Chapter 1

Updates in Human Papilloma Virus

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Introduction

Human papilloma viruses (HPVs) are members of a family of small, non-enveloped papillomaviruses having a double-stranded DNA. Approximately 200 HPV genotypes belonging to 49 species have been recognized by the International HPV Reference Centre. These genotypes have been sequenced and classified according to their phylogenetic position, biological niche and oncogenic potential with new types discovered regularly. The genera alpha, beta, gamma, mu and nu types infect the humans. Novel γ-HPV types are believed to be present in oral cavity of healthy individuals also [1]. Most recently, a novel HPV type 199 (HPV199) was identified in a nasopharyngeal swab sample [2]. Based on their oncogenic potential, 30–40 genotypes from the α-genus of HPVs that infect the human genital tract can be subdivided into low- and high-risk types. Low-risk HPV types include HPV6 and 11. These low-risk viruses have been associated with benign warts or condylomata. By contrast, at least 12 high-risk HPV types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59, have been associated with at least six different cancers as well as precursor neoplastic lesions.

HPV infection has been identified as a definite human carcinogen for many cancers including: cervix, vulva, vagina, anus, penis and oropharynx. Of the estimated 12.7 million cancers in the year 2008, roughly 610,000 (4.8%) could be attributable to HPV infection. The high-risk genotypes of human papillomavirus are believed to be the causative agents of up to 100% of cervical cancers, 50%
of penile cancers, 70% of vaginal cancers, 43% of vulvar cancers, 88% of anal cancers and 35% of oropharyngeal cancers. The Population Attributable Fraction (PAF) of cervical cancer, anal cancer, penile cancer, vaginal cancer, and vulvar cancer were set at 100, 88, 50, 70, and 43 respectively [3].

Research on HPV has seen a sharp surge in the last 10 years as seen by the number of articles published in PubMed. This rise in interest on HPV could partly be attributed to the apparent involvement of HPV in cancers other than that of the cervix, like oropharyngeal and anal cancers and the fascinating possibility of preventing these cancers before they occur.

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HPV Detection

The most important aspects of HPV detection include not just the presence or absence of HPV, but also identifying HPV integration events and determining HPV copy number with high sensitivity and specificity. As of 2015, 193 distinct commercial HPV tests and at least 127 test variants are available in the market for HPV detection [4]. Detection techniques of HPV have evolved a long way from the primitive and most commonly used reverse line blot technology (RLB) based tests, which is based on the hybridisation of PCR products to HPV-type specific probes on a membrane or Line Probe assay to more advanced tests based on real-time PCR and DNA sequencing. Roche Linear array (LA) is being considered the gold standard in HPV testing with its accurate genotyping [5] but is time consuming and costly. The principle of LA has been successfully duplicated and made more efficient in Hybribio HPV GenoArray which is quarter the cost of and takes half the time of LA test [6].

However, RLB-based tests are not able to determine viral load and hence unable to give a diagnosis for borderline cases in addition to being costlier and time consuming. The recent more advanced real-time PCR based detection assays are not only cost-effective, but also more sensitive and swift thus being more amiable for automation. BioPerfectus Multiplex Real Time (BMRT) HPV assay can detect 21 HPV subtypes, including 18 HR-HPV types of HPV-16, −18, −26, −31, −33, −35, −39, −45, −51, −52, −53, −56, −58, −59, −66, −68, −73, −82 and 3 LR-HPV types of HPV-6, −11, −81 and has been shown to be on par with the classical HPV genotyping. In addition, it
will also be useful to evaluate the clinical relevance of viral persistence at the genotype level, monitor disease recurrence, and examine the effects of widespread vaccination on prevalent HPV types [7]. The other popular multiplex real-time polymerase chain reaction (PCR) assays used for screening cervical cancer include Anyplex II HPV HR (Anyplex_HR; Seegene, Seoul, Korea), Cobas 4800 HPV(Cobas_4800; Roche Molecular Diagnostics, Pleasanton, CA, USA) and the Hybrid capture 2 (HC2; Qiagen GmbH, Hilden, Germany) [8].

In-situ hybridisation (ISH)-based test, RNAscope permits direct visualization of RNA in formalin-fixed, paraffin-embedded (FFPE) tissue with single molecule sensitivity and single cell resolution. The RNAscope HPV assay which was designed to detect the E6/E7 mRNA of seven high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 52, and 58) using a pool of genotype-specific probes not only shows tremendous sensitivity and specificity but the results also show strong clinical correlation to prognosis [9].

DNA sequencing based HPV genotyping is being attempted with mixed results. Nested PCR followed by Sanger sequencing cannot identify mixed infections with multiple types of HPV which is a clinically relevant aspect since women with mixed infections are at a higher risk of developing cervical cancer [5]. DNA sequencing by NGS is more sensitive than Multiplex-PCR and the Nested PCR followed by Sanger sequencing, [10] and shows promise in its ability to detect multiple infections. Moreover, NGS has the ability to detect population-specific variants of HPV which are missed by LA [11]. Whole genome sequencing (WGS) and whole transcriptomes sequencing (RNAseq) have the added advantage of identifying the status and site of HPV integration which is a clinically relevant entity. But the cost of the technique is on the higher side compared to other available techniques and interpreting WGS is still at its infancy. An alternative to WGS is the recently developed Mate-pair sequencing which is cheaper yet very powerful in detecting variations in copy number, structure of the genome and genome translocation information [12]. Other recently developed prominent methods of HPV genotyping include Clinichip HPV genotyping assay [13] and a PCR-based DNA microarray assay - 9G DNA chip test [14].

High Risk HPV Carcinogenesis

HPV has circular double-stranded DNA. It has three oncogenes, E5, E6, and E7 which modulate the transformation process [15]. The high risk HPV E6 and E7 oncoproteins inactivate the tumor suppressors p53 and pRB, respectively, which regulate the activities of the E2F family of transcription factors that control multiple cell cycle transitions as well as other cellular activities. The expres-
sion of these oncoproteins causes immortalization and genomic instability of the infected cells. However for the progression of the high-risk HPV-positive cervical lesions requires additional host cellular mutations. The genomic instability of the infected cell facilitates the acquisition of additional mutations rapidly, required for the malignant progression. Thus the expression of the high-risk HPV E6/E7 genes is necessary for the induction of premalignant changes and also directly contributes to malignant progression. HPV E7 can over-ride the growth-inhibitory action of the cyclin-dependent kinase inhibitors, including p21$^{\text{CIP1}}$ and p27$^{\text{KIP1}}$, which are critical regulators of cell cycle arrest during keratinocyte differentiation. High-risk E6 proteins causes rapid proteasomal degradation of p53. In combination with E7, high-risk HPV E6 proteins contribute to immortalization of primary human epithelial cells through the induction of telomerase activity. HPV E6 can activate hTERT transcription. Thus the HPV E6/E7 oncogenes expression provides a subset of the minimally required carcinogenic hits for full transformation of primary human epithelial cells [16].

HPV Infection and Clinical Manifestation of Disease

HPVs contain a double-stranded, closed circular DNA genome, which comprises approximately eight thousand base pairs with at least eight open reading frames. These viruses are epitheliotropics or mucosotropics, tend to infect the stratified squamous cells on the cutaneous or mucosal surfaces. HPV can be passed from one person to another during the skin-to-skin contact that occurs with sex, including vaginal, anal, or oral sex. About 14 million people are believed to acquire the infection annually and about 79 million people are believed to have prevalent infection [17].

The Clinical Manifestation of HPV Infections in Humans can be Broadly Classified into CUTANEOUS and MUCOSAL.

CUTANEOUS Manifestation of HPV Infection

Majorities of the HPV associated cutaneous lesion are benign and are relatively common in the general population, particularly in children and also in the immunosuppressed individuals. The spectrum of the cutaneous lesions along with their corresponding HPV subtype associations include:

a. **Common warts:** HPV 1, 2, 4, 27, and 57

b. **Plantar warts:** HPV 1 and 4 are frequently and HPV 57, 60, 63, 65, and 66 rarely

c. **Flat warts:** HPV 3 and 10
d. Filiform warts: HPV 1, 2, 4, 27, and 57 especially HPV 2

e. Pigmented warts: HPV 4, 60, and 65

f. Epidermoid cysts: HPV types 57 and 60

g. Skin cancers: Bowen’s disease (BD) is a squamous Cell carcinoma in situ of the skin; however in 3–5% of cases, it can progress to invasive carcinoma. The mucosal HPV types are commonly detected in lesions of extra-genital BD, especially in the peri-ungal region. The other HPV types detected in BD include HPV types 2, 6, 11, 54, 58, 61, 62, and 73. The link between HPV and non-melanoma skin cancers is not clear except in immunosuppressed individuals and in certain genetic backgrounds.

MUCOSAL Manifestation of HPV Infection

These include condyloma acuminatum, focal epithelial hyperplasia, cervical neoplasia and cervical cancer, other anogenital cancers and head and neck cancers.

a. Condyloma acuminatum/Warts: It is one of the most common manifestations of HPV in the genital areas which typically presents as papules, nodules or soft, filiform, pinkish, sessile or pedunculated growths. In men, genital warts more commonly involve the coronal sulcus, glands penis or the penile shaft. In women, the warts com-

monly affect the external genitalia and the cervix. The disease is most frequently caused by low-risk HPVs, (HPV 6 and 11) although many other genotypes have also been implicated. Another manifestation of the low risk HPVs is a condition called recurrent respiratory papillomatosis where in the warts to grow in the larynx and lungs. It is a rare condition, but can happen when a pregnant woman with genital HPV passes the infection to her baby during delivery. The lesion in the larynx can lead to breathing problems, hoarseness of voice or may rarely progress to laryngeal cancers.

b. Focal epithelial hyperplasia is a rare HPV-related disease (HPV 13 and 32) of the oral mucosa that is more common in children and women. The lesions of focal epithelial hyperplasia are mainly located in the lower lip; the upper lip, tongue, oral mucosa, oropharynx, palate, and floor of mouth are less frequently affected.

c. Cervical neoplasia and cervical cancer: The association between HPV and cervical cancer has long been established. HPV is detectable in a great majority of the cervical abnormalities, ranging from incipient cytological abnormalities and dysplasia to invasive carcinomas. HPV detection increases with an increase in the severity of the disease, i.e. with 50–70% positivity in CIN1/LSIL, 85 % positivity in CIN2 and a 90-100% positivity in CIN3 and invasive cervical cancer. Recent research in the field are aimed at development of cheaper and easily
available screening methods, the molecular characterisation of HPV induced cervical cancer and newer treatment options.

**Screening:** There have been two new developments since release of the 2012 guideline for cervical cancer screening by the American Cancer Society. The new management guideline recommended that women with HPV-negative ASC-US results return for screening in 3 years rather than 4 years and stated that HPV-negative ASC-US results are an insufficient basis to allow exit from screening at age 65 years. The second new development pertains to the approval by the US Food and Drug Administration (FDA) of one HPV test for primary cervical cancer screening based on the data on from the ATHENA trial which supports the use of HPV testing alone as an alternative screening strategy.

There are a number of shortcomings in the cisplatinum chemotherapy with concurrent radiation which has been used as the first-line of therapy for patients with stage IIB or greater of cervical cancer. Alternatives combined therapeutic strategies like HPV E6/E7 siRNA in combination with combined chemotherapy and its efficacy in alleviating tumors resistant to conventional treatments are currently being explored [18]. Research on HPV siRNA nanoparticles for targeted anticancer therapy have resulted in the development of 3 RNAi delivery systems for cancer therapy, namely, CALAA-01 (Calando Pharmaceuticals), ALN-VSP02 (Alnylam), and Atu027 (Silence Therapeutics). These candidates are being used to clinically validate the three distinct systemic delivery platforms on which they were based: the cyclodextrin-containing polycation RONDEL technology, the AtuPLEX lipoplex technology, and SNALP liposomes. Apart from these, nearly 30 RNA-i based candidates are in various stages of clinical trial [19].

d. **Other Ano-genital cancers:** These include cancers of the vulva, vagina, penis, and anus. A meta-analysis by De Vuyst investigated the prevalence of HPV in vulvar, vaginal and anal intraepithelial neoplasia (VIN, VAIN, AIN) grades 1-3 and carcinoma from 93 studies conducted in 4 continents. The overall prevalence of HPV infection was 67.8%, 85.3% and 40.4% among 90 VIN1, 1,061 VIN2/3 and 1,873 vulvar carcinomas; 100%, 90.1% and 69.9% among 107 VAIN1, 191 VAIN2/3 and 136 vaginal carcinomas; and 91.5%, 93.9% and 84.3% among 671 AIN1, 609 AIN2/3 and 955 anal carcinomas, respectively. HPV16 was found more frequently (>75%) and HPV18 less frequently (<10%) in HPV-positive vulvar, vaginal and anal carcinomas than in cervical carcinoma. HPV6 and 11 were common in VIN1 and AIN1, but not in VAIN1 [20].

In a more recent study including 1709 vulvar, 408 vaginal and 329 female anal cancer cases and 587 Vulvar Intraepitelial Neoplasia grade 2/3 (VIN2/3), 189 Vaginal
Intraepitelial Neoplasia grade 2/3 (VaIN2/3) and 29 Anal Intraepitelial Neoplasia grade 2/3 (AIN2/3) lesions, the HPV DNA prevalence was 28.6%, 74.3% and 90.0% for vulvar, vaginal and anal cancers, respectively, and 86.7%, 95.8% and 100% for VIN2/3, VaIN2/3 and AIN2/3, respectively [21].

The incidence of anal cancer continues to grow in all women, especially those living with HIV, despite the widespread use of combined antiretroviral therapy. Among males, anal cancer is more common in men who have sex with men, individuals with a history of anal warts and in immunosuppressed populations.

HPV infection is also detected more commonly in the basaloid and warty varieties of penile cancers, but only rarely in keratinizing SCC and verrucous cancers of the penis. In invasive penile cancers, HPV 16 is the most prevalent type (40–70%), followed by HPV 6 (22%), 52 (15%), and 11 (4%).

e. Head and Neck Cancers: There has been an global increase in the incidence of Head and neck cancers not associated to well-established etiological factors like tobacco and alcohol habits. Infection by the oncogenic HPV is thought to be the causative factor of this huge shift in paradigm. A recent meta-analysis showed that HPV prevalence in Head and neck cancers increased significantly from 41% in 2000 to 72% in 2004 [22].

Although the incidence rates of HPV among different head and neck cancer sites has largely been inconsistent with respect to geographical sites, it is widely accepted that HPV has been implicated in a majority of oropharyngeal cancers. HPV positive Oropharyngeal Squamous Cell Carcinomas (OPSCC) is a distinct neoplastic entity, characterised by non-keratinizing squamous cell carcinoma of low to intermediate T-category, exhibiting p53 degradation, retinoblastoma pathway inactivation and p16 up regulation, affecting mostly middle-aged men, having higher socioeconomic status and no or brief history of tobacco consumption with better responses to chemo-radiotherapy and better clinical outcomes [23,24].

There has been an alarming rise in the incidence of HPV associated OPSCC probably, as a consequence of changing lifestyles and sexual behaviors [25]. Yet the causative role of HPV in oropharyngeal squamous cell carcinoma (OPSCC) remains ambiguous as studies reveal that only 21.4% of OPSCCs had HPV integrated into the human genome which is remarkably less than the rates seen in cervical cancer [12]. The involvement of episomal and low copy number HPV to OPSCC carcinogenesis needs to be delved deeper.

The advent of Trans Oral Robotic Surgery (TORS) contemporaneously with an increasing incidence of early stage HPV-related OPSCCs has led to a renewed enthusiasm for exploring the role of surgery in oropharyngeal cancers. Well-designed trials are currently accruing and
the results of these trials will be critical to establish the role of TORS as a novel treatment approach in head and neck cancer. Further clinical studies such as de-escalation of chemo radiation treatment, HPV vaccine therapies and other targeted approaches are also being evaluated in this patient population. It is currently not advisable to change management for either HPV-positive or HPV-negative cancers as high-quality evidence to support such an approach is lacking. Further clinical trials are currently investigating the potential for de-escalation of radiation therapy in HPV associated head and neck cancers in the setting of various regimens of chemo-radiotherapy. (NCT01084083/ECOG1308, NCT01088802/J0988 and NCT01221753) [23].

The importance of HPV as a prognostic factor in OPSCC is reiterated by recent studies which reveal that OPSCC patients with an incidence of high risk HPV survive longer than patients without HPV, regardless of mode of treatment [26]. HPV-positive OPSCC also has a decreased risk of disease recurrence and late toxicity after treatment [27]. Among the HPV-positive OPSCC patients, the ratio of HPV E6*I and E6*II splice variants can predict poorer outcomes [28]. Taken together, these studies suggest that HPV status and prognostic profile of OPSCC patients can be used to design an optimal treatment regimen for them.

P16 as Surrogate Marker for HPV

Expression of p16 has traditionally been considered a surrogate marker for HPV in cervical cancer. But recent research highlight that the same is not true in all OSCC. Many studies have revealed that although many OSCCs show high expression of p16, it does not correlate with the presence or absence of HPV. Tobacco use shows a confounding effect in HPV positive cases. Hence p16 protein is not a reliable marker to assess the potential etiological role of HPV in OSCC in our population [29]. Although many studies confirm p16 protein expression and promoter methylation can serve as a prognostic marker for survival in oropharyngeal, non-oropharyngeal and tongue cancers [30-32] it is not always associated with positive HPV status.

Management and Prevention of HPV Infection

There is no specific treatment for the virus in itself, but there are treatments for the cell changes that HPV can cause. Most sexually active men and women will get HPV at some time in their life, but in most cases the body clears the infection without any treatment.

A new formulation of imiquimod (3.75% cream) is recommended for the management of ano-genital warts. Appropriate infection control, including performing excision using laser or electrocautery in well ventilated
rooms using standard precautions, is recommended to prevent possible transmission to healthcare workers who are involved in the management of condyloma acuminate [17]. The management of HPV associated cancers is generally similar to cancers that are not associated with HPV infection. Prevention is always better than treatment and there are a few things that a person can do to help decrease their risk of HPV infection and this essentially revolves around adopting safe sexual practices. Some cancers linked to HPV have screening tests that can be used to find HPV infection and cell changes early. For example there is a screening recommendation for women aged between 30 to 65 years to get both a HPV testing and Pap testing done every 5 years or a Pap testing done every 3 years for the primary prevention of cervical cancer. Another popular strategy is vaccination which is discussed below.

**HPV Vaccines**

HPV Vaccines were initially introduced to prevent cervical cancer known to be caused by infection with one or more of the high risk subtypes of HPV. Clinical trials have proven its efficacy in preventing cervical intraepithelial neoplasia (CIN); its effectiveness in preventing invasive cervical cancers has been explained by mathematical modeling programs. It is also believed to prevent other HPV related i.e. anogenital and oropharyngeal malignan-

cies in both sexes. HPV capsid forming protein, L1-based prophylactic vaccines against the high risk HPV-16 and HPV-18 have been in approved by the FDA and in use since 2006. HPV vaccines have courted many controversies ever since with regards to its efficacy, safety, ideal age of vaccination; use in HPV infected individuals and its use in males.

**Figure:** Progression towards Cervical Cancer from precancers with High risk HPV infection and molecular markers that can aid in detection of the disease along with HPV.

Newer vaccines are being developed to offer immunity against most high risk HPV subtypes. While the currently available Virus like particles (VLP)-based HPV
vaccines are only bivalent (GlaxoSmithKline, Cervarix*) and quadrivalent (Merck, Gardasil*) offering immunity against HPV-16/18 and HPV-6/11/16/18 respectively, a 9-valent HPV Vaccine has been developed and is under clinical trial. This new vaccine prevents infection and disease caused by HPV-6/11/16/18/31/33/45/52/58. This nonavalent vaccine, named V503 promises broader protection than the currently approved vaccines and has been shown to be effective in preventing precancerous lesions and persistent infections [25,33]. Quadrivalent HPV vaccines have been effective in the resolution of oral squamous cell papilloma although larger randomized clinical trial are required to emphasize the efficacy of the same [34]. Vaccines for specific subtypes are under animal trials like the recombinant DNA vaccine PVAX1-HPV58 mE6E7FcGB which is specific for HPV58 [35] although their practical applications remains to be seen.

Several second-generation HPV minor capsid protein L2-based preventive vaccines are under clinical trials. Vaccines targeting L2 induce broadly neutralizing antibodies, thus capable of blocking infection by a wide range of HPV types. Several new vaccine designs have been developed to optimize the display of L2 epitopes to the immune system and to reduce the cost of manufacture and distribution. L2-based vaccines show substantial assurance as a potential next-generation HPV vaccine [36]. Therapeutic vaccines for HPV including live/killed-vector-based, peptide/protein-based, nucleic acid-based, or cell-based vaccines although in clinical trials are not very popular due to their decreased potency [37]. HPV oncoproteins E6 and E7 are the targets for most therapeutic vaccines [38]. HPV vaccines can possibly aid not only in reducing the burden but also help in the management of HPV related cancers alone or as part of combination strategies. The safety and efficacy of the HPV vaccines are constantly being reviewed across different ethnic groups and age-groups.

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