cell cycle deregulation [77]. Other activities can also be impaired by E7, such as epigenetic cell function. This molecule has the capacity to displace histone deacetylases, specially HDAC 1, 4, and 7 blocking their binding sites to the HIF-1α promoter regions and leading to an upregulation of its expression. This, in turn, is considered a key step in angiogenesis and thus strongly contributes to tumor growth [78].

5. HPV gene expression and detection in lung tissue

The relation between HPV and lung cancer was postulated since the decade of the 1970s. In 1975, Roglic et al., followed by Rubel and Reynolds in 1979, observed

koilocytosis, a classical pattern in HPV infection, in sputum samples from benign bronchial lesions [79, 80]. Simultaneously, Syrjanen described that the epithelial changes seen in bronchial carcinoma closely resembled HPV-induced genital lesions [81]. Afterwards, several epidemiological data, particularly in non-smoking lung cancer patients, firmly established the relationship between HPV infection and development of lung cancer.

Although several studies showed the presence of oncogenic HPV in lung cancer tissue, demonstrating a causal role is necessary. Taking into account that integration of HPV DNA within the host is the critical point for oncogenic transformation, demonstration of the HPV DNA presence in lung cancer DNA cells is the main goal.

Cheng et al. demonstrated that HPV DNA was integrated into lung cancer DNA cells but not in the adjacent non-tumor cells and also demonstrated that HPV (+) lung cancer patients were predominantly non-smoking females, suggesting a role of HPV infection in the development of lung cancer in non-smokers (OR 10.12, CI 95%: 3.88–26.38 for non-smoking females), and it was one of the first proofs of concept of this causative role [82]. In this study, HPV DNA of high-risk serotypes 16 and 18 was determined by nested PCR and *in situ* hybridization (ISH). Taking this under consideration, PCR is an ideal method for determining HPV-Host DNA integration. The presence of HPV infection can be assessed through different methods, including the use of lung tissue samples (Frozen, Fresh or Formalin fixed and paraffin embedded tissue) but also serological samples (using techniques such as Bead-based multiplex serology method or Multiplex liquid bead microarray antibody assay) [24]. However, one must consider that serologic analysis is usually limited by the low amount of HPV circulating in the bloodstream and also by the low sensitivity and specificity of serological detection techniques; therefore, lung tissue is usually better accepted. In a previous meta-analysis, Xiong et al. [24] evaluated the association between HPV and lung cancer in over 6000 lung cancer patients and 24,000 HPV-exposed individuals. The results showed an association between lung cancer and HPV (OR 3.64; 95% CI: 2.60–5.08), with most studies (75% [28/37]) using polymerase chain reaction (PCR) analysis in lung cancer tissue for HPV detection. The sensitivity and false-positive rate of PCR is higher than with other methods, including *in situ* hybridization, Southern blot, dot blot, and sequencing [83, 84].

5.1 HPV detection and genotyping

In brief, DNA is extracted from lung cancer tissue and then different PCR methods can be used. One of the most common PCR methods used for HPV detection and genotyping is INNO-LiPA Genotyping Extra assay (Innogenetics N.V., Ghent, Belgium) [84]. This assay can detect 18 high-risk types using a reverse hybridization line probe (16, 18, 26, 31, 33, 35, 39, 45, 51–53, 56, 58, 59, 66, 68, 73, 82), 7 low-risk types (6, 11, 40, 43, 44, 54, 70), and some additional types (69, 71, 74). The assay also includes negative and positive controls (HPV6), as well as an internal control (HLA-DPB1 gene), to confirm DNA quality and the absence of PCR inhibitors. The results of trials conducted using each technique are summarized in **Table 1**.

In conclusion, the association between HPV infection and lung cancer should demonstrate the integration between HPV DNA and lung tumor cells DNA. The method usually performed for this assessment is PCR, and different techniques for genotyping have been used, mainly methodologies that include detection of high-risk serotypes but also low risk serotypes.

Author, year	Country	Method	HPV types	Sample type	Cases (n/N)	Controls (n/N)
Béjui-Thivolet, 1990	France	ISH	6, 11, 16, 18	Tissue	6/33	0/10
Li, 1995	China	PCR, DB	16, 18	Tissue	16/50	0/22
Fong, 1995	Australia	PCR	6, 11, 16, 18, 31, 33, 52b, 58	Tissue	2/104	0/104
Yang, 1998	China	PCR	6/11, 16, 31/33	Tissue	13/50	3/30
Niyaz, 2000	China	PCR	16, 18	Tissue	44/110	1/40
Cheng, 2001	China	PCR, ISH	16, 18	Tissue	77/141	16/60
Chiou, 2003	China	PCR	16, 18	Blood	71/149	22/174
Cheng, 2004	China	PCR, ISH	6, 11	Tissue	40/141	1/60
Jain, 2005	India	PCR	16, 18	Tissue (case) Blood (control)	2/40	0/40
Ciotti, 2006	Italy	PCR, sequencing	16, 18, 31	Tissue	8/38	0/38
Fei, 2006	China	ISH	16, 18	Tissue	23/73	2/34
Giuliani, 2007	Italy	PCR, reverse blot hybridization, sequencing	—	Tissue	10/78	0/78
Nadji, 2007	Iran	PCR, sequencing	—	Tissue	33/129	8/89
Buyru, 2008	Turkey	PCR, SB	16, 18	Blood	1/65	0/87
Wang, 2008	China	PCR, ISH, IHC	16, 18	Tissue	138/313	4/96
Yu, 2009	China	PCR	25 types	Tissue	43/109	16/71
Xu, 2009	China	ISH	16/18	Tissue	32/44	0/15
Krikelis, 2010	Greece	PCR	16	Tissue, BW	36/58	11/16
Wang, 2010	China	PCR	16, 18	Tissue	18/45	0/16
Joh, 2010	USA	PCR, sequencing	—	Tissue	5/30	0/21
Carpagnano, 2011	Italy	PCR, sequencing, INFINITI HPV-QUAD assay	16, 18, 30, 31, 33, 45, 35/68, 39/56, 58/52, 59/51, 6/11	Tissue, BW, EBC	12/89	0/68
Galvan, 2012	Italy, UK	PCR,DB	35 types	Tissue	0/100	0/100
Gatta, 2012	Italy	PCR	16, 18, 33, 35, 52, 58	Tissue	2/50	1/23

Author, year	Country	Method	HPV types	Sample type	Cases (n/N)	Controls (n/N)
Yu, 2013	China	PCR, reverse blot hybridization, SB	25 types	Tissue	75/170	21/91
Anantharaman, 2014	7 European countries	BMSM	6, 11, 16, 18, 31	Blood	791/1634	991/2729
Sagerup, 2014	Norway	PCR	15 types	Tissue	13/334	0/13
Sarchianaki, 2014	Greece	PCR, genotyping	37 types	Tissue	19/100	0/16
Yu, 2015	China	PCR	L1, 16, 18	Tissue	100/180	8/110
Fan, 2016	China	ICC	16	Pe	42/95	1/55
Gupta, 2016	India	PCR	16, 18, 31, 33, 45	FNAC, tissue	5/73	0/75
Lu, 2016	China	PCR	16, 18	Tissue	33/72	2/54
Robinson, 2016	USA	Microarray, oncovirus panel, genotyping PCR	28 types	Tissue	15/57	1/10
Xiong, 2016	China	PCR, reverse blot hybridization	21 types	Tissue	7/83	6/83
Simen, 2010	Finland	ELISA	16, 18	Serum	67/311	220/930
Anantharaman, 2014	10 European countries	BMSM	6, 11, 16, 18, 31	Blood	604/1449	601/1599
Colombara, 2015	USA	LBMA	6, 11, 16, 18, 31, 33, 52, 58	Serum	4/200	15/200
Colombara, 2016	China	LBMA	6, 11, 16, 18, 31, 33, 52, 58	Serum	8/183	8/2

Table 1.
Summary of reported trials conducted using each HPV detection and genotyping technique.

6. HPV in lung cancer clinical information and perspective

There are at least three postulated ways for HPV virus to reach the tracheobronchial tract and cause epithelial transformation and malignancy: (1) cervical to lung transmission, (2) from an infected reproductive system to the mouth, throat, and finally lungs, and (3) airborne transmission. All of these have been supported by solid epidemiological data [56, 59, 85, 86]. Once HPV reaches the tracheobronchial epithelium, several molecular and cytological changes can occur as consequence of proteins E6 and E7 from HPV. These oncogene proteins can regulate expression of

several target genes and proteins, which derives in promoted lung cell proliferation, angiogenesis, and cell immortalization. Among the genes and proteins affected are p53, pRb, HIF-1α, VEGF, IL-6, IL-10, Mcl-1, Bcl-2, cIAP-2, EGFR, FHIT, hTERT, HER-2, ALK, ROS1, and AhR [55, 87–90]. Evidence which supports the association between HPV infection and lung cancer continues to grow, but debate will likely continue due to heterogeneous methodologies for HPV detection in lung tissue. At least eight systematic reviews and meta-analysis have consistently found that HPV infection is a risk factor for lung cancer [22–24, 84, 91–94]. One of them included longitudinal studies: a nested case-control and a cohort study with high causality [24]. According to subgroup analyses from several trials, the HPV infection constitutes a risk for lung cancer, especially in non-smokers, similar to the findings in head and neck cancers, females and Asian race [22, 24, 82]. Additionally considering HPV affinity to squamous cells, HPV infection constitutes a risk for squamous cell lung cancer; however, other histologic subtypes including ADC and SCLC have also been related to HPV infection [23, 24, 94].

HPV serotypes 16 and 18, known as high-risk serotypes, are mainly associated with lung cancer risk, though low-risk serotypes are believed to cause benign, non-malignant, lesions [23, 24, 95]. However, this relationship has not been fully studied, and other serotypes including HPV 11 and HPV 31 have a less clear role in terms of lung cancer association. Currently available vaccines against HPV could theoretically prevent lung cancer development; however, this important issue has seldom been explored and more research is needed to draw robust conclusions. HPV status modifies treatment modalities and prognosis in head and neck cancers. Further research is necessary to determine whether lung cancer treatment should change according to HPV infection status. HPV coinfection in lung cancer favors the inclusion of E5 oncoprotein, which alters the mitogenic signaling downstream of Ras, EGFR, and PKC, as well as the constitutive activation of AP-1, which through c-jun may result in cell survival [96]. In the same way, E6 HPV protein blocks p53 activation causing an inhibition of p21 action, upregulating the expression of EGFR and inhibiting apoptosis by activating cIAP28. Additionally, the inclusion of the E7 protein leads to the downregulation of p16INK4 by hypermethylation and migration of tumor-infiltrating lymphocytes (TILs) [97]. Recently, Cheng et al. have found that HPV infection increases tumor activity via hypermethylation of the XRCC3 and XRCC5, an event that generates induced DNA [98]. In parallel, Zhang et al. proposed that inflammation related to HPV lung cancer is induced by increasing levels of HIF, VEGF and [90]. We previously reported a high HPV positivity rate in Hispanic patients suffering lung ADC; in addition, we described that presence of viral DNA leads to a better prognosis in *EGFR* and *KRAS*—mutated lung ADC and increases the expression of PDL1 [99]. Based on this information, HPV infection could modify host immune response and subsequently predict response to immunotherapy, which is currently a treatment modality in certain subgroups of lung cancer patients. Similarly, HPV infection appears to be associated with lung cancer in non-smokers, as are *EGFR* mutations; therefore, it is possible that a synergistic approach could be reached when treating the infection in *EGFR*-mutated lung cancer patients who receive targeted agents. In this regard, a previous study by Li et al. demonstrated that the presence of HPV DNA was significantly associated with *EGFR* mutations in advanced lung ADC. Interestingly, patients with both HPV infections and *EGFR* mutations have a reduced risk of progression compared to those without HPV infection or *EGFR* mutation (adjusted HR = 0.640; 95% confidence interval: 0.488–0.840; P = 0.001), suggesting a prognostic role for HPV status in this patient subgroup [73]. Another likely suitable target for therapy is MEK, a mitogenic signaling pathway protein

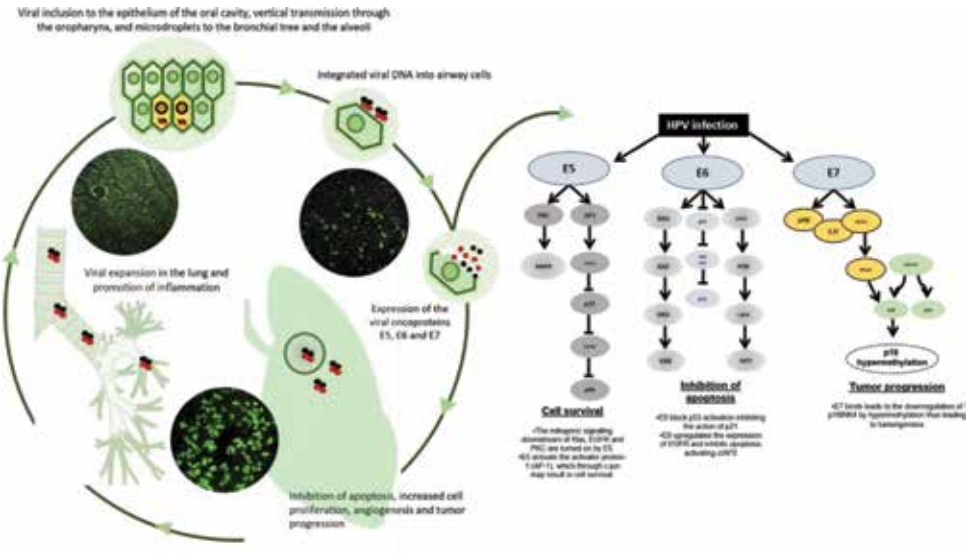

Figure 3.
Pathophysiology of HPV infection and development of lung cancer.

activated as a result of *KRAS* mutations in HPV, and some anti-MEK therapies have been tested in lung cancer [100]. Pathophysiology of infection and main molecular pathways compromised are presented in **Figure 3**.

If lung cancer patients with HPV infection need to de-escalate treatment, as in head and neck cancer patients, they requires further investigation. Some arguments are in favor of de-escalation considering, for example, the fact that lung cancer patients with HPV infection seem to have a better prognosis. Wang et al. described ADC with HPV 16/18 infections as having significantly higher survival rates compared to those that are HPV16/18 negative [101]. In a similar way, Hsu et al. reported survival benefits for stage I NSCLC patients who expressed the HPV16/18 E6 oncoprotein [102].

7. Conclusion

In conclusion, HPV infection constitutes a risk factor for lung cancer development, especially in patients infected with high-risk serotypes 16 and 18, non-smokers and females. HPV vaccination could have a potential role in the prevention of development of HPV-associated lung cancer. Furthermore, HPV status could modify some lung cancer treatment decisions; however, more information is needed to draw definitive conclusions.

8. Future perspectives

Future work in this field will likely include the validation of a screening test for HPV infection in lung cancer patients and also a strategy to follow HPV-infected individuals who might be at a higher risk of developing lung cancer. Additionally, the potential efficacy of anti-HPV vaccination for reducing the incidence of lung cancer must be adequately explored.

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Section 3

Molecular Pathogenesis of Human Papillomavirus

Chapter 5

Role of Extracellular Vesicles in Human Papillomavirus-Induced Tumorigenesis

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Abstract

Emerging evidence demonstrates a role of extracellular vesicles (EVs) in a variety of fundamental physiological and pathological processes ranging from antigen presentation to T cell to neurodegenerative diseases. In several types of malignancies, a variety of EVs can be isolated from bodily fluids of cancer patients, and it has been reported that the number of circulating EVs seems to be higher than in healthy subjects. This increase correlates with poor prognosis. Data obtained from different groups clearly point out a role of EVs in the transfer of bioactive molecules such as microRNAs and viral oncoproteins in human papillomavirus-induced malignancies of genital and oral tracts. This study summarizes these data in the context of relevant literature considering the EVs as carriers of oncogenic signatures in human cancer as well as their therapeutic potential in HPV-related tumors.

Keywords: extracellular vesicles, exosomes, microvesicles, human papillomavirus, tumorigenesis

1. Introduction

The extracellular vesicles (EVs) are nano- to micro-sized, cell-derived structures delimited by a double-layer lipid membrane. Even if the first reports describing the existence of "particles" derived from platelets with procoagulant effects and the presence in serum of the so-called platelets dust date back to 1946 and 1967, respectively [1], exosomes have been considered for a long time just as cell "rubbish" materials. Spotlight on EV functions have been turned on in the early 1980s when two different groups convincingly demonstrated the role of EVs in the transfer and recycling of transferrin receptor in reticulocytes [2, 3]. Since then, a clear role in several physiological and pathological processes has been ascribed to EVs. For instance, B lymphocyte-derived EVs present antigens and induce antigen-specific response in T cells, suggesting a role in adaptive immune responses [4]. EVs have also been implicated in almost all the neurodegenerative diseases through the spread of aberrant pathogenic peptides/proteins. This is the case of β-amyloid peptides and Tau protein in Alzheimer's disease [5, 6], prion proteins in Creutzfeldt-Jakob disease [7], αB-crystallin proteins in both Alzheimer's disease and multiple sclerosis [8], mutant

superoxide dismutase 1 and transactive response DNA-binding Protein 43 (TDP-43) in amyotrophic lateral sclerosis [9, 10], and α-synuclein in Parkinson's disease [11]. Furthermore, a variety of EVs can be isolated from bodily fluids of cancer patients in several types of hematological and non-hematological malignancies, and it has been reported that in these patients, the number of circulating EVs seems to be higher than in healthy subjects and correlates with poor prognosis [12].

Human papillomaviruses (HPVs) are responsible for around 33% of all human cancers related with infections. To date, more than 200 types of HPVs are classified into three main genera (α, β, and γ genus) and several species in the HPV phylogenetic tree. High-risk (HR) HPVs are grouped into a subgroup within the α genus. HR-HPVs are the etiological agents of cervical carcinomas and anal cancers and are involved in other genital tumors as well as in some head and neck tumors [13]. Twelve HPV types (i.e., HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59) were classified as oncogenic by the International Agency for Research on Cancer (IARC), being the HPV16 prevalent in HPV-positive malignancies. Among the HR-HPV-associated tumors, the large majority is represented by cervical carcinomas (i.e., more than 50,000 over 60,000 cases per year, data from GLO-BOCAN 2012; http://globocan.iarc.fr). Of note, the occurrence of HPV-positive oropharyngeal cancers is incrementing in the last decades [14].

Mucosal HR-HPV types belonging to the alpha genus are the only recognized to be related to human carcinogenesis by large epidemiological studies. Similar studies were performed on the involvement of cutaneous beta HPVs in cancer without achieving unequivocal results because these viruses, present in skin cancer cells only in small amount, are also present in the healthy skin. However, many functional studies on the E6 and E7 of several beta HPVs demonstrated their oncogenicity in vivo systems and in transgenic mouse models. These results lead to consider HPV5, HPV8, HPV20, HPV36 (β1 species), HPV22, HPV23, HPV38 (β2), and HPV49 (β3) as high-risk genotypes. In fact, it is well known that these beta HPVs are involved in disseminated infection and in squamous cell carcinoma (SCC) of immunocompromised subjects [15, 16]. However, the mechanism of beta HPV carcinogenesis is different from that of alpha HPVs; in fact, it has been shown that beta HPV types induce tumors in cooperation with other carcinogens such as UV ray and chemicals [17].

2. Extracellular vesicles

Previously defined in several ways as oncosomes, prostasomes, ectosomes, and microparticles, just to mention a few, EVs are currently named basing on both the biogenesis and particles size. According to these criteria, three main classes of EVs are identified: apoptotic bodies (ABs), microvesicles (MVs), and exosomes (Exos) (**Figure 1**). Both ABs and MVs are generated by plasma membrane blebbing; ABs are produced following cellular shrinking and fragmentation upon induction of programmed cell death (PCD) and are the larger vesicles with size ranging between 500 nm and 1 μm. Conversely, MVs are physiologically released during the cellular lifespan and are smaller than ABs (i.e., between 100 and 500 nm up to 1 μm). On the other side, Exos are generated by membrane invagination of intracellular corpuscles defined multivesicular bodies (MVBs) where they are stored as intraluminal vesicles (ILVs). Once MVBs fuse with the plasma membrane, ILVs are secreted in the extracellular space where they are called Exos. Giving the intracellular genesis of these EVs, Exos are the smallest class of vesicles being in size between 50 and 150 nm.

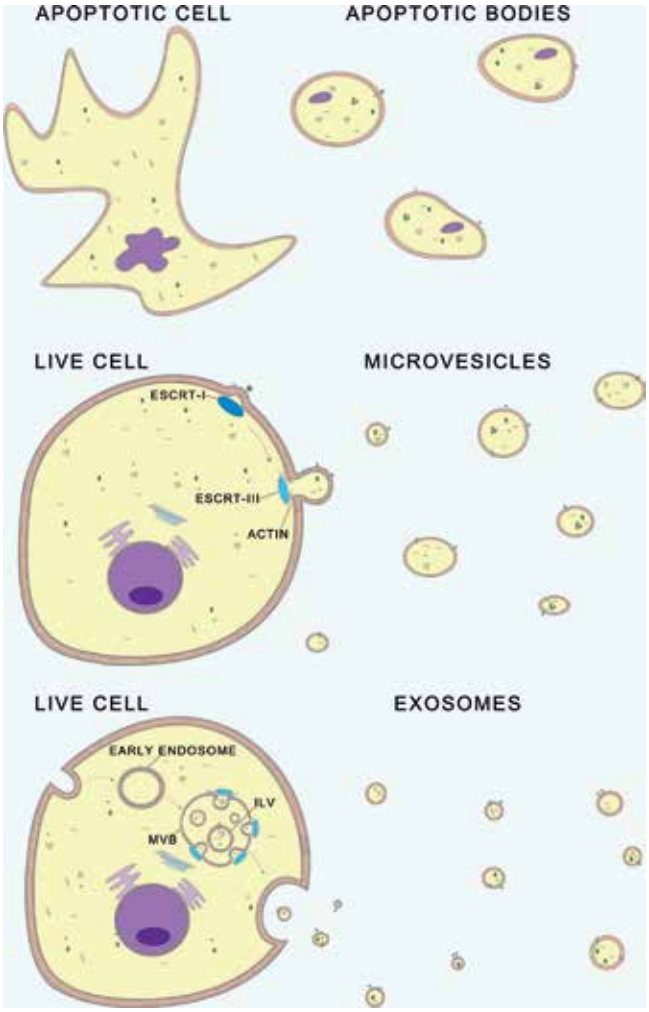

Figure 1.
EV biogenesis. Apoptotic bodies and microvesicles are generated by plasma membrane blebbing. Exosomes are generated by membrane invagination of intracellular corpuscles defined MVBs where they are stored as ILVs. ESCRT, endosomal sorting complexes required for transport; MVBs, multivesicular bodies; ILVs, intraluminal vesicles.

MVs and Exos biogenesis share common elements, particularly the endosomal sorting complex required for transport (ESCRT) proteins. Nevertheless, in the case of Exos, an ESCRT-dependent and ESCRT-independent machinery exists [18].

Depending on their biogenesis routes, the three classes of EVs carry different types of cargos even if all EVs contain lipids, proteins, as well as nucleic acid (i.e., RNA and DNA). Furthermore, specificity of EV cargoes depends on cell type and is associated with the physiological or pathological condition of the producer cell [19]. However, as a general rule, it could be estimated that ABs contain more DNA than MVs and Exos due to their release after PCD induction, whereas MVs are enriched in mRNA surface proteins and, owing to their synthesis, membranes enriched in cholesterol and saturated fatty acid lipids. Among the different classes of EVs, Exos are a very peculiar class because of their size and their intracellular synthesis. These features are of special interest for researchers because they allow the loading of smaller molecules (for instance, microRNAs and other small and long noncoding RNAs rather than mRNAs) and the cargo of proteins able to interact with a

specialized machinery [20]. Nevertheless, at present it is unclear if the elements of the machinery used to sort cargo into Exos are dedicated or rather shared with MVs.

As the three classes of EVs have overlapping size, it is not possible to isolate a single class of EVs in spite of the different methods utilized, which are based on differential centrifugation, density gradients of sucrose or iodixanol, size exclusion chromatography, or ultrafiltration. This is especially true in the case of Exos [21]. In fact, affinity chromatography-based methods that utilize antibodies or molecules able to bind to the phosphatidylserine overexpressed on EV surface [22] are useless as well. Indeed, the possible existence of surface proteins or other molecules specifically expressed by one class of EVs is already matter of debate. The International Society for Extracellular Vesicles has just issued some minimal experimental requirements for definition of extracellular vesicles and their functions, to allow standard assignment of specific biological cargo or functions to a single class of EVs [23, 24].

2.1 Role of EVs in tumorigenesis

EVs serve as shuttle vehicles for intercellular and intratissue communication by transferring a discrete and complex "data packet" consisting of proteins, lipids, and nucleic acids; as a consequence, cancer cells are able to shape tumor microenvironment (TME) through the release not only of soluble factors such as cytokines, chemokines, and growth factors but also of EVs. As previously reported, proliferation, angiogenesis, metastasis, inflammation, limitless replicative potential, resistance to apoptosis, and suppressive signal are considered hallmarks of cancer [25], and a clear role of EVs has been demonstrated in all these processes [26]. Surprisingly enough, this "flow of data" is bidirectional, going not only from transformed to normal cells (i.e., fibroblast, stromal, and immune cells) but also from normal to transformed cells. Exos released from cancer cells, for instance, induce neo-angiogenesis and increase vascular permeability, thereby facilitating metastasis, through the modulation of endothelial cell activities [27]. Fibroblasts are as well a target of tumor-derived EVs that trigger their differentiation into cancer-associated fibroblasts with pro-angiogenic and pro-tumorigenic properties [28]. Several in vitro tumor models demonstrated the ability of tumor cell-derived EVs to transform normal cells. For instance, stromal cells were transformed by Exos derived from colorectal cancer cells [29], adipose stem cells were transformed by EVs derived from prostate cancer cells [30], and nonmalignant fibroblast murine cells isolated from tumor of immunocompromised mice were transformed by breast cancer-derived MVs [31]. Further, Exos isolated from sera of breast cancer patients induce tumorigenicity of nonmalignant human breast cells when injected into immunocompromised mice [32]. However, it is a matter of debate if such a phenomenon occurs also during tumorigenesis. Tumor EV cargo can be represented by mutated oncoproteins [33, 34], mRNA codifying for fusion transcripts [35], oncogenic long noncoding RNAs (lncRNAs) [36], and miRNAs associated with chemotherapy resistance [37].

Conditions associated with tumor growth, like hypoxia, influence both quantitatively and qualitatively the EVs released from cancer cells. When co-injected into SCID mice with human glioblastoma cell line, hypoxic EVs increase both tumor growth and angiogenesis [38], whereas breast cancer cell lines preincubated with hypoxic EVs and injected into mice develop more metastases than the same cells preincubated with normoxic EVs [39].

When cancer cells leave the site of primary growth, they travel through the blood stream acquiring the ability to colonize other sites, thereby generating metastases. It is now evident that these circulating tumor cells (CTCs) are not able to colonize all tissues but only specific sites called pre-metastatic niches, where a favorable TME

has been pre-generated [40]. Due to their peculiar nature, EVs are one of the tools most used by tumors to create pre-metastatic niches. Interestingly, EVs derived from different types of tumor show different tissue tropism. Melanoma-derived EVs target the lung, liver, bone, and brain, whereas colorectal cancer-derived EVs form predominantly liver metastasis. EVs derived from most tumors show a tropism for the bone marrow, thereby generating pre-metastatic bone marrow niches [41, 42]. Although the surface receptors determining the specificity of EVs tropism are unknown to date, the resulting effect is a reprogramming of local target cells that produce soluble factors and extracellular matrix remodeling, necessary for the settlement of CTCs. In other cases, metastasis is dependent not on EVs derived from primary tumor but on EVs released by cells of the target site. For instance, it has been demonstrated that in the brain, but not in other tissues, breast cancer loses tumor suppressor PTEN; this effect is mediated by miR-19a contained in astrocyte-derived Exos [43]. Aging [44] and infection [45] are as well conditions able to create a favorable niche, called in that case active metastatic niche, independent of EVs derived from primary tumor but dependent on EVs produced in loco. This influence of EVs derived from normal cells on transformed ones could even have an opposite effect. Indeed, in some cases, EVs from normal tissues generate an unfavorable field for CTCs, thus inducing the so-called cancer cell dormancy. This is the case of bone marrow mesenchymal stem cell-derived Exos which induce dormancy of breast cancer cells through a miR-23b-mediated mechanism [46].

Another important feature of tumor-derived EVs is their immunomodulatory ability to subvert or evade immune recognition. To accomplish this task, EVs interact with surface immunoreceptors or are internalized within immune cells, thereby hindering activation and polarization into effector or cytotoxic T lymphocytes. Ligands of death receptors, as TNF-R1 and Fas, are engaged on CTL surface by ligands expressed on EVs, leading to the induction of apoptosis in these cells [47, 48]. Tumor-derived EVs also play a role in both T- and myeloid cell differentiation by inducing the generation of regulatory T cells and myeloid-derived suppressor cells, respectively [47, 49]. Finally, NK cells are also targets of tumor-derived EVs [50]. Immunomodulatory activity of tumor-derived EVs on NK cells is also improved by hypoxic conditions through a TGFβ and miR-23a mechanism [51].

3. HPV-induced tumorigenesis

The infection by HPVs that is very frequent in sexually active women can have a driving role in the improvement of tumor injuries of the uterine cervix. The uterine cervical carcinoma (CC) is the third most frequent tumor in women, and the high-risk (HR) HPV is found in about the totality of this tumor [52].

HPV can reach the deep layers of the epithelium through cervical microwounds and enter immature, multiplying cells, where the viral DNA is kept up as an episome and replicates through the host cell genome. As a consequence, the infected immature cervical cells remain in a proliferative state, obstructing their terminal differentiation [53].

During the HPV DNA replication, the HPV early genes, including those coding for the E6 or E7 proteins, are transcribed, but their expression is kept low by the HPV-E2 protein. However, E6 and E7 are produced at levels acceptable to impair the factors that regulate the growth and the differentiation of the host cell. HPV-E7 binds to and inactivates the retinoblastoma tumor suppressor protein (pRb), hampering infected cells to leave the cell cycle and differentiate. Meanwhile, HPV-E6 guides the host cell tumor suppressor protein p53 toward degradation through the proteasome of the cell with the consequence that E6 upregulates the intracellular

levels of the anti-apoptosis Bcl-2 protein, normally blocked by p53, and triggers the activity of telomerase that represses replicative senescence by stabilizing the length of the chromosomes end. Overall, the E6 and E7 activities block the apoptosis due to p53 in the HPV-infected cells, necessary to control cellular proliferation [54].

In around 80% of the cases, HPV-induced cell proliferation stays at subclinical level, developing cervical epithelium thickness or causing benign flat warts. In the remaining cases, HPV-E6 or HPV-E7 stimulates the growth rate of immature cervical basal and parabasal epithelial cells and their transfer to the superficial layer. This prompts the improvement of squamous intraepithelial injuries.

Usually, an immune reaction to HPV happens in few months after infection, with the consequence of viral clearance. From that point forward, the p53 and pRb function is restored in the basal and parabasal layers, and epithelial cell growth and differentiation return to normal. This event usually occurs in infections with low-risk HPV types, e.g., HPV6 or HPV11, whose DNA remains episomal.

On the other hand, persistent infection with HR-HPV, including HPV16 and HPV18, can be followed by the integration of viral DNA into the host cell genome. HPVs are kept up as episomes in precancerous lesions, while in some high-grade lesions, genomes can integrate into the host chromosome [55]. While there is no accord about the exact role of viral integration in HPV-induced cancer progression, it appears that the deregulation of E6 and E7 expression during viral integration contributes to the development of high-grade lesions. In this particular circumstance, cell key mitotic checkpoints are impeded, bringing to genomic instability, accumulation of mutations, and aneuploidy in infected cells. Subsequently, the entire cervical epithelium is replaced by poorly differentiated cells showing anomalous nuclei and atypical mitoses. The E6 and E7 proteins support this process by their ability to induce genetic instability of the host cell DNA and by deregulating cell factors related with epigenetic reprogramming. Recently, a number of studies have enhanced the information of phenotypic effects induced by the HPV-E6 and HPV-E7 oncoproteins. It was demonstrated that oncogenic HPVs contribute to all the main phenotypic changes of cancer cells defined as "hallmarks of cancer" [25], from sustained proliferative signaling, sidestepping growth suppressors, and activating tissue invasion and metastasis to empowering replicative immortality, initiating inflammation and angiogenesis, and repressing cell death.

In any case, the expression of the HPV-E6 and HPV-E7 oncogenes is necessary but not sufficient for HPV-induced carcinogenesis. This statement is supported by the proof that only few women infected with an oncogenic HPV type will develop cervical cancer. Likewise, this process requires a very long time after infection and is characterized by the presence of premalignant precursor lesions with expanding grades of cell dysplasia (cervical intraepithelial neoplasias, CIN, stages 1–3), which either spontaneously relapse or, in the minority of cases, progress to invasive cancer [56].

The risk to develop invasive CC is increased by the use of oral contraceptives, smoking, early sexual practice, different sexual partners, and coinfections.

In addition, HPV utilizes several mechanisms to contrast the host immune response, for example, the suppression of innate immunity, inhibition of T-cell effector function, frequent loss of human leukocyte antigen (HLA) expression, and genetics events, all of which can lead to immune evasion. If the immune system fails to clear persistent HPV infections, after several decades, progression to cervical cancer appears [57].

It is important to underline that the extra alterations required for HPV tumor progression are not restricted to epigenetic changes inside the cell but also with the crosstalk with external cofactors. For example, the microbiome at individual body sites can affect tumor development [58]. Surprisingly, during progression of HPV-positive lesions to cervical tumor, microorganisms resident in cervicovaginal microbiome increase, and *Lactobacillus* spp. diminish [59].

Moreover, different investigations have been performed to analyze the microRNA (miRNA) expression profile in cervical cancer, and important relationships have been found between miRNA patterns and cervical tumor.

MicroRNAs are short RNAs that control the transcriptome and proteome at posttranscriptional level. To deeply understand the role of miRNAs in cervical cancer progression, meta-analysis and gene set enrichment analysis have been utilized to examine studies already published on miRNAs in cervical cancer [60].

It has been demonstrated that some of the dysregulated miRNAs are connected with specific phases of cervical growth development. To study the impact of miRNAs on the pathogenesis of cervical cancer, a miRNA-mRNA interaction network on chosen pathways was created by incorporating viral oncoproteins, dysregulated miRNAs, and their predicted/validated targets. The study has demonstrated that miRNAs deregulated at the different stages of cervical cancer are functionally associated with several key cancer-related pathways, for example, cell cycle, p53, and Wnt signaling pathways. These dysregulated miRNAs may have an important role in tumor development. Some of the stage-specific miRNAs can also be utilized as biomarkers for tumor characterization and checking of cancer development.

It has been demonstrated that miRNAs are discharged into the extracellular space or in the circulation framework in either microvesicles or exosomes [5]. In particular, exosomes have the ability to convey their load to recipient cells through receptor-mediated interactions. Unexpectedly, some studies have shown that some miRNAs are not transported by exosomes, but rather can be fairly recognized in a free soluble form, protected by RNA-binding proteins [61, 62]. The type of delivery of circulating miRNAs could depend on the type of tissue damage, suggesting an alternative role for every kind of transport and demonstrating that different types of cells, for instance, the endothelial cells, could contribute to the delivery of circulating inflamma-miRNAs.

3.1 Role of EVs in HPV-induced tumorigenesis

In spite of the fact that a connection between tumorigenesis and synthesis/release/function of EVs had been shown before, the role of EVs in the pathogenesis of HPV-induced malignancies began to be observed just lately (**Table 1**). The first confirmation of the involvement of EVs in the pathogenesis of HPV tumorigenesis dates back to 2009, when the presence of extracellular Survivin in HPV-18 positive cells HeLa was suggested [63]. Cell medium containing Survivin shows anti-apoptotic, proliferative, and pro-metastatic potential with respect to inactive T34A mutant [63]. After 2 years, the same researchers showed that extracellular Survivin was integrated in Exos and that proton irradiation caused synthesis and release of these Exos [64]. These Survivin-positive Exos were then investigated for their protein cargo content by examining the stress-induced proteins. Therefore, the presence of other inhibitor of apoptosis proteins (IAPs) as XIAP, c-IAP1, c_IAP2, and Livin/ML-IAP was shown [65, 66]. The presence of IAPs relied upon HPV oncoproteins, since Exos obtained from E6- and E7-silenced HeLa cells showed a decrease in the expression of these inhibitors; surprisingly, E6-/E7-silenced HeLa secreted more Exos than control cells [66]. The cargo content of HeLa-derived, Survivin-positive Exos was additionally described at the level of miRNAs [67]. The researchers demonstrated that 52 miRNAs were deregulated and the expression of 23 out of these was influenced by E6/E7 silencing. Most of the upregulated miRNAs play a pro-proliferative, anti-apoptotic, and anti-senescent role. The downregulated ones play instead opposite functions. Very important is also the fact that miRNA content of Exos showed a comparative but not superimposable profile of expression, since 11 out of 46 miRNAs found in Exos are not deregulated

References		Type of EVs	EV source	Purification method	Cargo type	Cargo hallmark
Aromseree et al.	[77]	Exos	EBV-infected LCLs	Differential centrifugation	Viral mRNAs	EBER1 EBER2
Chiantore et al.	[68]	Exos	E6-/ E7-transduced primary human keratinocytes	Differential centrifugation	miRNAs	miR-222
Carolis et al.	[82]	EVs	Sera	Differential centrifugation	DNA	Circular HPV DNA
Gaiffe et al.	[75]	ABs	HeLa CaSki	—	DNA	E6 and E7 DNA
Gezer et al.	[70]	Exos	HeLa	Differential centrifugation plus filtration	lncRNAs	lincRNAp21 CCND1-ncRNA HOTAIR, TUG1 GAS5, MALAT1
Harden et al.	[69]	Exo-EVs	Primary human keratinocytes	Total exosome isolation reagent	miRNAs	miRNAs connected to apoptosis, necrosis, and cell viability
Hermetet et al.	[76]	ABs	HeLa CaSki	—	—	—
Honegger et al.	[66]	MVs	HeLa	Differential centrifugation	Proteins	Survivin, XIAP, c-IAP1, Livin
Honegger et al.	[67]	Exos	HeLa	Differential centrifugation	miRNAs	Several miRNAs deregulated
Kannan et al.	[81]	Exos	Sera UM-SCC-104	Commercial kits	Proteins	HPV16-E7 MUC16 SIRPA
Khan et al.	[63]	Exos	HeLa S	Differential centrifugation	Proteins	Survivin
Liu et al.	[79]	Exos	Cervicovaginal lavages	Differential centrifugation	miRNAs	miR-21 miR-146a
Rana et al.	[72]	EVs	Primary human keratinocytes	Differential centrifugation	Proteins	IL-36γ
Valenzuela et al.	[65]	Exos	HeLa	ExoQuick Kit	Proteins	Survivin, c-IAP1, c-IAP2, XIAP
Zhang et al.	[80]	Exos	Cervicovaginal lavages	Differential centrifugation	lncRNAs	HOTAIR, MALAT1, MEG3

Table 1.
EV type and cargo in HPV+ cells/specimens.

in cells, proposing the presence of specific mechanisms for incorporation of these miRNAs into Exos. The analysis was also performed in the HPV-16-positive cell line SiHa with superimposable results, proposing that HPV deregulation of miRNA

expression is not genotype-specific [67]. The study of miRNAs performed in Exos obtained from primary keratinocytes transduced with E6 and E7 from HPV-16 or HPV-38 confirmed the results obtained in the cell lines by Honegger et al. and showed in these vesicles the presence of mRNA coding for E6 and E7 and the capacity of Exos to transfer these mRNAs to non-transduced keratinocytes [68]. Focusing on a panel of some tumor-related miRNAs, Harden and Muller acquired comparable expression profiles between cell- and Exos-related miRNAs [69]. Other researchers demonstrated the presence of long noncoding RNAs (lncRNAs) into HeLa-derived Exos checking the presence of lincRNA-p21, CCNDA1-ncRNA, HOTAIR, TUG1, and GAS5 [70]. Significantly, lincRNA-p21, the most overexpressed lncRNA in Exos compared to parental cells, is a repressor of p53-dependent transcriptional responses [71] suggesting that his horizontal transfer may influence gene expression in acceptor cells.

EVs derived from HPV-positive cells are likewise able to horizontally transfer cytokines and mRNA thereof, consequently assuming an immunomodulatory role in the cancer microenvironment. Rana et al. showed the presence of proinflammatory cytokine IL-36γ into EVs isolated from Poly(I:C)-treated keratinocytes [72]. Since it has been reported that HPV-16 suppressed the Poly(I:C)-induced expression of several proinflammatory genes [73], it is conceivable to think that IL-36γ expression in EVs is suppressed by HPVs. According to this anti-inflammatory function, the expression of many proinflammatory cyto- and chemokine mRNAs is deregulated in E6-/E7-transduced keratinocytes, and as in the case of miRNAs, this profile is sufficiently conserved in EVs with a statistically significant reduction of IL-1α, IL-1β, CCL27, CXCL1, CXCL3, and angiogenin mRNAs [74].

Different classes of EVs have been also related to HPV-induced tumorigenesis. It has been shown that ABs transfer viral DNA to nonprofessional phagocytic cells [75]. Specifically, these researchers showed that human primary fibroblasts could internalized ABs derived from apoptotic Hela or CaSki (HPV16+) cells and following this internalization showed a transformed phenotype (i.e., development in soft agar, aneuploidy, diminished expression of p53 and p21). This internalization of apoptotic cells, made by both professional and non-professional phagocytic cells and called efferocytosis, happens in a time- and stage-dependent manner since only late apoptotic fragments were able to be taken up by normal fibroblasts; on the contrary, professional phagocytic cells internalize with high effectiveness and degrade both early and late ABs without indications of transformation [76].

EVs, and especially Exos, may be involved in the viral crosstalk, as in the case of coinfection with HPV and Epstein–Barr virus (EBV). It has been reported that Exos from EBV-positive lymphoblastoid cell lines and containing EBV small noncoding RNAs, called EBERs, are delivered to HPV-positive keratinocytes [77]. In EBV-positive cells, EBERs can affect innate immunity and cell proliferation [78], but in the case of their horizontal transfer to HPV-positive keratinocytes, their role remains elusive [77].

The delivery potentiality of EVs, in terms of cargo content and possibility to recover them from virtually all the bodily fluids, makes them an ideal diagnostic and prognostic tool to monitor tumor onset and progression as well as therapy effectiveness while avoiding invasive procedures. The liquid biopsy approach has been recently applied also in HPV-positive carcinomas. The first attempt to study EVs in specimens collected from HPV-positive cancer dates back to 2014 [79] when it was demonstrated that exosomal miR-21 is overexpressed in HPV-positive normal specimens and even more in HPV-positive cervical cancer specimens compared to normal HPV-negative ones. In addition, Exos from cervicovaginal lavages of both HPV-positive normal and cancer patients contain more HOTAIR, MALAT1, and MEG3 lncRNAs [80]. Moreover, in HPV-16-associated oropharyngeal cancer

(HPVOPC), serum collected from patients contains Exos expressing key altered proteins, namely, mucin-16 (MUC-16) and signal regulatory protein α (SIRPA) as well as E7 oncoprotein [81]. Similarly, Exos derived from a HPVOPC cell line displayed the ability to induce epithelial-mesenchymal transition (EMT) and invasiveness of two human mammary epithelial cell lines [81]. Finally, the presence of EVs containing HPV DNA was analyzed in the serum of patients with breast cancer. The authors found two out of eight HPV DNA-positive specimens among patients with ductal carcinoma in situ with a complete correspondence between tissue and serum-derived EV specimens and one out of fourteen HPV DNA-positive specimens among benign breast disease-affected women [82].

4. Engineered exosomes in immunotherapy of HPV-related tumors

Exosomes, in virtue of the properties of stability, biocompatibility, and low immunogenicity and of the possibility to be loaded with a cargo, are increasingly explored as therapeutic delivery agents for drugs and small molecules in a number of pathological conditions. Importantly, exosomes hold good stability while preserving the activity of the molecules shipped, which are fundamental characteristics for delivery, while the outstanding hallmark concerns safety, being a cell-free and very controllable system. In the recent past, exosomes have been investigated for the delivery of siRNAs, miRNAs, shRNAs, and anti-inflammatory/anticancer agents as curcumin and doxorubicin and paclitaxel.

Working either through the parental cells or the environmental milieu, exosomes can be tailored to target definite diseases, e.g., by expressing specific surface ligands and receptors and loading them with therapeutic agents. In alternative to the manipulation of exosomes through the biogenesis in the parental cell line, exosomes can be purified and then modified for incorporating therapeutic molecules. Exosomes delivered by systemic route preferentially accumulate in the liver, spleen, and kidneys, but by scaling the dosage and changing the route of administration, it is possible to affect biodistribution. As the exosome tropism is affected by the donor cell type which determines the membrane composition, attention must be paid to the fact that tumor exosome content is enriched in molecules that can promote tumorigenesis.

A number of applications of therapeutic exosomes in pathologic conditions such as diabetes, cancer, and cardiovascular and neurological diseases are under investigation, with some of them in phase I and one (advanced non-small lung cancer) in phase II clinical trial. For their part, HPV tumors represent *per se* targetable lesions because of their confined localization and lend themselves to being attacked by an exosome-based immunotherapeutic approach that can be either active or passive.

The first studies utilizing exosomes in immunotherapy are dated 20 years ago and used B-cell-derived exosomes to induce MHC-restricted T-cell responses [4]. In the next studies, exosomes were proposed as tumor vaccines for a number of cancers such as melanoma, glioma, hepatocellular carcinoma, and renal cell carcinoma [83–89], due to their capability to deliver antigens from professional APCs such as DCs to other APCs.

Cervical cancer immunotherapy using dendritic cells pulsed with exosomes derived from HeLa cells was recently proposed. Monocyte-derived dendritic cells from cervical cancer patients were matured in vitro to DCs and pulsed with exosomes purified from HeLa culture medium with an opportune protocol. These HeLa-exo pulsed DCs in co-culture experiments with autologous lymphocytes showed the ability to stimulate a strong T-cell activation and a CTL-specific cytotoxic response [90]. In a mouse animal model, HPV-specific cytotoxic activity useful in cervical cancer immunotherapy was also demonstrated using exosomes [91].

Proteins localized into the membrane compartment generating exosomes can be used as carriers for delivering other proteins into the exosomes: this is the case of the HIV-1 Nef protein. A Nef mutant (Nef^{mut}) was generated from a natural defective HIV-1 expressing a Nef variant with the property to accumulate in abnormal quantities in the raft membranes and then in exosomes, compared to the wild-type protein [92]. This 27 kDa Nef^{mut}, both myristylated and palmitoylated, was appropriately engineered to work as a carrier for other proteins long up to 630 amino acids, once fused to its COOH terminus. It has been demonstrated that the fusion products can be incorporated not only into genome-free viral particles of HIV-1, working as a vaccine [93], but also into exosomes of cells transfected with the corresponding DNA constructs.

To develop a therapeutic vaccine for the cure of HPV16-related tumors, the HPV16-E7 and HPV16-E6 tumor-specific antigens were fused to Nef^{mut} and expressed in HEK 293 T cells into the exosomes, anchored to the membrane by Nef^{mut}. Mice harboring subcutaneous HPV16-specific tumors, when immunized with recombinant Nef^{mut}-E7 or Nef^{mut}-E6 exosomes, developed a strong specific cell-mediated immune response, which were able to block the growth and reduce the burden of the tumors generated by injection of TC-1 tumor cells [94, 95]. The production of recombinant exosomes and their purification for therapeutic vaccine development are nevertheless multistep procedures difficult to scale-up. For this reason, the Nef^{mut} expression in exosomes was also pursued and obtained from Nef^{mut}-E7 or Nef^{mut}E6 DNA plasmids in vitro and in vivo. The results allowed to develop an upgraded HPV16 therapeutic vaccine based on the DNA inoculation of Nef^{mut}-E7 and Nef^{mut}-E6 plasmids [96].

The peculiarity of the Nef-fused antigens is to be cross presented to the T cells once they reach the dendritic cells in form of exosomes, forming the platform for the genesis of CTL vaccines [97].

Nef^{mut} is also being investigated for the delivery of therapeutic intracellular antibodies against the HPV-E6 and HPV-E7 oncoproteins. Anti-HPV16-E6 and anti-HPV16-E7 antibodies in single-chain format were previously shown to exert anti-proliferative activity in HPV-positive cells in vitro and therapeutic efficacy against HPV-positive tumors in animal models [98]. Preliminary experiments show that exosomes purified from cells transfected with plasmids expressing the Nef^{mut}-anti-16E7 scFv fusion protein contain an antibody in an active conformation able to bind to the target E7 (Accardi et al., unpublished), exactly as what occurs for the scFv delivered as DNA or protein [98, 99]. In view of the translational application of these antibodies in the clinic for the treatment of preneoplastic HPV-related lesions localized in the anogenital area or even in the oropharynx, exosomes can represent a safe and easy delivery tool. These results represent an incentive to continue the studies, albeit with the awareness of the limits of this strategy, mainly consisting of the lack of reliable standardized methods for the control of exosome contents and purification and of the limited number of exosomes that can be produced.

5. Conclusions

Our knowledge of the EV biology and role, mainly with respect to virus infection, is still in its infancy. Studies have demonstrated that the delivery of viral and cell factors by EVs empowers the control of neighboring unaffected cells. Communication by MVs enables the virus to interact with, and control, cell microenvironment. Various reports proposed that virus infections use the cell vesiculation pathways for virus maturation, immune evasion, and intercellular correspondence. In addition, there is expanding proof of tumorigenic properties

of Exos and MVs derived from cancer cells, depending on either a direct effect of oncogenes transferred to beneficiary cells or an indirect effect of modification of the TME. Importantly, in virus-induced cancer cells, the Exos cargo is different from that of the non-infected cells. These EVs seem to be enriched with viral or cell oncogenic factors as oncoproteins and oncomiRs. Thus, viral-modified Exos may function to horizontally transfer oncogenic molecules among neighboring cells, but they are also armed to manipulate the TME by favoring angiogenesis and inflammation or exerting immunosuppressive effects.

6. Executive summary

- Extracellular vesicles are mediators of intercellular and intra- and intertissue signals that transfer discrete cargo composed by nucleic acids, proteins, and lipids.
- Extracellular vesicles are involved in both physiological and pathological processes.
- Tumors exploit extracellular vesicles to reshape the microenvironment, reprogram normal neighboring cells, evade cell-mediated immune responses, and prepare pre-metastatic niches.
- Human papillomavirus infection has a deep impact on extracellular vesicle cargo in terms of protein expression, mRNA, lncRNAs, and miRNA content as well as viral DNA transfer via apoptotic bodies.
- Mediators transferred by extracellular vesicles released from HPV-positive cells are involved in proliferative, anti-apoptotic, and anti-senescence pathways.
- Extracellular vesicles released from HPV-positive cells contain mRNAs for cytokines and chemokines suggesting a role of these mediators in the reshape of tumor microenvironment.
- Due to their stability and low immunogenicity, extracellular vesicles represent an ideal tool for the delivery of chemotherapeutic drugs to tumor cells as well as for the stimulation of antitumoral immune responses.
- In the next few years, tumor-specific and tumor-associated extracellular vesicles will represent a versatile diagnostic and/or prognostic marker to be detected in blood samples in the liquid biopsy.

7. Future perspective

EVs may represent an innovative tool for the diagnosis, the prognosis, and the follow-up of malignancies as well as for the delivery of anticancer drugs. The ability to vehiculate and protect their cargo from lytic enzymes as well as immune effector mechanisms makes EVs an ideal support for the so-called liquid biopsy and for the next-generation cancer therapy. Indeed, valuable markers as cancer-associated DNAs, mRNAs, miRNAs, and other lncRNAs are protected from the activity of nucleases present in the plasma. Tumor-specific or tumor-associated antigens could be more efficiently presented to immune cells or transferred to antigen-presenting

cells using EVs rather than artificially synthesized liposomes. These scenarios are predictable for all the malignancies, especially those associated with oncoviruses where tumor-specific antigens are well expressed (i.e., E6 and E7 in the case of HR-HPV). Nevertheless, little is known about the biogenesis and release as well as the cargo content of EVs especially in terms of expression of specific tumor- or tissue-associated EV antigens. In the next few years, "benchtop" research will be focused on these aspects of EV biology, thereby leading to "bedside" diagnostic and clinical application of EVs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Section 4

Novel Insights in Human Papillomavirus Infection

Chapter 6

Human Papillomavirus (HPV) Infection in Males: A Need for More Awareness

Mohammed M. Manga, Adeola Fowotade and Mohammed Yahaya

Abstract

Globally, human papillomavirus (HPV) is the most common viral sexually transmitted pathogen, which is significantly associated with high morbidity and mortality in both sexes. Except those vaccinated, virtually all sexually active individuals will be infected with HPV in their lifetime. Although most HPV infections are transient, association with anogenital warts, cervical, penile, and other malignancies have been reported. HPV can be transmitted from one person to another through contact especially during sexual contact including anal, vaginal, or oral. Although HPV infection affects both males and females, its causal association with cervical cancer has made most literature to be mainly on females. In view of its sexual transmissibility and the increasing prevalence of HPV-related malignancies among males worldwide, there is need for more awareness on the infection in males. Most developed countries offer HPV vaccination for girls, but vaccine recommendations for boys are still relatively uncommon especially in developing countries where the burden of HPV-related malignancies is still very high. The current discourse highlights the need for increased awareness on HPV vaccination among this neglected gender group.

Keywords: human papillomavirus, males, awareness, anogenital, malignancies

1. Introduction

The concern on male HPV infection stems from both the disease burden and the potential risk of its transmission from males to females [1]. To date, prevalence and incidence of HPV infection in males is much less established compared to females [2]. In males, infection with high-risk HPVs is associated with penile intraepithelial neoplasia (PIN) in addition to others such as anal and oropharyngeal cancers [3, 4]. The incidence of HPV-related anal and oral cancers is generally on the increase but especially among individuals who are immunocompromised [1, 5]. In some developed nations, the prevalence of oropharyngeal/anal squamous cell carcinoma (SCC) among both men and women was reported to be on the increase [4]. The range of HPV prevalence among males is between 1.3 and 72.9% and is minimally affected by age as against the observed trend in females. Females tend to have a higher probability of acquiring high-risk genotypes compared to males whose risk for acquiring both high- and low-risk types appear to be similar [6].

Based on successes recorded in females, HPV vaccination among males was introduced and had shown much promises so far [6]. However, acceptability/uptake and awareness of HPV vaccines among different male populations have continued to face challenges even in some developed countries despite the successes in female programs [7–12]. This is worst in developing nations where even female immunization programs are almost nonexistent [13–15].

2. Historical background

Over a century ago, an increased risk for the development of cervical cancer was observed among prostitutes as against nuns. Subsequently in the early 1980s, the suspected linkage between sexual behavior and the development of cervical neoplasia was confirmed to be due to genital infection with HPV [16]. In 1983 and 1984, HPV 16 and 18 were isolated from cervical cancer specimens [17].

Currently, there are more than 300 human and animal papillomaviruses which constitute the *Papillomaviridae* family out of which over 200 have been described and organized into 5 phylogenetic genera named alpha, beta, gamma, mu, and nu [17, 18]. However, even as at 1970, it was assumed that there was only one HPV which was thought to be the cause of various warty lesions that infected different tissue sites in humans. Initial perceptions about HPV were mainly as the etiology of transient and trivial/unsightly excrescences. This assertion was changed with the advent of recombinant DNA technology which revealed the presence and effect of multiple HPV types with tropism for different mucosal/cutaneous squamous surfaces and associated development of warts. It further became obvious that some of the HPV genotypes infecting the anogenital tract were oncogenic and causally associated with cancer of the uterine cervix [19].

Evolution of papillomaviruses have been closely linked with their relevant animal hosts over millions of years. The life cycle of HPV genotypes also reflects the differentiation of its respective epithelial target including different parts of the skin and oropharyngeal mucosa [20]. In view of the assertion that humans evolved from nonhuman primates in Africa, origin of HPV types was also linked to Africa phylogenetically. Additionally, the phylogeny of HPV variants (three lineages: European, Asian American, and African) reflects the migration patterns of *Homo sapiens*. The spectrum of diseases associated with HPV infections (anogenital malignancies and warty lesions) have also accompanied humans throughout evolution [21].

3. HPV structure and morphology

HPVs belongs to *Papillomaviridae* family which comprises a diverse family of non-enveloped, small circular double-stranded DNA viruses of about 55 nm in size and consists of about 72 capsomeres [22–24]. The HPV genome is made up of 8000 base pairs. They are relatively stable and could maintain infectivity over a long period in moist environment [25].

It has three functional coding regions: E, a gene coding early viral function; L, a gene coding late viral function; and LCR, a long control region (also referred to as noncoding regulatory region "NCR" or "upstream regulatory region" (URR)) which lies between E and L [24, 26]. Genes are designated as "early" or "late" on the basis of their functional action timing [16].

The genome is organized into eight open reading frames: a long local control region, six early proteins (E1, E2, and E4–E7) and two late proteins (L1 and L2). E1, E2, E5, E6, and E7 are expressed early in the differentiation, E4 is expressed

throughout, and L1 and L2 are expressed during the final stages of differentiation [20]. The early genes are involved in DNA replication, transcriptional regulation, and cellular transformation, whereas late genes encode the viral capsid proteins (the capsomeres) which accounts for 80% of the viral particle [3, 16, 25, 27].

Two of the viral proteins, E6 and E7, are consistently expressed in HPV-positive cervical cancers. The high-risk HPV E6/E7 expression is rate limiting for cervical cancer development. These oncoproteins contribute to tumor initiation and also play important roles in malignant progression through the induction of genomic instability and other mechanisms [26]. E1 and E2 play direct roles in viral replication [3]. The viral gene expression also correlates with the differentiation stages of the epithelium [16]. The viral genome is maintained at the basal layer of the epithelium, where HPV infection is established [20]. The virally infected cells differentiate as they move upward from the basal layer toward the surface of the epithelium with associated induction of high-level viral replication and gene expression followed by virion assembly/release [18].

The phylogeny of HPV variants revealed three lineages: European, Asian American, and African. The evolutionary process stemmed from greater adaptability of certain intra-type HPV variants to specific human population groups. They remained stable viruses over time and have neither changed host species nor reorganized themselves. HPVs have maintained their basic genomic organization for a period exceeding 100-million-year period [21].

4. HPV genotypes

More than 200 types of HPV have been identified by DNA sequence data, and 85 HPV genotypes have been well characterized to date [25]. Classified under the *Alpha papillomavirus* genus are about 40 HPV genotypes that commonly infect the genital tract and are subdivided in to low- and high-risk types [28]. The high-risk types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Others which include HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, 89, and CP6108 are the low-risk group and are frequently detected in benign lesions such as condylomata acuminata [22, 23, 29]. HPV types 26, 53, 66, 68, 73, and 82 are considered as probably carcinogenic [29, 30]. However, HPV types 68, 73, and 82 were occasionally grouped under the high-risk types, while HPVs 34, 57, and 83 are of undetermined risk [30]. Another approach to classification of HPVs on the basis of different oncogenic potentials grouped them in to high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), intermediate-risk (HPV 26, 53, and 66) and low-risk (HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81) types [31].

The distinction between high- and low-risk HPV genotypes is constantly being revised as greater details about the virus become clearer. It therefore follows that classification based on oncogenicity of some HPV types could change over time, with consequent implications on diagnosis and management of HPV-related infections [32].

5. Pathogenesis of HPV infection

Oncogenic human viruses generally infect, but without killing their host cells. They have the tendency to establish long-term persistent infections. Malignant progression of hrHPV-associated lesions usually occurs in the presence of other risk factors, such as decreased immune function and/or after a long latency period after other genomic alterations in the host cell DNA has occurred [17].

In biological evolution, HPVs are successful infectious agents which induce persistent infections without frequent and serious complications for the host and shed virions for transmission to other naive individuals. They avoid the host's defense systems through several processes which include lack of viral-induced cytolysis or necrosis and absence of inflammation, lack of blood-borne or viremic phase, poor access to vascular and lymphatic channels and to lymph nodes where immune responses are initiated, and having mechanisms for inhibiting interferon synthesis and receptor signaling [21].

5.1 Cell cycle and HPV-induced carcinogenesis

Series of phases including the G0, G1, S, G2, and M constitute normal cell cycle which are modulated by cell cycle regulatory genes. These phases are under strict control during transition and are also well coordinated during progression with different cell signals. Cyclin-dependent kinases (CDKs), CDK inhibitors, p53, p27, and p21, and the retinoblastoma gene product (Rb) are the main regulators of cell progression with p53 and Rb as the two most important tumor suppressor genes in the human body. Minor mutations could alter the concentration of p53 with resultant arrest of mitosis and failure of DNA repair, while major damage is associated with apoptosis [16].

The life cycle of HPV follows the differentiation of keratinocytes and begins with expression of E6 and E7 oncoproteins by the virus in the affected epithelium. In the acute phase, viral DNA which is usually present as an episome within the affected host cell is cleared by IFN-β. However, cells with integrated HPV DNA are resistant to this antiviral effect, and the virus completes its life cycle and produces new infectious viral particles, using the host's DNA and RNA polymerase [16, 19]. This mechanism of viral reproduction which is tightly controlled by E2 and regulated by E6 and E7 does not cause cancer [16]. But in high-risk HPVs, T-cell responses to E2 and E6 are lost or reduced, and E7 proteins bind to pRB more efficiently than in low-risk HPV. The E2 oncoprotein usually functions as a transcriptional repressor of the promoter that drives expression of both the E6 and E7 genes. With abrogation of E2 expression, there is dysregulation in E6/E7 expression due to loss of the transcriptional control and resultant suppression of the killer defense response and loss of p53-induced apoptosis increasing chromosomal instability and cancer development [3, 19, 26].

6. Epidemiology of HPV infection

HPVs remain a serious global public health problem due to their association with anogenital/oral cancers and warts [22]. Approximately 630 million individuals are infected with HPV worldwide, while 30 million genital HPV infections are diagnosed each year. It is estimated that in the United States alone, there are 20 million people infected with HPV, and 6.2 million individuals become newly infected each year. Over half of sexually active men and women are infected at some point during their lives [26, 30]. The overall HPV transmission rate was estimated to be 58.8 per 100 person-years from penis-to-cervix and 208.8 per 100 person-years from cervix-to-penis [2]. The estimated total cost for the clinical management of HPV-related diseases in the United States is greater than $3 billion per year; the majority of this sum is spent on the management and treatment of premalignant lesions [26].

The widespread presence and acceptability of many risk factors (including early/polygamous marriages, high parity, and poverty) have made HPV infection to

be endemic in Africa. This is due to both increase in acquisition and promotion of the oncogenic effect of the virus [33].

About 15 HPV types have been classified as oncogenic. Among the oncogenic viruses, HPV 16 and HPV 18 are the most prevalent [20]. HPV types tend to be transmitted together, resulting in a high proportion (20–30%) of concurrent infections with different types [20]. HPV 16 is the most prevalent type worldwide. HPV 18, 45, 31, and 33 are the next most prevalent types [23].

HPVs have both pronounced tropism for certain epithelial cells in addition to being specie specific and have been detected in a wide range of animal species [3]. The hrHPVs are causally related to anogenital cancers including cervix, vulva, and anus in women and penis and anus in men [19, 34]. Low-risk HPV types 6 and 11 are most commonly detected in genital and anal warts, representing 90% of these cases [22].

Although the predominant mode of viral transmission occurs through sexual contact, HPV also has been found in individuals prior to first coitus suggesting the possibility of vertical transmission from mother to child [35]. However, the viral mode transmission in children is still being elucidated [36].

6.1 Risk factors for HPV infection in males

Several studies [37–41] have reported different factors significantly associated with HPV infections which include current and past sexual behavior (including lifetime number of female sex partners (FSPs)), MSM, female partner with positive cervical HPV infection, early sexual debut, absence of circumcision, lack of condom use, immunosuppression, history of other sexually transmitted infections, race/ethnicity, educational level, and smoking cigarettes.

6.2 Prevalence of HPV in males

Different studies have reported varying prevalence rates from different countries. In the United States, prevalence was found to be 65.4% for any HPV, 29.2% for oncogenic HPV, and 36.3% for non-oncogenic HPV among males [37]. Another study revealed a prevalence of 49% of any type of HPV and 35% of hrHPV [38]. Overall prevalence in Europe was found to be 12.4–28.5% in general population and 30.9% in high-risk population [42]. Approximately 90% of anal cancers are associated with HPVs out of which 90% are due to types 16 and 18. This is in contrast to cervical cancers in which about 70% are due to these predominant high-risk genotypes [43]. Although women have higher rates of anal cancer than men in the general population, the greatest risk is seen among HIV-infected men who have sex with men (MSM) who also have higher prevalence of anal HPV [43–45].

Variation in prevalence has also been observed based on differences in the infected sites. In a Greek population, it was highest at anal sites (33%) compared with 23% at penile sites and 4% at oral sites [39]. In another study, the prevalence of HPV infection was 73% at anal site, 26% at penile site, and 16% at oral site [46]. MSM had higher prevalence (84 vs. 42%) at anal site and a lower clearance rate than heterosexuals [46]. Globally, HPV DNA was detected in 33.1% of penile cancers [47]. Prevalence of HPV-related malignancies have been found to be 22.4, 4.4, and 3.5% for oropharynx, oral cavity, and laryngeal cancers, respectively [48].

In Africa, the prevalence of anal HPV was 69.1% in Central Africa and 40.6% in Nigeria [49, 50]. Up to 82.7% of hrHPV was reported in Central Africa out of which 52.0% were multiple infections and more prevalent among HIV-positive MSM [49]. The prevalence of anal hrHPV among HIV-positive MSM was 91.1% in Nigeria [50].

In South Africa, HPV genotypes were detected in 72.8, 11.5, and 15.3% of anal, oropharyngeal, and urine specimens, respectively [51].

7. HPV-associated diseases in men

Neoplasias associated with HPV in men include genital warts, penile, anal, and oropharyngeal and other head and neck cancers [43]. Although there were studies suggesting possible association between HPV and prostate cancer in males, none have reached universal acceptability [52, 53]. Recently, a causal association between hrHPV and HPV-related multiphenotypic sinonasal carcinoma (HMSC) has been described [54–57]. Other malignancies associated with HPV include SCC of the skin/nose tip and skin appendages [58–62]. Association between HPV and bladder cancer even though without uniform conclusions has also been reported in several studies [63–69].

8. Diagnosis of HPV infection

Specimens for detecting HPV in males could be collected from any or all of the following parts of the genital tract, glans, coronal, penile shaft, scrotum, and anal region [43]. Other specimens could be based on the part of the body affected.

The diagnosis of human papillomavirus (HPV) can be inferred from morphologic, serologic, and clinical findings. HPVs cannot be cultured, and the detection of virus relies on a variety of techniques used in immunology, serology, and molecular biology [70].

9. Immunity against HPV

Men do not develop adequate immune responses to maintain protection. Studies have shown that at all ages, antibody levels are lower in men than women [43]. Natural history studies of HPV in men show that HPV clears quite rapidly compared to females [43].

9.1 Natural immunity against HPV

Despite HPV's ability to evade the host's immune system and to downregulate innate immunity, a primary HPV infection is cleared naturally in approximately 90% of cases within 2 years mainly because of cell-mediated immune responses directed against the early HPV proteins particularly E2 and E6. Seroconversion only occurs in about 60% of women, and men are much less likely to have HPV antibodies detected. CD4+ T-helper cells are crucial in avoiding persistent HPV infection, as well as inducing wart regression [2, 19, 21].

The host's immune response to HPV infection (humoral immunity, mainly IgG) is usually slow, weak, wane over time, and varied considerably with many women not seroconverting [17, 19]. Generally, close to half of the individuals seroconvert to L1 protein of HPV 16, 18, or 6 within 18 months. Other HPV antigens [E1, E2, E6, and L2] do not evoke any antibody responses in patients with acute or persistent HPV infection [21]. Natural infection-elicited antibodies may not provide complete protection to HPV over time. A recent WHO position paper stated that host antibodies, mostly directed against the viral L1 protein, do not necessarily protect against subsequent infection by the same HPV genotype [21].

9.2 Immune response to HPV vaccine

The evidence from animal papillomavirus infections, including some of the earliest published works, showed very clearly that neutralizing antibodies were protective. The experiments showed that if rabbits were infected systemically with the cottontail rabbit papillomavirus (CRPV) by direct injection of virus; papillomas did not arise on the skin of the challenged animals, and neutralizing antibodies were generated. The animals were completely resistant to subsequent viral challenge by abrasion of the epithelium [19]. Immunization also facilitates the regression of existing lesions [25].

Technological advancement leading to production of virus-like particles (VLPs) is the prelude to development of effective HPV vaccine. Highly immunogenic VLPs capable of mimicking natural infection and eliciting high titers of long-lasting virus-neutralizing antibodies (significantly more than natural infection) could be generated using recombinant DNA. This is because the antigenic dose in VLPs is much higher than what obtains in natural infection as the capsids are directly exposed to systemic immune responses. This leads to better quality and the quantity of the immune response generated by vaccines compared to natural infection. The intramuscular administration of HPV vaccines leads to rapid access to the local lymph nodes with subsequent evasion of immune avoidance strategies of the virus [19, 21].

A rapid, potent, and sustained immunologic response due to the administration of both quadrivalent vaccine (targeting HPV 6, 11, 16, and 18) and a bivalent vaccine (targeting HPV 16 and 18) has been reported. These vaccines can elicit an immunological response against the two most common oncogenic types (16 and 18) but not against all the high-risk types except for cross-neutralizing antibodies in some individuals [19, 21]. The duration of protection afforded by the vaccines revealed greater than 98% protection over a 5- to 6.4-year period against HPV 16, 18, 6, and 11 [19].

9.3 The HPV vaccines

Strategies for the control and treatment of genital HPV infections are a matter of high priority typically because of their relationship with anogenital and other malignancies. Traditionally, vaccines have remained a cost-effective means of preventing many viral diseases including HPV in recent times [19]. Sexually naïve adolescents are routinely being vaccinated in many countries as recommended by the World Health Organization (WHO). The effectiveness of these vaccines is most pronounced in unexposed as against previously infected individuals [24]. This is because the current vaccines are not therapeutic against existing infections or lesions, and cross-protection against other HPV types is partial or nonexistent. Therefore, the greatest public health benefit of the current HPV vaccines is when given at an age before sexual debut [20].

Recommendation for routine vaccination of adolescents at ages 11 or 12 years has been in place.

Since 2006 for females and since 2011 for males [71]. The United States was the first country to adopt a gender-neutral routine HPV immunization policy in the year 2011 for both males and females [72]. The time of commencement of vaccine administration determines the minimum number of doses recommended based on the age of recipient. Two doses are recommended for those who initiate between ages 9 and 14 years, while three doses are for those initiating at ages 15 through 26 years and for immunocompromised individuals [71]. A three-dose regimen could be for all the three vaccines at time intervals of 0, 1, or 2 and 6 months [71].

The effect of all vaccine types is higher for HPV 16-/18-associated lesions than for others. It is also greater in those who are high-risk HPV negative at initiation compared to those with unknown HPV status [73]. The effect of HPV vaccination in males is also moderate against persistent anogenital infection and high-grade anal intraepithelial lesions if given to HPV infected males as against those without the infection. This supports a recommendation for vaccination of boys also before sexual debut so as to ensure maximum protection [74].

10. Conclusion

Although global attention has been more toward HPV infection in females, it is equally important in males due to increasing prevalence especially among the high-risk populations. Many developed countries have commenced routine immunization of males for HPV but virtually no low-income country followed the same pathway. There is need for more awareness especially in developing countries where the burden of HPV is highest.

11. Future perspectives on HPV in males

The current trend suggests a relatively low level of awareness on HPV-associated diseases in males and acceptability of vaccination against the virus in most countries but worst in developing nations [75–79]. There are efforts from varying perspectives by different groups to improve the current situation based on findings from some studies [80, 81]. Technological advancements will see the use of various means such as mobile computer applications to influence the knowledge about HPV and acceptability/uptake of the vaccines [82]. There might be a need for changes in policies even in developed countries to accommodate more challenges as they unfold [83]. Culture and beliefs may be explored to further strengthen the level of awareness and acceptability of HPV vaccines in different populations [84]. Design and development of more potent and user-friendly vaccines for both preventive and therapeutic purposes will continue with resultant wider acceptability/improved safety [31]. With the successes observed in countries who have implemented structured programs for HPV vaccination, more nations may embrace similar/improved approaches for better outcomes [85].

Abbreviations

CIN	cervical intraepithelial neoplasia
CDKs	cyclin-dependent kinases
CRPV	cottontail rabbit papillomavirus
DNA	deoxyribonucleic acid
FSPs	female sex partners
HIV	human immunodeficiency virus
HPV	human papillomavirus
HR	high risk
LCR	long control region
LR	low risk
MSM	men having sex with men
NCR	noncoding regulatory region
PIN	penile intraepithelial neoplasia

SCC	squamous cell carcinoma
STIs	sexually transmitted infections
URR	upstream regulatory region
VLPs	virus-like particles
WHO	World Health Organization

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Section 5

Prevention of Human Papillomavirus

Chapter 7

Results of a Survey Concerning Cervical Cancer Risk Factors among Women in Western Kazakhstan

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Abstract

During 2014–2017, a survey concerning risk factors for cervical cancer involving 1166 clinically healthy women and 65 having CaCx was conducted in Western Kazakhstan. Only 34.7% of interviewees constantly participated in state-sponsored screening program, while 37.3% ignored screening in free state-sponsored clinics. Favorable attitude toward vaccination stated 22.9% of the respondents, whereas 38.8% knew nothing, and 33.6% could not clarify their position in this issue. Education is a key factor for better perception of preventive measures—69.2% of the respondents with higher education are aware of vaccination ($p \leq 0.00001$, Cramer's V 0.18, χ^2–23.1). Social profiles of HPV-infected and CaCx-diseased women differ significantly and, mainly, by standard of living and occupational status. The likelihood of the CaCx onset in Western Kazakhstan decreased by 14 times at relatively high standard of living (OR 0.0713, $p = 0.024$) and by 3.3 times provided at least irregular participation in screening (OR 0.3384, $p = 0.0304$). Overall, the findings are quite able to contribute to an understanding why women become affected by CaCx. Low standard of living due to lack of education, low attendance of screening, and low awareness on preventive measures-all these reasons are interacted and constitute a set of universal triggers for vulnerability toward CaCx.

Keywords: cervical cancer, human papillomavirus, awareness, vaccination, screening, risk factors, Kazakhstan

1. Introduction

For cervical cancer (CaCx), the number of diagnoses could "rise by at least 25% to over 700,000 by 2030, mainly in low- and middle-income countries," said a statement from the Lancet [1]. Some sources mention areas of Western Asia as countries with the lowest CaCx rates [2], while just a few sources are available on the disease-related issues in borderline Central Asia, where Kazakhstan and some other post-Soviet states are located [3, 4]. Reportedly, the annual incidence rate of cervical cancer for Kazakhstan was calculated as 14.5 ± 0.3 with 8.0 ± 0.1 mortality

for the period 1999–2008 [5]. Data of the International Agency for Research on Cancer (IARC) on cervical cancer incidence in 2012 for the global network resource Cancer Today (formerly Globocan) indicated the highest incidence of CaCx in the Republic of Kazakhstan among borderline countries—29.4 per 100,000 of the female population standardized by age, while the corresponding index for the Russian Federation was 15.3, for Uzbekistan 13.5, and 7.5 for China, respectively [6]. Despite definite progress achieved, issues of cervical cancer prevention have still remained tense in the country. According to data of the ICO Information Centre on HPV and Cancer (the Catalan Institute of Oncology HPV center) as of December 23, 2015, there were 6.72 million women aged 15 years and older at risk of developing cervical cancer, and estimates indicated that every year 2789 women were diagnosed with cervical cancer and 982 died from the disease [7]. Morbidity, according to the ICO experts, has been roughly estimated 32.8 per every 100,000 women standardized by age, i.e., increased several times as many for the period less than a decade. Meanwhile, cervical cancer is a real object for early detection because of its belonging to a number of visual forms and can be largely prevented by both effective screening and vaccination [8].

A system of the cervical cancer screening has been implementing in our country since 2008, and in frames of this nationwide program, all women aged 30–70 years are subjected to mass cytological examination every 4 years. Age of women has been increased from 60 to 70 years, and the interval has been diminished from 4 to 5 years according to the latest regulation no. 995 as of December 25, 2017. With that, screening coverage (attendance), which had been about 72% for the first years upon implementation [3], i.e., in line with the WHO recommendations, then began to decline, reaching about 50% by the present time, as leading scientists of KazIOR (Kazakh Research Institute of Oncology and Radiology) recorded.

Furthermore, the other large problem is related to the CaCx screening routine in the country. To date, the majority of specialists in management of women with atypical cytological results are guided by the joint recommendations of the ACS (American Cancer Society), ASCCP (American Society for Colposcopy and Cervical Pathology), USPSTF (US Preventive Services Task Forces), and other leading institutions [9]. Regrettably, these recommendations still have not been adopted by the health policymakers in our country, despite the existing HTA (Health Technology Assessment) reports and leading experts' opinions confirming advantages of HPV-based screening in a co-testing way, i.e., collectively with cytology [10–14].

To implement worldwide-accepted screening in a co-testing way, any countries should first create their nationwide maps of HPV prevalence and type distribution, as HPV is an apparent causative factor for the CaCx development, and its various types differ by carcinogenic potential [15–19]. And meanwhile, data on HPV leading types across Kazakhstan still are limited with a few publications, and far not all the regions have been studied [20–23]. Currently, 14 types are referred to as the types of highly carcinogenic risk (HR-HPV) [24]. Listed researches on HPV prevalence reported high dissemination of HR-HPV types, within 25–28.3% across examined regions.

According to world's leading experts' opinion, only implementation of universal HPV vaccination with enhanced screening would maximally reduce the burden of cervical cancer in post-Soviet countries, albeit options for reducing the HPV-related disease burden are resource-dependent [4].

Revised in Melbourne (2014), the WHO tactics on the CaCx prevention has confirmed that HPV vaccination of girls aged 9–13 years still remains the primary principle of prevention [25].

High rates of cervical cancer along with wide dissemination of HR-HPV types in Kazakhstan entail the need to renew the state-scale program of universal

compulsory vaccination of adolescents. Successfully launched in Kazakhstan in 2013, a pilot vaccination program then was discontinued, largely due to the negative attitude of parents who were not yet ready to the challenges of modern world. However, further efforts are needed to overcome prejudices in primary prevention of cervical cancer. According to the estimates of specialists, stated in the press release of the Centers for Disease Control and Prevention (CDC), in the USA there was an impressive decrease in the prevalence of vaccine types of HPV by 56% in the group 14–19 years old for 7 years of the introduction of vaccination against cervical cancer in adolescent girls (2006–2013) [26]. Recommendations for vaccination are developed by the world's leading cancer institutes not only for girls but also for boys 11–12 years old. Effectiveness of vaccination now is convincingly proven and is no longer questioned [27].

Thus, a wide circle of issues on the CaCx prevention is to be solved in Kazakhstan in the nearest time, and specific information of the relatively targeted audience of these efforts would serve as a basis for positive changes in this direction.

2. Risk factors for HPV infection and cervical cancer development

According to WHO and CDC, the following conditions are considered the risk factors for the cervical cancer development:

- Inaccessibility of the screening program or rare participation in it.
- Persistent HPV infection.
- States causing immunosuppression, such as HIV, high-dose steroid use, etc.
- Lower genital tract neoplasia irrespective of the area: vulvar, vaginal, and anal.
- Increasing the number of sexual partners (increases risk of HPV acquisition) along with early age of sexual debut.
- Presence of sexually transmitted infections, such as *C. trachomatis* and possibly herpes simplex virus (HSV).
- Tobacco smoking (current and, to a lesser extent, past tobacco smoking) increases the risk of cervical squamous cell carcinoma.
- The use of birth control pills: long-term use increases the risk of cervical squamous cell carcinoma.
- More than three full-term pregnancies.

It is worthwhile to emphasize that the risk factors for HPV infection do not coincide in full with the risk factors for cervical cancer. Only persistent HPV infection constitutes fundamental condition for the CaCx development, while other mentioned risk factors such as smoking play a supporting role [13, 28].

To our knowledge, the peak incidence of HPV infection occurs in 20-year-olds, the peak incidence and detection of CIN-III is characteristic for the age group of 30-year-olds, and the peak incidence of cervical cancer occurs at the age of

40 years or more. According to estimates, cervical cancer can occur in about 3–5% of women with high-risk HPV infection unless secondary prevention (screening) implements [29].

3. Survey as an instrument to get information

Survey, being the most cost-effective and quick tool to recognize needs, intentions, and perception of the targeted audience, serves for specific purposes, but its design depends not only on the aims claimed but often on standard of living and concomitant features of the sample tested, such as educational level, availability and quality of healthcare, etc. One may observe quite noticeable differences in designing the surveys depending on economic status of the countries where those tools applied. Mostly, in high-income countries, more detailed surveys designed to reveal more complex context are used, due to relatively long practicing. For example, in Italy, surveys aimed for obtaining baseline data on risk factors have been widely practiced for at least 30 years [30]. Besides, in high-income countries, web-based survey, or computer-aided self-administered interviewing (CASI), appears to be frequently used, as well as applying mail and telephone surveys, due to providing better confidentiality for an individual, despite relatively low response rate (65% considered acceptable) [31–33]. Personal interviews usually are conducted upon facing "difficult cases," i.e., where obtaining complex information is needed. Direct interviewing provides opportunities for best control, surveillance, and on-site verification. Meanwhile, direct interviewing, being a relatively expensive and time-consuming way to obtain data, nonetheless, applies more frequently in low-/middle-income countries, where there are many illiterate or low-educated people or in sites where sociocultural customs, different from western lifestyle, are practiced [34–36]. Overall, all these generalizations are quite arbitrary, as specialists choose a way of operating mostly based on purposes and capabilities of their research.

As to the models for questionnaire development, the two most cited and used approaches seem to be most popular, according to literature sources.

One of these approaches constitutes a conception of the Theory of Planned Behavior (TPB) as applied to the behavioral researches on cervical cancer issues [32, 37, 38]. According to the theory, the author Ajzen I. stated, "a more favourable attitude makes a person more attentive toward a recommendation made by significant others" [39].

The second approach refers to the Health Belief Model, on the basis of which Robert DeVellis developed guidelines summarized in his book *Scale Development*. Based on these guidelines, a principally new questionnaire, CPC-28, has been developed by Maria Teresa Urrutia and R. Hall [40]. The questionnaire includes six domains: "the barriers to take a Pap test," "the cues to action," "the severity," "the need to have a Pap test," "the susceptibility to cervical cancer," and "the benefit" domain. CPC-28 has been used by many researchers as an example for development of their own questionnaires [41, 42].

These approaches suggest development of questionnaires aimed to reveal perception, intentions, beliefs, and possible attitudes of the individual tested. As applied toward HPV infection and cervical cancer issues, such models gave a lot to reveal prejudices relatively CaCx preventive measures—screening and vaccination—throughout almost all strata of the female population.

The following step in the questionnaire developing is testing for validation purposes, often including "pretest-test-retest" stages. Testing is the key factor for checking the tool's validation and reliability. Usually, outer experts are involved to check the questionnaire. Field-testing in specially selected representative groups for

the trial interview purposes is combined with the testing of its internal consistency by Cronbach's alpha coefficient. During the trial interviews combined with Cronbach's alpha calculation, the amount of items may be changed. For example, in CPC-28 53 initial items then were decreased to 28, and other researchers reported cutting down their items from 69 to 26 in order to reach optimal Cronbach's alpha within 0.7 and higher [43]. It should be noted that when evaluating the survey specific results, it is not appropriate to rely on Cronbach's alpha index solely. Reliability of the interviewees' responses does not depend on Cronbach's alpha directly. In listed researches the number of items varies from 12 [36] to 26–29 [32, 40, 43] and up to 64–65 [41, 44].

A separate domain of surveys concerning CaCx is presented by studies addressing the issues of quality of life (QoL), information needs, sexuality, and other problems in patients with cervical cancer or its precursor, who had undergone the treatment [45–49].

Overall, creating an effective tool allows for obtaining a lot of valuable data for timely renewal of cervical cancer prevention strategies, including issues of selecting the most rational information sources for the targeted audience.

4. The survey on cervical cancer risk factors conducted in Western Kazakhstan: aims, methodology, and findings

Findings of the survey presented below are quite indicative and to a definite extent may reflect the current situation with awareness of the CaCx preventive measures not only in Kazakhstan alone but, in a broad sense, in post-Soviet Central Asian states.

General information about the country: the Republic of Kazakhstan is a leading state in Central Asia and refers to middle-income countries. The country ranks 9th in terms of territory in the world, 64th in terms of population, and 184th in terms of density (6.3 per sq. km). The population of the country as of January 1, 2016, is 17,417,673; the ratio of men and women is 48:52%. Share of the population aged 15–65 is 71%. The national composition of Kazakhs is 66.1%, Russians 21.5%, and other ethnic group. 12.4% (data are taken from the information source of the Agency of Statistics of the Republic of Kazakhstan). The western region is industrially developed and consists of four large provinces: Aktobe, West Kazakhstan, Mangystau, and Atyrau. All provinces are involved in oil industry, with the presence of atomic industry in Mangystau.

4.1 Aims, materials, and methods of the research

During 2014–2017 a multipurpose scientific project on HPV infection and cervical cancer issues was carried out across the region by the West Kazakhstan University's research team.

The interview constituted a part of the mentioned research and aimed to determine qualitatively and quantitatively a group at risk for possible cervical cancer development. Therefore, tasks of the survey were the following:

- Identifying women of general female population who are infected with HPV in order to allocate those who are exposed to the CaCx development risk factors

- Comparing women infected with HPV but not having CaCx and those diagnosed with cervical cancer by matching, to establish dominant risk factors in the region

Design and protocol of the study were approved by the University's Institutional Review Board (October 9, 2014). The work was carried out in accordance with the Helsinki Declaration principles. All participants who signed the informed consent form were fully informed on the objectives of this analysis.

4.1.1 General sample (clinically healthy women)

In determining the sample size for general female population, the following points mattered:

- According to a pilot study of the West Kazakhstan University on HPV as of 2014, N for HPV genotyping was 1098 with valid statistical results at the prevalence HR-HPV 26.04% ($p \leq 0.043$) [21].

- Statistical data on the number of urban female population living in western cities of regional importance and suburbs.

In total, N according to calculations (two-side type I error of $p \leq 0.05$, 95% CI) was counted 1152, of which 417 in Aktobe, 253 in Uralsk (West Kazakhstan), 237 in Atyrau, and 245 in Mangystau.

Data were collected in medical settings in cities of regional importance, including the nearest vicinities. To reach maximally possible scope of female population and avoid possible bias, all kinds of outpatient clinics were involved: state-sponsored, insurance, and private ones. Enrollment of women was held either during their routine visit to the gynecologist, by ads placed in the clinics lobby, or by the invitation of sentinel specialists. Inclusion criteria for general sample were the following: age 18–60+ years, resident of Western Kazakhstan of any ethnicity, and no vaccination history.

The exclusion criteria are nonresidents of Kazakhstan and vaccination history.

HIV status and pregnancy of the first trimester were not exclusion criteria.

4.1.2 Cervical cancer sample

As to the sample size of the patients with CaCx first time diagnosed, the number of adult (18+) female population of the republic along with the incidence of cervical cancer in Kazakhstan equaled to 4.8% (data of the Agency on Statistics as of 2013) was applied in the formula:

$$N = \frac{p \times q \times Z_{\alpha}^2 \times N}{\Delta^2 \times N + p \times q \times Z_{\alpha}^2} \quad (1)$$

where $Z_{-}(\alpha) = 1.96$ is the critical values of the normal standard distribution for a given $\alpha = 0.05$, N is the number of female population of the republic (6,700,000), $p = 0.048$ is the incidence of cervical cancer, $q = 1–p = 0.952$, and $\Delta = 0.05$ is the sampling error.

According to calculations, the needed sample size was within 67–80.

All consonants to participate in the study were selected among women with first-time-diagnosed cancer across all regional oncology centers.

Inclusion criteria are any age, any stage of the cancer process, and histological verification of the diagnosis.

Exclusion criteria are nonresidents of the Western Kazakhstan and presence of the previous medical intervention—radiotherapy, chemotherapy, and surgical treatment.

Qualitative detection and quantification of human papillomavirus were performed in both samples by PCR real-time method based on the Russian test systems and equipment ("DNA-Technology" LLC, Russian Federation). Production of the company "DNA-Technology" was certified (ISO 13485: 2012).

4.1.3 Statistical processing

SPSS Statistics 20 software (IBM, Armonk, NY, USA) and the program Statistica 10 (Dell software, USA) were applied for calculations. For all tests a two-side type I error of $p = 0.05$ or less at 95% CI was assumed statistically significant. Nonparametric operational tests were used due to a priori missing a normal distribution. To identify the dominant risk factors for CaCx development, appropriate statistical tests were carried out: an analysis of the Pearson $\chi 2$ contingency tables to identify significant links (with the definition of the Cramer's V criterion), analysis of the quantitative variables in two independent samples (Mann-Whitney test), and logistic regression analysis with odds ratio calculation (OR).

4.2 Questionnaire designing and survey conducting

4.2.1 Questionnaire designing

The questionnaire was developed in two languages, Kazakh and Russian (optional), in a semi-structured manner, with questions, mostly closed, to collect data reflecting a role of the most known risk factors in the development of CaCx.

Overall, the questionnaire included three conditional domains: the first one for collecting social/demographic information, such as age, ethnicity, education, occupation, and family (per capita) income of women who were being interviewed. This domain also included issues related to the number of pregnancies and the presence of cervical cancer in close relatives irrespective to the time period, at present or in the past (not in terms of hereditary, but to assess differences in perception). The conditional second part of the questionnaire concerned behavioral/social settings: attitude toward smoking, the number of sexual partners during life, age of sexual activity onset, and the method of contraception currently used, with focus on the birth control pills (BCPs). The third conditional domain included questions devoted to perception of the CaCx preventive measures: attendance of municipal PHC clinics (in terms of availability of state-sponsored free healthcare), screening activities, and attitude toward vaccination against cervical cancer. This part consisted of closed questions, to reveal the women's perception of nationwide measures, given a mentioned decreasing of the screening coverage and discontinuation of the pilot vaccination program in adolescents, started in 2013. As previously stated, adolescents' parents perceived the program mostly negatively.

As to the content of the questionnaire, models described in the literature were not applied when designing, since all available examples were intended for relatively homogeneous audience, whereas in this questionnaire, the list of questions was identical both for women from the general sample (i.e., clinically healthy) and women who were diagnosed with CaCx. Besides, another consideration was mattered. Such a study was the first in its kind in medical practice of the region and the country, and its response rate was unknown. So, it was decided to develop a light version of the tool consisting of 14 most important items. Eventually, this number of questions did not burden the participants and allowed the stated objectives of the survey to be solved.

4.2.2 Validation of the questionnaire

Validation of the questionnaire was performed through Cronbach's alpha (α) calculation, and findings were summarized in **Table 1**.

The item "contraceptive use" knocked down the total row due to negative r (−0.06). When removing the item, a total Cronbach's α increased from 0.53 (bad) to 0.58, i.e., eventually was recognized "doubtful." Despite the fact that reliability properties of the questionnaire did not meet accepted requirements (α 0.07 and higher), it was decided not to modify the tool for increasing its internal consistency due to considerations described above. Preliminary testing and retest also were not performed.

Initial calculation for all items			**Calculation upon deleting the item "contraception methods"**		
Result for the scale, mean = 21.2652			Result for the scale, averaged = 19.7543		
Std. dev. = 4.07863			Std. dev. = 3,85,041		
N items, 14			N items, 13		
Alpha Cronbach, 0.452699			Alpha Cronbach, 0.567090		
Standardized alpha, 0.525177			Standardized alpha, 0.578369		
Mean interposition correlation, −0.080197			Mean interposition correlation, −0.096674		
Items	**General position correl (r)**	**α upon removal**	**Items**	**General position correl (r)**	**α upon removal**
Age	0.171210	0.430272	Age	0.254605	0.541922
Ethnicity	0.148982	0.441428	Ethnicity	0.146472	0.561391
Education	0.351550	0.376869	Education	0.364908	0.511785
Employment	0.370232	0.346615	Employment	0.396442	0.497283
Income	0.374762	0.378943	Income	0.420856	0.502972
Number of pregnancies	0.018588	0.464434	Number of pregnancies	0.021628	0.585372
Close relatives with CaCx	0.075294	0.451611	Close relatives with CaCx	0.087969	0.567689
Duration of sexual life	0.227343	0.415536	Duration of sexual life	0.301034	0.529433
Number of sexual partners	0.263014	0.414043	Number of sexual partners	0.290278	0.536063
Contraceptive use	−0.064162	0.568096	Contraceptive use	—	—
Smoking	0.155715	0.443014	Smoking	0.161773	0.561118
State PHC facilities attendance	0.281290	0.407147	State PHC facilities attendance	0.292777	0.533949
CaCx screening attendance	0.015578	0.471991	CaCx screening attendance	0.020295	0.595756
Vaccination awareness	0.161580	0.437350	Vaccination awareness	0.162059	0.558797

Table 1.
Results of Cronbach's α calculation.

4.2.3 Allocation of interviewees according to the "per capita income"

Data for the "per capita income" item were taken from the website of the Statistics Committee of the Ministry of National Economy for the fourth quarter of 2014 (data on the standard of living, www.statgov.kz). The amount of the subsistence minimum determining the poverty line was within or slightly more than 100 USD (according to a currency rate).

Overall, three grades were allocated: from less than 100 USD per month up to 200 USD per capita (category of "poor"), from 200 USD up to 500 USD per capita (category of "satisfactory income"), and from 500 to 1000 USD and higher (the category of "relatively well-off people"). Allocation of the respondents in this questionnaire ("poor," "satisfactory income," "well-off") was made based on statistical publications on the standard of living formed on the basis of a sample survey of households and posted on the website of the Statistics Agency of the Republic of Kazakhstan ("Monitoring of incomes and living standards of the population in the Republic of Kazakhstan". Analytical notes of the Agency of the Republic of Kazakhstan on Statistics of the Department of Labor and Living Standards. Astana, 2011–2013). Based on the above information, it was decided to calculate per capita income within the twofold subsistence minimum amounting to 200 US dollars, as a threshold of a relatively satisfactory income, and revenue of 500–1000 USD as a threshold of a conditional "well-off income."

4.2.4 Survey conducting

Direct interviews have been held on site by the research team without participation of the local staff for providing a better confidentiality of the information obtained. To motivate a better veracity, researchers allowed not to indicate a real name and provided relevant explanations on filling in the most "problematic" items—smoking, number of sexual partners, and income. At the same time, active assistance to interviewees when filling in the questionnaire was not permitted.

4.3 Results and discussion

A total of 1166 clinically healthy and 65 having CaCx women were interviewed across the region. A set of data on the survey across both samples, including descriptive statistics, is presented in **Table 2**.

Some obtained data have been cross-checked through the available sources. Information on such indicators as the age of sexual debut, number of pregnancies, specific gravity of smokers, and number of women who use BCPs has been presented in the mentioned report on Kazakhstan by the ICO group on monitoring cervical cancer [7]:

- Average age of sexual debut in women in the Republic of Kazakhstan—20.7 (20.8 in the present survey)
- Average number of pregnancies—2.7 (3.0 in the present survey)
- Total number of women applying birth control pills—7.1% (4.8% in the present survey)
- Total number of smoking women—9.5% (10.8% in the present survey)

Parameters (the questionnaire items)	Cronbach's α for each item	Detailing	General sample, N 1166	CaCx sample, N 65	Notes
Age categories	0.54	18–29	37.7%	1.5%	
		30–39	34.0%	21.9%	
		40–49	17.8%	34.4%	
		50–60+	10.5%	42.2%	
Average age of the interviewees					
General sample: 34.5 ± 9.9 (31.2;36.1, 95% CI) Range 16.0–63.0 M 33.0 (27.0–41.0 by 25/75 quartile)		CaCx sample: 49.0 ± 12.4 (45.9;52.1, 95% CI) Range 28.0–80.0 M 47.5 (40.0–58.5 by 25/75 quartile)			
Ethnicity	0.56	"Asian"	85.3%	79.7%	Representatives of Turkic-speaking people
		"European"	13.6%	20.3%	Representatives of the Slavic diasporas, Germans
		Other (mostly Caucasus ethnic groups)	1.1%	—	Azerbaijanis, Dagestanis, Koreans, etc.
Education level	0.51	School education	31.4%	65.6%	
		Professional college	22.9%	17.2%	
		Higher education (university)	45.7%	17.2%	
Employment	0.50	Not occupied	33.6%	48.4%	Unemployed, housewives, retired
		Low-skilled labor	13.7%	26.5%	
		Medium-sized proficiency sector	20.7%	9.4%	
		Highly-skilled occupations	32.0%	15.6%	
Monthly income per capita	0.50	From less than 100 USD and up to 200 USD	40.1%	50.0%	Category of "poor" people
		From 200 USD up to 500 USD	39.4%	46.9%	Category of "satisfactory income"
		From 500 to 1000 USD and >	20.5%	3.1%	Category of relatively well-off people
Total number of pregnancies	0.59	None	10.7%	3.1%	This refers to childbirth, abortion, ectopic pregnancy
		1–2	36.1%	25.0%	
		3 and more	53.2%	71.9%	
Average number of pregnancies in the history					
General sample*: 3.0 ± 2.2; range 0–16; M 3.0 (2.8–4.4 by 25/75 quartile)		CaCx sample: 4.5 ± 3.3; range 0–14; M 4.0 (2.0–6.0 by 25/75 quartile)			

Parameters (the questionnaire items)	Cronbach's α for each item	Detailing	General sample, N 1166	CaCx sample, N 65	Notes
Presence of close relatives with CaCx	0.57	Yes	5.1%	9.4%	Irrespective to the time period: at present or in the past
		No	94.9%	90.6%	
Age of onset of sexual activity					
General sample*: 20.8 ± 3.4 Range 13.0–45.0 M 20.0 (18.0–22.0 by 25/75 quartile)			CaCx sample: 20.3 ± 2.3 (19.4;20.8, 95% CI) Range 19.0–21.0 M 20.0 (15.0–27.0, 25/75 quartile)		
Lasting of sexual life	0.53	0–10 years	47.2%	3.1%	Regardless the marriage or relationship lasting
		11–20 years	29.9%	31.3%	
		20+ years	22.9%	65.6%	
Average lasting of sexual life					
General sample: 13.5 ± 9.2; range 1.0–45.0 M 12.0 (6.0–20.0)		CaCx sample: 26.5 ± 10.8 (23.3;29.7, CI 95%) M 22.0 (7.0–59.0)			
Number of sexual partners during life	0.54	1 partner	64.7%	60.9%	Regardless of the relationship lasting
		2–5 partners	28.2%	28.1%	
		6 and more	7.1%	10.8%	
Average number of sexual partners during life					
General sample: 2.2 ± 2.9 (1.9;2.7, CI 95%); range 1–30			CaCx sample: 3.0 ± 3.4 (2.1;3.9, CI 95%); range 1–15		
Current application of contraceptive methods (at the time of interview)*	—	I do not apply	43.8%	89.0%	Only the age category ≤49 years old was considered
		Birth control pills*	4.8%	—	
		IUD (intrauterine device)	12.4%	4.7%	
		Condoms	23.0%	6.3%	
		Other (tubal ligation, calendar method, coitus interruptus)	16.1%	—	
Attitude toward smoking*	0.56	I smoke (smoked)	10.8%	9.4%	Regardless of the smoking lasting
		I do not smoke	89.2%	90.6%	
Attendance of municipal PHC facilities (outpatient clinics at the place of residence)	0.53	I visit constantly	40.7%	31.3%	
		I visit sometimes, irregularly	46.3%	39.0%	
		I do not visit, as I attend only private clinics	13.0%	29.7%	
Participation in the nationwide screening program for cervical cancer (in state-sponsored clinics)	0.60	I participate constantly	34.7%	39.0%	Age category < 30 years old was not considered as not included in the screening routine
		I participate irregularly (missed the last/	28.0%	15.6%	

Parameters (the questionnaire items)	Cronbach's α for each item	Detailing	General sample, N 1166	CaCx sample, N 65	Notes
		previous examination)			
		I do not participate (ignore, as I attend gynecologists in private clinics only	37.3%	45.3%	
Awareness of vaccination against cervical cancer	0.58	I know nothing about vaccination	38.8%	60.9%	
		I have heard about vaccination, but do not know how to percept	33.6%	25.0%	
		I welcome vaccination against cervical cancer	22.9%	10.9%	
		I am set against vaccination/I consider it unnecessary/ dangerous	4.7%	3.1%	

**An asterisk indicates some indicators of general sample, for which there are republic-wide data from other sources.*

Table 2.
Total data for both samples across the region with inclusion of descriptive statistics.

In another authoritative source [50], published in the framework of the UNICEF international research and summarizing data of the Republic of Kazakhstan on many medical and social indicators, the share of women 15–24 years old who had sexual intercourses with the "unofficial partner/partners" (promiscuity) during the last year was 16.6%, while the proportion of smoking women aged 15–49 years—8.4%.

Overall, data from these authoritative sources in fact coincided with those obtained in the present work, which to a definite extent might indicate reliability of the information provided by participants of the interview.

4.3.1 Social profile of women infected with HPV in the western region of Kazakhstan

A total of 25% of women from the general sample in frames of the present research appeared to be infected either with HR-HPV types or with non-HR types (22.3; 27.7 CI 95%, p = 0.05), N 291. One of the tasks of the present study was to compare those infected with HPV with those who are not infected in order to identify links between the risk for HPV infection and social/behavioral parameters. Results of the analysis are presented in **Table 3**.

This analysis made it possible to outline the social profile of women infected with HPV in the western region of the country. These are women with satisfactory financial status (monthly per capita income 200–500 USD), occupied with highly skilled work, who had up to five sexual partners and more than three pregnancies in

№.	Parameter or potential risk factor	Achieved level of significance, p-value (≤0.05)	Cramer's V value	Maximum contribution to the final statistics, Pearson's χ^2
1	Age	0.062	0.09	—
2	Ethnicity	0.78	0.03	—
3	Education	0.4	0.05	—
4	Employment	0.002	0.11	χ^2—18.03 9.1% out of 25.0% HPV-infected are representatives of highly skilled occupations
5	Level of per capita income	0.00007	0.13	χ^2—19.1 12.1% out of 25.0%—a group with an income of 200–500 USD per month per capita ("satisfactory income")
6	Number of pregnancies	<0.00001	0.14	χ^2—24.0 11.2% out of 25.0% had three and more pregnancies
7	Presence of close relatives with CaCx	0.52	0.02	—
8	Sexual life duration/age of onset of sexual activity	0.062	0.07	—
9	Number of sexual partners	<0.00001	0.16	χ^2—30.7 23.5% out of 25.0% had up to five partners
10	Application of birth control pills	0.33	Phi 0.00096	—
11	Smoking	0.47	0.02	—
12	Municipal outpatient clinic attendance	0.46	0.02	—
13	Participation in screening program	0.19	0.05	—
14	Awareness of vaccination	0.54	0.03	—

Table 3.
Analysis of the links between HPV infection and social/behavioral parameters.

their history. These women constitute a group at risk for further development of the process, i.e., persistent infection and possible invasive cancer. Increasing awareness of CaCx prevention among young women should rank first in making policy concerning CaCx issues.

4.3.2 Relationship between the level of education and perception of CaCx preventive measures

Further analysis has been performed with the aim of clarifying the relationship between the level of education and perception of preventive measures for cervical cancer. As mentioned before, attendance of state (municipal) PHC facilities implies accessibility and sufficiency of a national free healthcare. In a broad sense, opportunity to attend state-sponsored free outpatient clinics is also to be considered as a prevention of socially significant diseases.

In the general sample (**Table 1**), only 13% of respondents indicated that they do not visit state-sponsored clinics at the place of residence, while among respondents with higher education, this indicator has increased up to 35.1% ($p \leq 0.00001$; Cramer's V 0.14; χ^2–23.1). Only 62.7% of interviewees (34.7% constantly and 28.0% sometimes) respond to an invitation to visit free screening in state (municipal) facilities, and 37.3% of respondents do not attend free screening program at all, preferring either opportunistic screening in private physicians or not undergoing Pap test at all. Among the educated subjects, this indicator has increased up to 51.3% ($p = 0.002$, Cramer's V 0.1, χ^2–18.1). Among respondents in the general sample, 40.7% regularly visit the state PHC facilities, but only 34.7% of all interviewees treat toward screening activities responsibly.

More than two-thirds (69.2%) of subjects with higher education are aware of vaccination against cervical cancer ($p \leq 0.00001$, Cramer's V 0.18, χ^2–23.1), whereas in the total sample, this figure amounted to 56.5% (33.6% have heard, but cannot clarify their attitude—positive or negative, 22.9% are aware and welcome).

Ideally, close to 100% of educated subjects of this research had to welcome mass screening and nationwide immunization program against cervical cancer. For example, according to a large-scale survey conducted in Brazil (n = 54,000), a high correlation was found between the level of education/standard of living and the attendance of mammography and cytology (Pap test): up to 70–80% of educated interviewees constantly visited screening events—$r = 0.52$ and $r = 0.66$, respectively [51]. In general, Kazakhstan belongs to a group of countries with high Human Development Index (HDI). According to the results of HDI evaluation in 2016 [52] when these data were collected, our country ranked 56th in the international rating between Belarus and Malaysia.

Given the relatively high HDI of the country with a large stratum of enlightened women, the findings suggest that measures for primary (vaccination) and secondary (screening) prevention of cervical cancer are insufficient and do not meet the needs of population, especially of its educated part. The same applies to situation with municipal PHC facility attendance (35.1% of educated subjects avoid visit and 51.3% of them avoid free screening there). In this context, relatively low attendance found in the present survey in educated population can be indicative of unsatisfactory quality of services, which eventually may result in bringing down a prestige of the national healthcare.

4.3.3 Overall awareness of CaCx preventive measures: role of information sources

Overall awareness of the broad circle of the issues on CaCx prevention varies depending on the countries, age groups, and education level. Though 71–78% adults aged 50–70 in England knew that the main aim of the screening programs was to catch cancer early, but only 18% of them were aware that cervical screening is primarily preventive [53]. The low level of Pap screening awareness was found among the students in South Korea [32], about 65% female Saudi teachers were considered less-knowledgeable about CaCx risk factors [33], only 13% of interviewed Uyghur women heard about vaccine against CaCx [34], and 30.1% of female students in Poland were unaware of vaccination as a prevention method [44]. In the present research, the obtained data on awareness of vaccination in general sample are approximately similar with the mentioned: 38.8% knew nothing about vaccines against cervical cancer, while 33.6% heard, but could not decide how to percept it. These findings evidence a deficit of information apprehensible for a majority of female population.

A trend, to a definite extent confirming the mentioned TPV model, according to which most of people in issues of health are guided by opinion of significant others

showing to them more favorable attitude (close relatives, etc.), might be traced in findings of the present survey. A group of interviewees which collided with cervical cancer in their families were analyzed in order to compare their awareness with a baseline level in general sample. Among relatives of women who fell ill or died from cervical cancer, the awareness of vaccination has reached 76.8% ($p = 0.01$, phi 0.1, χ^2–6.0), i.e., even higher than in the stratum of highly educated interviewees (69.2%), which implied that a part of them purposefully had sought information regarding prevention/treatment of CaCx. These findings involve the issues on information sources. According to the mentioned survey conducted across the country's households [50], a share of women aged 15–24 which use the Internet (social networks, messengers) is 94.6%, while the proportion of women aged 15–49 years, at least once a week consuming mass media (newspapers, magazines, radio, TV), is only 16.1%. Results of this research concerning preferences in information seeking in young women would be worth to arrange CaCx prevention awareness campaign via the Internet across the country.

4.4 Cervical cancer-diseased women in the western region of Kazakhstan: likelihood of the disease onset

A total of 65 women aged in average 49.0 ± 12.4 diseased with CaCx (just diagnosed and not yet undergoing treatment) have been interviewed during a survey. Overall description of this sample has been summarized in **Table 1**. What is the most inherent to them comparing to the general sample: most of them (65.6%) have just school education (compulsory for all population in Kazakhstan) vs. 31.4% in the general sample, the share of the employed in highly skilled occupations is 15.4 vs. 32% in the general sample, only 3.1% of them refer to a "relatively well-off" in terms of income, a part of them never visited municipal PHC facilities (31.3%) vs. 13% in the general sample, and they never heard about vaccination (60.9%) vs. 38.8% of clinically healthy women, respectively.

In order to reveal the dominant risk factors for cervical cancer and select a control group, matching was conducted among those infected with HPV but not affected with cervical cancer and those having CaCx. Matching was carried out in proportion 1:1 (65 vs. 65), i.e., for each case of the disease, there was one case from the control group. Selection of the control group for matching was made according to the age criterion and also with the help of the random number generator, i.e., each HPV-infected had equal chances to get into the control group. Thus, 65 respondents from HPV-infected group were randomly selected for analysis to identify risk factors.

An analysis of the Pearson χ^2 contingency tables to identify significant links (including the Cramer's V criterion) is shown in **Table 4**.

Table 5 presents results of the Mann-Whitney test, detailing the analysis of quantitative variables.

Thus, social profile of women with CaCx was defined: they are mostly aged 50–60 + years old, in overwhelming majority infected with HPV 16, poorly educated, unemployed, mostly living within the poverty line, with lasting of sexual life over 20 years, not participating in the screening program, and not aware of the cervical cancer prevention measures (vaccination). A large number of pregnancies and high level of viral load also mattered in their profile.

To assess the likelihood of the disease onset, a logistic regression model was developed. As a "positive effect," the onset of the disease was accepted, and as a "negative effect"—the absence of cervical cancer. The logistic regression was performed by the "forward" method, provided that the variables were introduced, if $p < 0.05$, and removed, if $p > 0.1$. The sample size was 130 cases, where 65 (50%)

№.	Qualitative parameter or potential risk factor	Achieved level of p-value (≤0.05)	Cramer's V criterion	Maximum contribution to the summary statistics, Pearson's χ^2
1	Age	0.003	0.29	11.3—age 50–60+
2	Ethnicity	0.5	0.09	1.19
3	Education	0.00075	0.33	14.4—poorly educated
4	Employment (occupation)	0.00053	0.37	17.6—not employed (unemployed, housewives, retired)
5	The level of per capita income	0.001	0.33	13.6—low income
6	Number of pregnancies	0.23	0.15	2.9
7	Presence of close relatives with CaCx	0.13	0.13—phi	2.2
8	Sexual life duration	0.007	0.28	9.9—over 20 years
9	Number of sexual partners	0.19	0.16	3.3
10	Methods of contraception	0.32	0.007	0.0009
11	Smoking	0.97	0.002	0.0007
12	Attendance of the state clinics	0.09	0.19	4.8
13	Participation in screening program	0.006	0.28	10.2—not participating in screening program
14	Awareness of vaccination	0.026	0.27	9.2—not aware of vaccination
15	Type of HPV*	0.00007	0.35—phi	15.9–72.6% of women with CaCx are infected with typ. 16

** For other genotypes of HPV p-value 0.05 has not been revealed.*

Table 4.
Results of contingency table analysis.

were positive and 65 (50%) were negative ones. The logistic regression model was evaluated through the Nagelkerke R^2 (0.3881, $p < 0.0001$) and recognized "working." Coefficients, standard errors, and a chance, including the odds ratio (OR), have been calculated by commonly accepted methods, and the risk group for CaCx begins at a value >40. Results are summarized in **Table 6**.

Thus, likelihood of the disease onset:

- Decreases by 14 times at a per capita income level of 500–1000 USD + (category of relatively well-off)
- Increases by 0.9 times with the lasting of sexual life over 20 years
- Increases by 0.16 times provided lack of attendance in the state (municipal) clinics
- Decreases by 3.3 times provided at least irregular participation in screening for cervical cancer

Calculation of the morbidity prognosis based on OR in both groups (HPV-infected but not affected with CaCx and having CaCx) was performed.

Variables	U Mann-Whitney test Criteria are significant at the level of p < .05000							
	Summary rank CaCx	Summary rank control	U	Z	p-Level	Z correct.	p-Level	Two-sided exact p
Age	4931.5	3453.5	1308.5	3.63184	0.000281	3.63469	0.000278	0.000233
Age of onset of sexual activity	3845.0	4540.0	1765.0	−1.48147	0.138482	−1.49867	0.133960	0.138856
Number of partners	4049.5	4335.5	1969.5	−0.51816	0.604346	−0.56433	0.572531	0.603861
Duration of sexual life (exposure)	4955.0	3430.0	1285.0	3.74254	0.000182	3.74551	0.000180	0.000148
Number of pregnancies	4595.0	3790.0	1645.0	2.04674	0.040685	2.06837	0.038606	0.040401
Viral load level	4785.0	3600.0	1455.0	2.94174	0.003264	2.94231	0.003258	0.003057

Table 5.
Results of the Mann-Whitney test.

Variables	Coefficient	Std. error	Wald	p	OR	95% CI
Income per capita 500–1000 USD + (3)	−2.64144	0.86882	9.2432	0.0024	0.0713	0.0130–0.3912
Sexual life lasting >20 years (3)	0.083917	0.023797	12.4349	0.0004	1.0875	1.0380–1.1395
Attendance of state clinics (lack of attendance) (3)	1.80433	0.63020	8.1974	0.0042	6.0759	1.7667–20.8954
Participation in screening (irregular) (2)	−1.08362	0.50041	4.6892	0.0304	0.3384	0.1269–0.9023
Constant	−1.69108	0.58494	8.3581	0.0038		

Table 6.
Calculation of a chance and OR for the disease onset.

Overall, prognosis is justified for 73.9% infected with HPV, but not affected by cervical cancer and for 70.3% for women having CaCx (correctly predicted cases—72.09%, at a cutoff value of p = 0.5).

4.5 What was learned from a survey on cervical cancer risk factors in Western Kazakhstan

Based on the data collected in 1166 clinically healthy women, of them 291 (25%) infected with HPV, and 65 women having cervical cancer, one may conclude that the main reason for a chance of the CaCx onset is a low understanding on what are the measures of preventing CaCx.

Only 34.7% of interviewees constantly participate in nationwide screening program, while 37.3% fully ignore nationwide screening in free state-sponsored PHC facilities. Favorable attitude toward vaccination against cervical cancer stated 22.9% of respondents, whereas 38.8% knew nothing, and the rest 33.6% could not clarify their position in this issue.

Education is a key factor for better perception of preventive measures—more than two-thirds of respondents with higher education are aware of vaccination against cervical cancer ($p \leq 0.00001$, Cramer's V 0.18, χ^2–23.1).

And meanwhile, the same stratum of educated women mostly negatively treats to state-sponsored PHC facilities, avoiding visit (35.1 vs. 13.0% in the general sample, $p \leq 0.00001$, Cramer's V 0.14, χ^2–23.1). Moreover, 51.3% of educated women avoid nationwide free screening in state PHC facilities ($p = 0.002$; Cramer's V 0.1; χ^2–18.1). This fact evidences insufficient quality of medical care in state-sponsored clinics.

Lack of relevant information on the CaCx in interviewees who had close relatives with CaCx made them seek and eventually reach a higher awareness level concerning preventive measures—76.8% vs. 56.5 in the general sample ($p = 0.01$, phi 0.1, χ^2–6.0). These findings evidence a deficit of information apprehensible for a majority of the female population.

Though a more number of sexual partners contributed to the risk of being infected with HPV ($p \leq 0.00001$, Cramer's V 0.16, χ^2–30.7), but this factor played no role in the risk of CaCx development. Overall, social profiles of HPV-infected and CaCx-affected women differ significantly and, mainly, by standard of living and occupational status.

Social profile of women having CaCx is mostly aged 50–60 + years old, in overwhelming majority infected with HPV 16 (72.6% of them), poorly educated, unemployed, mostly living within the poverty line, with the sexual life lasting over 20 years, not participating in the screening program, and not aware of the cervical

cancer prevention measures (vaccination). A large number of pregnancies and high level of HPV viral load also mattered in their profile.

The likelihood of the CaCx onset under conditions of Western Kazakhstan decreases by 14 times at relatively high standard of living, income not less 500 USD per capita (OR 0.0713, p = 0.024) and decreases by 3.3 times provided at least irregular participation in screening for cervical cancer (OR 0.3384, p = 0.0304).

Overall, the findings suggest that measures for primary (vaccination) and secondary (screening) prevention of cervical cancer are insufficient and do not meet the needs of the population, especially of its educated part.

5. General conclusion

Findings obtained in this first survey arranged in Kazakhstan are quite generalizable for post-Soviet Central Asian states and, to a lesser extent, for the overwhelming majority of Asian developing countries with high incidence of CaCx. These findings are quite able to contribute to an understanding why women become diseased with CaCx. Low standard of living due to lack of education, low attendance of screening, and low awareness on preventive measures, all these reasons, are interacted and constitute a set of universal triggers for vulnerability toward CaCx.

Kazakhstan is not an exclusion within a wide range of middle-income countries, which need drastic changes in approach to prevent cervical cancer and in revision of a set of applied measures. Population-based surveys, being a very effective tool for studying needs of the targeted audience, should serve as the first step toward diagnostically optimal and cost-effective updated nationwide program for the CaCx prevention.

Elaboration and implementation of a new program should focus on a significant increase of awareness in female population on cervical cancer consequences and a role of HPV infection as a causative factor.

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Conflict of interest

All authors declare that they have no competing interests.

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This book gives a comprehensive overview of recent advances in human papillomavirus (HPV) infection, as well as general concepts of infections, immunopathology, diagnosis, treatment, epidemiology, and etiology. It examines current clinical recommendations in the management of HPV, highlighting the ongoing issues, recent advances, and future directions in diagnostic approaches and therapeutic strategies. The book focuses on various aspects and properties of HPV, whose deep understanding is very important for safeguarding the human race from further loss of resources and economies due to HPV infection. I hope that this work will increase the interest in this field of research and that the readers will find it useful for their investigations, management, and clinical usage.

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