Chapter 9

Serological Biomarkers for the Prediction and Detection of Human Papillomavirus Associated Cancers

Lourdes Gutierrez-Xicotencatl,
Azucena Salazar-Piña, Lilia Chihu-Amparan and
Adolfo Pedroza-Saavedra

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Abstract

High-risk human papillomavirus (HPV) types are not only associated to uterine cervical cancer, but also to a fraction of cancers of the vulva, vagina, penis, anus, head and neck. An HPV infection generates a protective humoral immune response against the capsid proteins L1 and L2; however, an immune response against other HPV early proteins is also generated. This latter is not a protective response, but those antibodies can be useful as biomarkers of the status of the infection and/or the stage of the cancer lesion. Until now, there are no conclusive results regarding the use of anti-HPV antibodies as biomarkers in diagnosis. In this review, we hereby summarized the actual panorama of the humoral immune response against different HPV early proteins during the development of the disease as possible biomarkers for the prediction and detection of HPV-associated cancers.

Keywords: serological biomarkers, human papillomavirus, humoral immune response, HPV-associated cancers, cancer diagnostic

1. Introduction

Prevention of cervical cancer (CC) and other related human papillomavirus (HPV) diseases constitutes a public health priority worldwide [1]. Primary prevention has been achieved through the introduction of the prophylactic HPV vaccines, but the target groups are only adolescent girls and young women (up to 25 years old) [2]. Secondary prevention has been

implemented through screening methods to prevent precancerous lesions from progressing to cancer [3]. The CC prevention programs in the world are based on cervical cytology and colposcopy to detect precancerous lesions, which have helped to reduce significantly the incidence of this illness in countries with well-organized programs and good coverage of the target population, but this is not the case in developing countries [4, 5]. The main problems are the lack of qualified personnel, the poor quality of the screening tests, lack of follow-up colposcopy and treatment, and over-saturation of the health system facilities, estimating that less than 20% of the CC cases are detected opportunely in these countries [6]. The HPV has been the target for the new molecular diagnostic technologies to detect high-risk (HR)-HPV DNA in cervical cells, but these tests have not been sufficient to discriminate women with precancerous lesions in progression to cancer, from those that eliminate the infection and the lesion. Thus, the increasing incidence of HPV-related cancers worldwide, the inefficacy of the cancer prevention programs in developing countries, and the lack of efficient HPV diagnostic tests, make this a priority health problem worldwide [7].

For this reason, it is important to develop new screening methods, which should achieve high sensitivity, specificity, and should be inexpensive for developing countries. These new diagnostic methods could be used in triage with the cytology or HPV-screening tests to detect opportunely women at risk to develop CC. In addition, it would be important to develop new technologies and to identify new biomarkers that allow the early detection of other HPV-related cancers. In this sense, antibodies against HPV antigens have become the new biomarkers that can be used to detect persistent HPV infection that in combination with other molecular tests could be useful for early detection of HPV-associated cancers.

2. Differential expression of human papillomavirus proteins during the viral cycle

The HPV is a non-enveloped icosahedral virus of approximately 50 nm in diameter that contains a double-stranded circular DNA genome of around 8 Kb, which is divided into three regions: the long control region (LCR) that regulates the viral DNA transcription and replication; the early region (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7* genes) that controls the transcription and replication of the viral genome as well as to control the carcinogenesis; the late region (*L1* and *L2* genes) that contains the genes that expresses the viral capsid proteins [8, 9]. Differential expression of HPV proteins during the viral cycle is important for virus replication and evasion of the host immune response. In new infected cells, the HPV replication starts with the expression of low levels of HPV E6 and E7 viral oncoproteins that generates cellular proliferation and genome instability [10, 11]. First, the major viral oncoprotein E7 binds to the retinoblastoma tumor suppressor protein (pRb), which allows the cell to continue into the cell cycle [12]. Simultaneously, the E6 oncoprotein is expressed, and binds to the cellular ubiquitin ligase E6AP, which in turn results in degradation of the p53 protein, a transcription factor for cell cycle arrest, and in extreme situations, for induction of apoptosis [12]. As the infected basal cells migrate to the upper layers and differentiate, viral DNA replication is favored by the binding of E1 and E2 proteins to the LCR to regulate viral proteins expression [13, 14].

Once the viral DNA replication ends, the E2 protein represses the expression of the E6 and E7 oncogenes to allow the continuation of the viral cycle [15]. In the middle of this process, the E5 oncoprotein is expressed to maintain for a longer time the S-phase of the cell cycle and to delay the differentiation process to allow the complete expression of the viral proteins and the viral DNA replication [16, 17]. In the upper layers, E4 protein interacts with the cytoskeleton collapsing the cytokeratin filaments and enhancing the liberation of viral particles [18, 19], and it is also involved in the viral DNA replication [20]. Finally, the two viral capsid proteins L1 and L2 are expressed in terminally differentiated cells, once the replication of the viral genome has been completed, and ending with the release of the viral particles [21–23].

On the other hand, during a HPV persistent infection, there is a gradual loss of regulation of the E6 and E7 expression genes, which allows the development of early cervical lesions (CIN 1–3; cervical intraepithelial neoplasia grade 1–3) [24]. However, more than 70% of the CIN lesions are eliminated by the immune system. Progression to CC occurs due to an over-expression of E6/E7 oncoproteins, as a result of integration of the viral genome, which leads to the loss of the regulator *E2* gene [15]. This is an important event in the carcinogenesis of CC, as the over-expression of E6 and E7 oncoproteins generates cellular immortalization [25], stop cellular differentiation that generates dysplasia, the cells became anergic for TNF-α (tumor necrosis factor) and TGF-β (transforming growth factor) [25, 26], and chromosomal rearrangements could occur, as has been observed with the *c-myc* gene [27].

During a normal viral cycle, all the early HPV proteins carry out their functions inside the cells, and the viral antigens are poorly exposed to the immune system of the host. However, persistence of HPV infection allows the production of antibodies. Although the antibodies generated against the early HPV proteins are not of neutralizing type, these are suitable for their study as possible biomarkers, which recently is under investigation.

3. Cancers associated with HPV infection

Among all human cancers, 15% are caused by viral infections. HPV infection is recognized as one of the major causes of infection-related cancers in both men and women. Generally, HPV has been associated with more than 90% of anal and cervical cancers, about 70% of vaginal and vulvar cancers, 70% of oropharyngeal cancers, and more than 60% of penile cancers [28].

The HPV is the most common sexually transmitted virus and the HR-HPV types 16 and 18 are more prevalent in CC (approximately 70%) [28]. This type of cancer has been a major public health problem among adult women in developing countries. The last worldwide report for CC identified more than 440,000 incident cases and over 230,000 deaths due to this disease [1]. HR-HPV infection is necessary but not sufficient to cause this cancer, which develops over a long period of time through precursor lesions at the squamocolumnar junction cells near the transformation zone [29]. These cells shown to be multipotent residual embryonic cells have also been identified at the anorectal junction similar to the cervix [29]. Although, the cellular origin and the HPV-DNA prevalence are similar in the anus and in the cervix, the incidence ratio of cervical/anal cancer is quite different (17:1) [30]. The majority of low-grade squamous

intraepithelial lesions (LSIL) do not progress to high-grade lesions (HSILs) or carcinoma, which suggests that the HPV infection alone is not sufficient to generate cancer, as other cofactors such as immune deficiency, host genetic factors, among others are involved [30].

In anal cancer (AC), the HPV infection is detected in 80–90% of the cases, and HPV16 is the predominant type (80%) [31, 32] with a frequency higher than in other anatomical sites [32, 33]. This high frequency of HPV16 may reflect a differential tropism of this type that leads to malignant transformation in the anal mucosa. The prevalence of HPV in AC differs by geographic region, with the highest prevalence in North America and Europe and the lowest in Africa [31]. From the gastrointestinal tract malignancies, the prevalence of AC is around 2–3%, with 27,000 new cases reported worldwide in 2008 [31].

Vulvar cancer (VC) is originated from a precursor in intraepithelial lesions named vulvar intraepithelial neoplasia (VIN) and this type of cancer accounts for >90% of the malignant tumors in the vulva [34]. Recently, there is increasing evidence that suggests two different etiopathogenic pathways for the development of VC, one that is associated to HR-HPV and the second that is HPV independent. The prevalence of HPV-DNA in VIN lesions varied from 52 to 100%, but it is over 90% in VC [32, 34]. Over the last decade, the incidence of HPV-associated VC has increased mainly in young women, probably because of high-risk sexual behavior and a better recognition of these lesions due to HPV-DNA detection [32, 35].

On the other hand, penile cancer (PC) has been considered a relatively rare malignancy in the western world, although recent reports indicate an increase in incidence rates in developing countries (from 0.8 to 1.4/100,000) [36]. The etiology of PC is multifactorial with multiple established risk factors including infection with HPV. Epidemiological studies found that 48% of evaluated PC samples were positive for HPV-DNA and the type 16 or 18 was implicated in approximately 31% of these tumors, with HPV16 being the predominant type [37, 38]. In men, HPV infection can result in a spectrum of genitourinary manifestations that can cause genital warts, penile intraepithelial neoplasia (PIN), and PC. However, most HPV infections are asymptomatic, and up to 70% are cleared within 1 year [38].

The final group of the HPV-associated cancers is the one related to the head and neck cancer (HNC) that is the fifth most common cancer in the world [1]. Every year, there are more than 640,000 cases of this cancer reported and it causes over 350,000 deaths [1]. Squamous cell carcinoma is the most frequent type of neoplasia lesions affecting the head and neck area [39]. The laryngeal cancer (LC) is the most common among head and neck neoplasia and it accounts for about 60% of all cancers in the head and neck area [39]. LC may result from late complications of squamous cell papilloma (SCP), although most of those malignant changes develop without papillomas. Generally, squamous laryngeal cancer development begins with dysplastic changes within the epithelium of mucosa membrane lining the organ, this is followed by an intraepithelial neoplasia and finally the development of the pre-invasive cancer (carcinoma in situ) [39, 40]. However, HPV involvement in LC etiology has not yet been fully evaluated [41].

Within this group of cancers, oropharyngeal carcinomas (OPC) are the most dependent on HPV. The incidence of HPV-positive OPC has been markedly increasing in North America and Europe, with a higher rate in men than in women in North America [30], and HPV16 has been detected in the majority of these cancer cases [40]. Until now, little is known about the transmission and immunogenicity of HR-HPVs within the oropharynx. There is a strong

association with having performed oral sex and the number of lifetime partners [42], suggesting that initial infection of HPV in the oropharynx is related to high-risk sexual activity. HPV nucleic acid examination in rinse and gargle samples showed a prevalence of 4.7% of HR-HPV infection in the general population among the ages 45–65 years old. However, it is still unclear the implications of the viral infection in the development of OPC [42].

Moreover, esophageal cancer (EC) is the leading cause of cancer mortality worldwide, with approximately 500,000 incident cases and more than 400,000 deaths each year [1]. There are two types of EC; the most common is the squamous cell carcinoma (ESCC), which is highly prevalent in Eastern countries and in developing countries. The second type is the adenocarcinoma (EAC), which is associated with Barrett's esophagus, and its incidence has raised by 5–10% each year, in developed (Western) countries [43].

4. Immune response to the HPV infection

Mucosal HPV infections frequently arise in the anogenital tract and in the head and neck region, and these sites of infection have high threshold of immune tolerance [44].

The infection and replication of HPV is restricted to differentiating epithelial cells, where there is a limiting presentation of viral antigens to the host immune system. As a result, there is a low but detectable humoral immune response in most infected individuals [45]. HR-HPV types 16 and 18 mainly induce persistent infections without frequent serious complications for the host; they are also highly successful in releasing viral particles transmissible to others [46]. This virus takes the host to a point of balance where the infection does not represent a serious drawback, and viral replication is not limited by the host immune response [46], because the virus does not have a blood-borne phase or viremia. The HPV infection does not induce necrosis, cytolysis, or inflammation, and as a result, there is little or no release of pro-inflammatory cytokines in the local environment [47]. The HPV viral cycle occurs in cells that are destined for death by anoikis (detachment), and because of this, there are no danger signals to alert the immune system to generate an efficient response to eliminate the infection [48].

It is well documented that more than 80% of the genital lesions caused by HPV infections are cleared as a result of a successful cell-mediated immune response, during which cells of the innate immune system such as keratinocytes, dendritic cells (DCs), Langerhans cells (LgCs), macrophages, natural killer (NK), and NKT cells, may play an important role in clearing the infection by promoting a pro-inflammatory process [49]. In the female genital track, the natural host of the HPV infection, there are keratinocytes that could act as immune sentinels, as it has been shown in skin [50]. These cells express Toll-like receptors (TLRs, belonging to the pathogen recognition receptors (PRR) family) on the cell surface (TLR-1, -2, -4, -5 and -6) and in the endosomes (TLR-3 and -9). Specifically, TLR-9 is activated by unmethylated double-stranded CpG-rich DNA [51], allowing the secretion of interferons (IFNs) to activate the NK cells [52], which in turn kill the HPV-infected cells [53]. However, if the HPV infection becomes persistent, there is a downregulation of the innate immune response, which facilitates the virus to escape from the immune system. This mechanism could be through the downregulation of the IFN response by the oncoproteins E6 and E7 that interfere with different molecules involved in the signal transduction pathways of these cytokines [54].

Conversely, the LgCs and DCs (antigen-presenting cells) initiate the adaptive immune response to eliminate the HPV infection, through antigen-specific presentation to B and T cells in the lymph node. In this process, there is a generation of a Th1-type microenvironment by secretion of pro-inflammatory cytokines, which helps to activate Tc cells (directed against the early HPV proteins E2, E6 and E7) to kill the infected cells [55]. This immunological response is complemented by the generation of neutralizing antibodies against the L1 capsid protein to further inhibit the spread of the viral infection, but the virus uses several strategies to evade this adaptive immune response [56]. Recently, it was demonstrated that E2, E6, and E7 proteins upregulate the expression of IL-10 and TGF-β by interacting with their promoters, which allows an immunosuppressive environment [56]. Additionally, the E5 oncoprotein regulates the antigen presentation to the Tc cells by retaining the MHC-I in the Golgi apparatus, and preventing the MHC-I complex transportation to the cell membrane [57]. Besides, the antigen presentation to the Th cells is also regulated by E5, since this protein prevents the acidification of the endosomes, where the MHC-II restricted antigen is processed [58]. At the end, the immune system fails to clear the HPV infection as a result of an imbalance between Th1 (pro-inflammatory) and Th2 (anti-inflammatory) cytokines, which allows a persistent infection with a high risk for the development of CC [56, 59].

Finally, the humoral immune response against the HPV infection is driven through the activation of the B-cell receptor by recognition of HPV antigens and stimulation by the CD40 Th cells receptor that allows the differentiation into plasma cells to produce antibodies against HPV proteins [48]. In this way, a differential antibody response against different HPV antigens (E1, E2, E4, E5, E6, E7, L1, and L2) is generated and detected in the sera of HPV-infected women [60], and specifically, anti-E7 antibodies have been identified and associated to CC, and suggested as a possible markers for late stages of this disease [61–65].

5. Humoral immune response against human papillomavirus antigens

For several decades, the humoral immune response against HPV proteins has been used to study the viral cycle, and more recently as markers of HPV-associated cancers at different anatomical sites [66]. In this regard, some studies showed that seroconversion and the presence of anti-HPV antibodies were associated to the occurrence of precancerous lesions at different anogenital sites such as CC, AC, and from other sites like oral cancer (OC) [66, 67].

The presence of anti-HPV antibodies has been investigated through several epidemiological studies in several populations with different exposures to the virus and found a great variety in the prevalence and kinetics of these antibodies. The variations observed in the antibody response could depend on the population type, anatomical site of infection, viral antigens present, among others, but also the detection method may influence the antibody results observed (**Tables 1** and **2**). The immune response to the HPV proteome (or lack thereof) may provide some biological clues required to answer some of these questions. The HPV oncoproteins E6 and E7 are early viral proteins that drive neoplastic transformation, are reliable indicators of an HPV-associated neoplasia, and can lead to detectable serum antibodies prior

to and at time of diagnosis, as well as post treatment [68]. To evaluate the serum antibodies against HPV antigens, generated during infection, precancerous lesions and cancer, several laboratory technics have been used, such as the ELISA (enzyme linked immunosorbent assay), western blot, radioimmunoassay (RIA) and more recently, Luminex multiplex. These methods use different antigens such as L1 virus like particles (VLPs) from different HPV types; synthetic peptides from L1, L2, E2, E4, and E7, bacterial recombinant proteins, or *in vitro*-translated viral antigens [7, 69].

A large number of epidemiological and clinical studies have been carried out to search for the presence of antibodies against HPV proteins and to identify associations of these serological markers with different types of anogenital cancers. In this sense, antibodies against proteins L1, E4, E6, and E7 from HPV16 are the most explored and are most frequently associated with different HPV-associated cancers (**Table 1**) [62, 66, 69–89].

Until now, the anti-L1 antibodies are more commonly associated to anal, penile, vulvar, vaginal, and cervical cancer, but the results are contradictory. Some researchers found similar prevalence of anti-L1 antibodies in controls and in penile, vulvar, and vaginal cancer cases (17-38%) [66, 79], while others have shown higher prevalence of anti-L1 antibodies in AC (~54%) and CC (~68%) than in controls [79, 82]. These variations could be the result of different sensitivities in the tests used, as well as the purity and the origin of the viral antigens [55, 90]. Even though there are anti-L1 antibodies present in different types of cancer, these antibodies do not differentiate the anatomical site of the HPV infection or the lesion site. Still some differences have been identified, as anti-E7 antibodies are good markers for CC and anti-E6 antibodies for OC [62, 91]. In this way, several studies have been carried out and showed that antibodies against E7 have been commonly associated with AC and CC, with prevalence that goes from 45% in the anus [78] and up to 75% in the cervix [62, 69], while in the penis, vulva, and vagina, the antibody prevalence was under 15% [79]. In contrast, although serological antibodies against E6 prevalence were high in CC patients by using different tests (from 37 to 44%) [70, 86], the association of anti-E6 antibodies with this type of cancer has not been very well defined [70, 74, 75, 88].

One of the most studied HPV proteins is E4, and this is probably due to its abundance (20–30% of total protein in condylomas) and to its differential production along the viral cycle [92]. During HPV DNA replication in low-grade lesions, high expression levels of E4 protein are observed, while in high-grade lesions, this protein is almost absent [24, 92, 93]. As a result of these observations, the E4 protein is proposed as a marker of viral replication [92, 93]. However, the methodology to detect E4 protein relies on biopsy samples, which are difficult to obtain. For that reason, the detection of HPV antibodies has become a more sensitive system to indirectly follow-up the expression of viral proteins. Several epidemiological studies have shown higher prevalence of anti-E4 antibodies is observed in women with premalignant lesions than among CC cases or in the general population [62, 75, 94, 95]. Previously in our laboratory, we showed that anti-E4 antibodies were in low prevalence in healthy women (11%), but the prevalence increased in subjects with CIN1-3 lesions (70%), and slightly decreased in CC (60%), which suggests an early recognition of this protein by the immune system [61, 62], and postulated as early markers of the disease (**Table 1**).

Cancer type	Method	Population	Serum antibodies (%)						Ref.
			E1	E2	E4	E6	E7	L1	
Anal	ELISA	AC cases		70			45	50	[78]
	ELISA	Hospital controls and AC cases						25–52	[71, 72]
	Multiplex GST	Anogenital cancer cases	21	13	29	29	33	54	[79]
Penile	ELISA VLPs	Hospital controls and PC cases						63	[84]
	ELISA L1	Hospital controls and PC cases					11	24–38	[66, 71, 72]
	Multiplex GST	Anogenital cancer cases	8	13	17	8	13	17	[79]
Vulvar	ELISA VLPs	Hospital controls and VC cases						27	[84]
	ELISA L1	Hospital controls and VC cases						43	[72]
	Multiplex GST	Anogenital cancer cases	5	8	16	2	8	27	[79]
Vaginal	ELISA VLPs	Hospital controls and cancer cases						27	[84]
	ELISA L1	Healthy controls and cancer cases						44	[72]
	ELISA	Hospital controls and cancer cases					0	26	[66]
	Multiplex GST	Anogenital cancer cases	0	0	0	8	0	25	[79]
Cervical cancer	Multiplex GST	Healthy controls and CC	10	12	17	32–37	28–42	19–44	[70, 74, 75, 88]
	Western blot	Healthy controls and CC			60		75		[62]
	ELISA	Healthy controls and CC				19–54	13–53	28–68	[63, 70, 76, 77, 81–83, 86, 87]
	RIA	Healthy controls and CC				50–51	33–39	56	[76, 80]
	Luminex multiplex	Healthy controls and CC				11–44	14–61	21–35	[70, 73, 89]
	Slot blot	Healthy controls and CC			73		80	40	[69]

AC, anal cancer; PC, penile cancer; VC, vulvar cancer; CC, cervical cancer.

Table 1. Antibodies against HPV16 antigens in different types of anogenital cancers.

In contrast, little is known about the presence of anti-E4 antibodies in other anogenital HPV-associated cancers. The study of Kreimer and coworkers [79] carried out in AC, VC, and PC patients identified prevalence of anti-E4 antibodies under 30%, but they did not look for these

Cancer type	Method	Population	Serum antibodies (%)						Ref.
			E1	E2	E4	E6	E7	L1	
Tongue	ELISA	Healthy controls and TC					25	4–21	[66, 84]
Oral cavity	ELISA	Hospital controls and OC						12–25	[84]
	ELISA GST	Hospital controls and OC				9	10		[100]
	Multiplex GST	Hospital controls and OC	8	6	8	1	6–14	5–23	[101, 102]
Oropharyngeal	ELISA GST	Healthy controls and OPC	63–74	36–72	24–42	42–90	12–80	6–33	[66, 100, 103–105]
	Luminex GST	Healthy controls OPC and Partners	73	80–83	43	50	63	23	[106]
	Multiplex GST	Healthy and Hospital controls and ADC	16–21	24–25	11–13	30–35	20–25	14–42	[101, 102]
		OPC and Non-OPC	37–70	45–77	34–45	61–85	47–72	55–61	[107, 108]
		OPC	64	84	36	90	71	70	[91]
Laryngeal	ELISA	Healthy controls and LC					12	20	[66]
	Multiplex GST	Hospital controls and ADC	9	5	12	1–2	6–12	3–24	[101, 102]
Esophageal	ELISA	Hospital controls and EC cases				17	0	8–31	[66, 84, 109–111]
	Multiplex GST	Hospital controls and EC cases	6	5	8	0.3–3	5–9	2–23	[101, 102]

TC, tongue cancer; OC, oral cancer; ADC, aero-digestive cancer; OPC, oropharyngeal cancer; LC, laryngeal cancer; EC, esophageal cancer.

Table 2. Antibodies against HPV16 antigens in different types of head and neck cancers.

antibodies in early lesions of these anogenital cancers, where this early HPV marker should be the prevalent, as it was observed for CC (**Table 1**).

Antibodies against E1 and E2 have been analyzed in anogenital cancers and only in AC, the anti-E2 antibodies have been observed with high prevalence (70%), which is through the use of peptides in ELISA [78]. However, a more recent study using a multiplex assay showed that the prevalence of these anti-E2 antibodies was fewer than 15% [79]. In the case of CC, the observed prevalence of anti-E1 and anti-E2 antibodies was under 15% [75] (**Table 1**), but the prevalence of anti-E2 in different degrees of cervical lesion by ELISA was high in CIN1-2 lesions (64%), and it decreased with the increasing severity of the cervical lesion (CIN3, 31%) [96, 97]. Overall, these data suggest that anti-E2 antibodies could constitute a good biomarker for CIN lesions, but these need further studies.

The presence of anti-E5 antibodies in cervical lesions has been described in only one report by using a microarray assay, but no associations were identified with any stage of the uterine cervical lesions [98]. It is necessary to characterize better anti-E5 antibody response, with a more sensitive and specific assay, as this could be an interesting serological marker, since by looking for the presence of E5 mRNA, this was associated with low-grade anogenital lesions [99].

Studies of anti-HPV antibodies in other HPV-associated cancers are underway, but the most recently studied are those localized in the head and neck sites, where different antibodies against E1, E2, E4, E5, E6, E7, and L1 viral proteins have been studied to try to identify associations with the presence of some type of cancer lesion in the oral cavity, in the tongue, pharynx, larynx, and even esophagus (**Table 2**) [66, 84, 91, 100–111].

The study of anti-HPV antibodies in sites such as the mouth and the esophagus began in the late 1990s, but more recently, the study of these antibodies has focused on lesions caused in the oropharyngeal area. In TC, low prevalence of anti-E7 antibodies (25%) and anti-VLPs (4–21%) antibodies were observed [66], while in OC, the prevalence of anti-E7 was under 15%, but it was very variable for anti-VLPs antibodies (from 5 to 25%), differences that could depend on the methodology of antibody detection used (**Table 2**) [79, 84, 101].

One of the cancers in which the presence of anti-HPV antibodies has been analyzed in more detail is the OPC, in which ELISA and multiplex assays have been used with different HPV antigens. These studies strongly showed that anti-E6 antibodies are highly prevalent in OPC (from 30 to 85%), but this prevalence increased up to 90% when the OPC cases were HPV16 positives [91, 102, 106]. However, this was not the case for anti-E7 antibodies, where the prevalence varied from 12 up to 80% [66, 103]. Also, some studies measured the antibodies presence before and after cancer treatment, and they showed that seropositivity to E6 and E7 significantly decreased after treatment. However, only anti-E6 antibodies showed an increased risk of disease recurrence, making these anti-E6 antibodies good biomarkers for disease prognostic [100, 103–105].

The other anti-HPV antibodies with high prevalence in OPC have been those against E1 (~74%) and E2 (~77%) viral proteins [66, 100, 103–105, 107, 108], but this is not the case for other head and neck cancers, where the highest prevalence of antibodies against these viral antigens was under 10% (**Table 2**) [101]. Although the E4 protein is proposed as a marker of viral replication, in the case of OPC, the prevalence of anti-E4 antibodies was under 45%, and this was using the ELISA-GST system that is a highly sensitive method (**Table 2**) [104]. These studies suggest that the same serological markers are not present at the different anatomical site where the HPV-associated cancers appear. These results are very promising in the search for serological biomarkers, which in combination, they generate profiles that could differentiate the anatomical sites where the HPV-associated cancer is present, as it has been the case for the profile anti-E1/E2 + anti-E6 that has been associated to OPC [91], and for CC, the suggested antibody profile is anti-E4 + anti-E7 [69].

There are few studies that have analyzed anti-HPV antibodies in LC and showed low prevalence of the antibodies that varied from 2% for anti-E6 antibodies to a maximum of 24% for anti-L1 antibodies (**Table 2**) [66, 68, 101, 102]. Similar results have been reported for EC, and

the prevalence of anti-HPV antibodies fluctuated from 31% for anti-L1 antibodies and were under 10% for the rest of the anti-HPV antibodies [66, 68, 84, 109, 111]. At this moment, the identification of serological markers for LC and EC are inconclusive, and more studies need to be carried out with more sensitive methodologies such as the slot-blot system, but also a restriction to HPV positive cancer cases should be considered, as a low proportion of these two types of cancers are associated to HPV.

In addition, most of the studies of anti-HPV antibodies in different HPV-associated cancers have been carried out in the late stages of these cancers. It would be of great interest to study early stages of the HPV-associated cancers as some of these anti-HPV antibodies seem to be important in early diagnostic and during follow-up as possible prognostic markers. In the case of CC, there are several studies of anti-HPV antibodies carried out with precancerous uterine cervical lesions, where it has been suggested that anti-E4 antibodies are important markers for CIN1-2 [61, 62], while the profile anti-E4 + anti-E7 is a good marker for CC [69]. In the case of other HPV-associated cancers, there are only few studies that measured anti-HPV antibodies in early stages of the disease, as it is the case of anus and oral cavity lesions, where anti-VLPs antibodies presented the highest prevalence (43 and 30%, respectively) [111–115]. However, more studies are necessary to characterize the humoral immune response in the different HPV-associated cancers and their related precancerous lesions.

It is important to mention that differences in methodology and concerns about HPV misclassification, aside from the heterogeneous responses in the antibody patterns seen in the various studies, and in the different HPV-associated cancers require further evaluation. Besides, there are other confounding variables such as HPV type, viral load, viral exposure history, host immune system factors, and clinical risk factors. Therefore, prospective evaluations of anti-HPV serum antibodies should be controlled for as many of these factors. In addition, it is likely that an antibody signature consisting of a panel rather than a single antibody may provide the highest yield to be able to differentiate anatomical site, as well as early detection of these HPV-associated cancers.

6. Diagnostic and prognostic tests for HPV-associated cancers

Essentially all cervical cancers, most anal and oropharyngeal cancers, and some vaginal, vulvar, and penile cancers are caused by HR-HPVs, but until now, there are no general guidelines for screening of all these HPV-associated cancers. Recently, the availability of new tests and ongoing research are changing the approach to screening and diagnosis of these types of cancers. However, most of the studies have been carried out in CC.

6.1. Cervical cancer

For CC, there are specific guidelines that have been modified in the last years, which include the introduction of new testing technologies, which have improved the early diagnostic of this disease. The cytology is the primary screening system for precancerous lesions and can

be done using Papanicolaou-stained smears (Pap), although this test has a low sensitivity (50–80%) [116, 117], and a high percentage of false positives, due to the fact that inflammatory cytology is considered abnormal [7, 117]. To confirm the presence of a uterine cervical lesion, a colposcopy test is required, which has a high sensitivity (80–95%) and a low specificity (23–63%), because the test becomes positive in the presence of an inflammatory process, and it is not useful to detect early uterine cervical lesions [118].

The introduction of molecular tests to detect DNA from HR-HPVs has shown to be highly sensitive and makes them good screening systems. Recently, there are different HPV molecular technics that are FDA (Food and Drug Administration, USA) approved to use in conjunction with cytology in CC screening programs. Among these tests are the Hybrid Capture® 2 (HC2) (Qiagen, GmbH, Hilden Ger) and Cervista® HR-HPV Test (Hologic, Inc., MA, USA) that amplify the positive hybridization signal and allow the detection of multiple HR-HPV types in one step [119, 120]. The Cervista® HR-HPV and Cervista® HPV types 16/18 tests have shown to be complementary with a 100% of sensitivity for detection of CIN3+ and of 98% for CIN2+ in women with diagnostic of ASC-US (atypical squamous of undetermined significance) by cytology and HR-HPV positive [121], system that has been approved by FDA.

On the other hand, HC2 has been tested at the general population for the detection of HPV worldwide. This system detects 13 HR-HPV types (-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68) and 5 of low risk (LR) (-6, -11, -42, -43, and -44), and it is semi-quantitative (detects up to 1 pg HPV-DNA/ mL). The HC2 was FDA approved to detect women who have been referred to colposcopy with an ambiguous cytology of ASC-US and for the screening of women over 30 years old in conjunction with the cytology test [120]. Several epidemiological studies have shown the high predictive value of the HPV test for early detection of CC. This test is highly sensitive (93–98%) and specific (60–85%) to detect high-grade lesions, which makes the HC2 ideal for screening, and in combination with the cytology test increases the possibility to identified correctly women in risk to develop CC [3, 122, 123].

There are other systems to detect HPV-DNA by using reverse hybridization, some of which are INNO-LiPA® (Line Probe Assay, Innogenetics, Ghent, Belgium), CLART® HPV2 (Genómica, Madrid, Esp), Clinical Arrays® HPV (Genómica, Madrid, Esp) and the Linear Array® (Roche Mol Diagnostics, CA, USA), and this last one detects 37 HR- and LR-HPV types [119, 124]. The difference between these tests is the sensitivity as the LiPA detects from 10 to 100 DNA viral copies. These genotyping methods have a high sensitivity (95%) to detect CIN2+ lesions, making these tests suitable for screening the general population [119].

The detection of the HPV-DNA as indicator of the viral presence does not determine the presence of an active infection, and because of this, a complementary diagnostic test is necessary. Evaluation of viral load has been used as a biomarker of persistent infection, because cytological abnormalities are more frequently observed in CIN2-3 and CC with high viral load [125]. The viral load is determined by Real-time PCR and this system is highly sensitive and specific, and genotyping can be carried out in the same assay. Two diagnostic tests have been developed that use this technology, the Abbott Real-Time HR-HPV (Abbott Mol GmbH & Co. KG, Germany) and the COBAS® 4800 HPV (Roche Mol Diagnostics, CA, USA). These systems detect 14 HR-HPV types in one reaction, and use a colorimetric detection system to differentiate

the types 16 and 18; however, the sensitivity of COBAS® 4800 to detect CIN2 is higher (97%) than the one from Abbott (95%) [123, 126], and just recently have been FDA approved.

In the case of CC, the integration of HPV to the cellular genome seems to be an important part of the carcinogenesis, as this generates an abortive infection with high-level production of the E6 and E7 oncoproteins due to the loss of the *E2* gene expression. This event causes the high expression of mRNA of the *E6* and *E7* oncogenes in the upper layer of the stratified epithelium in CIN2-3 and CC [127]. Thus, the E6/E7 mRNAs have been suggested as specific markers for precancerous lesions. In this way, several commercial tests have been developed to detect mRNAs from E6/E7 such as PreTect® HPV Proofer (NorChip AS, Norway) and APTIMA® HPV (Gen-Probe CA, USA). Both tests detect E6/E7 mRNAs by Real-time PCR, but PreTect® detects only 5 HPV types while APTIMA® detects 14 HR-HPVs, and this is carried on samples from liquid-based cytology. The APTIMA® assay has a similar sensitivity (98%), but higher specificity for HR-HPVs than HC2 (90% vs. 85%, respectively) to detect CIN2-3, making these molecules potential markers for the detection of high-grade lesions, but this is still under investigation [119, 128].

For more than 15 years, it was established that the treatment of early lesions (CIN1, ASC-US, and LSIL) was through follow-up patients with cytology and colposcopy for up to 2 years. However, this procedure is costly, and several visits to the medical office are required, and saturation of the colposcopy system, and loss of patients during the follow-up have made unable to give adequate treatment to those women at risk for developing CC. Because of these problems, it has been necessary to look for new biomarkers that allow the early identification of uterine cervical lesions that could progress to CC. From these efforts, some biomarkers have been identified and are described below.

The $p16^{INK4a}$ is an inhibitor of the cell cycle by inhibiting the hyperphosphorylation of pRb, through blocking the activity of the cyclin D-CDK4/6 complex. It has been observed that the inactivation of pRb by E7 results in the over-expression and accumulation of $p16^{INK4a}$ in the cells, making this protein a surrogated marker of E7 expression, which is associated to the CC development [129, 130]. The detection of $p16^{INK4a}$ is carried out in liquid-based cytology samples and in tissue biopsies by immunohistochemistry (IHC). Different epidemiological studies have demonstrated that $p16^{INK4a}$ is a good marker to identify HPV positive women with CIN 2-3 [129, 130] and it is a useful marker to clarify 90% of ambiguous histopathological diagnostics [131, 132]. The disadvantage of this biomarker is that in other non-associated HPV cancers also it is over-expressed [133].

The Ki-67 proliferation nuclear antigen (cell proliferation marker) is localized in the parabasal layer in the normal stratify uterine cervical epithelium, but during the development of CIN lesions, the expression of Ki-67 is extended all along the cervical epithelium. This marker is ideal to identify tumor cells, which correlates with the clinical stage of the lesion and the development of cancer. The detection of this Ki-67 marker is carried out in liquid-based cytology samples by IHC, and it is useful to detect CIN 2-3-associated HPV lesions [131]. Epidemiological studies in Europe and USA showed that Ki-67 can be used in combination with $p16^{INK4a}$ and helps to increase the sensitivity to a similar level as for HPV detection, but research is still under way to better characterize and validate these biomarkers [131, 132, 134].

6.2. Anal cancer

The AC has similar features with CC, and natural history studies showed that high-grade anal intraepithelial neoplasia (AIN) is a precursor to invasive anal cancer. Because of these similarities, several features of the CC screening program have been taken for routine screening to detect precancerous anal lesions. In the general population, the Pap test to screening for precancerous anal lesions is carried out by taking a sample from the anal canal and the lesions are classified by using the Bethesda nomenclature. At the moment, anal cytology seems to be useful for screening of high-risk individuals, including HIV-positive patients [135].

In contrast, HPV testing has limited utility for AC screening because of the high prevalence of the infection and multiple HPV types in the anal canal of women and HIV-infected men. Only when the test is restricted to HPV16/18, the specificity of the test increased (77%), but the sensitivity dropped (62%), making this diagnostic system not suitable to detect individuals at risk to develop AC [136].

In AC, the high-resolution anoscopy is the standard procedure to diagnose this type of cancer, although several biomarkers such as Ki-67, $p16^{INK4a}$, and others have been investigated to improve the accuracy of histologic diagnosis. Up to date, only the $p16^{INK4a}$ IHC test has been very well documented to increase the predictive positive value of the histopathology test to identify correctly AC cases [137].

In the case of other anogenital HPV-associated cancers such as vulvar, vaginal, or penile, the diagnostic is carried out by identifying a lesion by visual examination from the genital site to the perianal area, and confirming by performing a biopsy. Screening tests are not available and there are no recommended screening methods to detect HPV infections in these types of cancers. From the surrogated markers of HPV infection, $p16^{INK4a}$ was the only marker with sufficient data to support its utility in the evaluation of lower anogenital tract lesions [138].

6.3. Head and neck cancers

HNCs are often detected at late stages, as conventional visual and tactile examination is a way to diagnose this type of cancer. Substantial efforts to develop oral lesion detection systems such as those based on autofluorescence or tissue reflectance (e.g., the Dentlight Oral Exam Light kit, Microlux DL, Orascoptic DK, Sapphire Plus, Trimira Identafi 3000, and ViziLite-Blue and VELscope) have been developed. However, the ability of these tests to discriminate between cancer and benign mucosal lesions is limited and because of this, the OC screening guidelines still do not recommend these diagnostic tests for routine screening of asymptomatic adults for HNCs [139]. The presence of HPV DNA in saliva was thought to be promising for early detection, but the test showed to have low sensitivity and specificity. For this reason, more studies are required to define the populations where the HPV test could have a positive impact and to evaluate the clinical value of this test [140].

On the other hand, the variable prevalence reported for HPV in HNCs could be attributed to the anatomical site where the sample is taken, but approximately 25% of all HNCs are HPV-DNA positive, and type 16 is the most prevalent. The variations in viral prevalence among different studies may be due to a combination of lesions of the different head and

neck anatomical subsites, sample sizes, sampling techniques (frozen, formalin-fixed or paraffin-embedded sections, scraping or oral rinses), and the methodologies used to detect HPV. Because of this, rigorous criteria should be considered for the separation of samples from the various anatomical subsites of the head and neck, as well as to increase sensitivity, specificity, accuracy, and reproducibility of the HPV tests for this type of cancer [140, 141].

Recently, it was suggested that HPV-DNA status in HNC should be analyzed together with other specific markers of active infection such as E6/E7 mRNAs transcription or $p16^{INK4a}$ expression, thus to better characterize these types of HPV-associated cancers. In this sense, the presence of E6/E7 mRNAs and $p16^{INK4a}$ expression was detected mostly in OPC [142]. Numerous other markers such as Ki-67, over-expression of epidermal growth factor receptor, p53, and others, have been studied in HNCs, but none of them have been consistently reliable [140, 141].

6.4. Serological biomarkers for the detection of HPV-associated cancers

The diagnostic of HPV infections for the detection of HPV-associated cancers has been difficult as there is no general screening test that alone or in combination with others allows the early identification of the disease. Because of this, it is necessary to look for tests that would be highly sensitive, specific, less invasive, and inexpensive, and that could be implemented at the general population.

It has been very well described that during the viral cycle, there is a sequential expression of the HPV proteins, which has been associated to different infection stages such as replication (associated to E4 protein), transformation (associated to E6/E7 proteins), and past infections (associated to L1 protein). Thus, the organism generates an antibody response against the viral antigens, in the same way as they are expressed during the viral cycle, letting the identification of different infection stages. Therefore, the humoral immune response naturally amplified the reaction against HPV antigens so that this becomes a good source of new biomarkers to detect HPV-associated premalignant lesions at risk of developing cancer. As a result, the presence of antibodies against E4 protein has been related to viral replication, whereas anti-E7 antibodies are considered markers of a current HPV-associated malignancy [7, 63, 143]. In this context, the use of serological markers has been constantly studied to identify patients with different types of cancer associated with HPV. At present, most of the studies have focused mainly on CC and HNCs.

To study these HPV serological biomarkers, different techniques and reagents have been developed; as for instance, recombinant fusion proteins have been used as antigens in Western blot; synthetic peptides with important immunogenic epitopes for B cell are used in ELISA tests and modifications of this technique have been used to increase the specificity and sensitivity of the assay (**Tables 1** and **2**). Other systems developed to detect HPV antibodies involve the *in vitro* protein transcription and translation, and this is used for radioimmunoassays and also for a novel slot-blot system [7, 61, 62, 69, 143]. The use of these tests to measure anti-HPV antibodies in different populations have shown the utility of these as biological markers of different types of lesions not only at the uterine cervix, but also at other anatomical sites.

In the uterine cervix, antibodies against HPV16 E6 and E7 were detected late during the development of CC with a new streptavidin-biotin capture ELISA, and were pointed as bad prognosis markers [81]. In a retrospective study, by using an ELISA-GST, the presence of anti-E6 and anti-E7 IgG antibodies were identified between 0.5 and 5 years before CC diagnosis, suggesting the usefulness of these antibodies as disease predictors [144]. More recently, in a study conducted by Salazar-Piña and coworkers, by using a novel slot-blot system, they showed that anti-E4 + anti-E7 antibodies discriminate CC from CIN 2-3 lesions with high sensitivity (80%) and a low false negative rate (20%), which corroborate the usefulness of these antibodies as markers for early detection of CC. In this study, they also observed that anti-E4 antibodies alone could be useful as HPV exposure markers at early stages of the disease [69].

Similar results were also observed with OPC, where a bead-based multiplex serology method (multiplex-GST) was used to detect different anti-HPV antibodies and showed a strong association between HPV16 E6 seropositivity and the disease, which suggests that these antibodies can be predictive markers of the disease as they were present more than 10 years before the cancer diagnosis [101, 102]. The sensitivity and specificity of this multiplex-GST system for anti-E6 antibodies was close to 100%, and because of that these antibodies have been proposed as a tool for diagnosis and prognosis of HPV-OPC [91, 145]. This multiplex-GST system also showed a high sensitivity (82%) and specificity (100%) for anti-E2 antibodies in the diagnosis of HPV-OPC. However, low sensitivity and specificity were observed for anti-E7, anti-E1, anti-E4, and anti-L1 antibodies [145].

All this together suggests that anti-HPV antibodies are promising diagnostic, prognostic, and potentially screening markers of HPV-associated cancers, as the presence of anti-HPV serum antibodies can vary according to the anatomical site where the cancer is generated by the HPV infection, however, more studies of anti-HPV antibodies are needed to validate them as serological markers for HPV-associated cancers.

7. Conclusions

This review of serological biomarkers is not intended to be an exhausted one, but rather to bring together the most important findings in the field and to point out the usefulness of these biomarkers in the diagnostic and prognostic of the different HPV-associated cancers. Numerous methods are being developed to detect HPV and related biomarkers that alone or in combination can be used to improve the positive predictive value of current screening methods, and to be able to identify precancerous lesions with a high risk of progression to cancer.

At present, serological anti-HPV antibodies are promising diagnostic, prognostic, and potentially screening markers of HPV-associated cancers. It is likely that a combination of anti-HPV antibodies will generate profiles that could discriminate precancerous lesions in progression to cancer, and also to differentiate the presence of HPV-associated cancers at different anatomical sites. For instance, it was shown that anti-E4 antibodies are associated to CIN1-2 lesions and that the profile anti-E4 + anti-E7 antibodies are useful for early detection of CC, while the presence of anti-E1/E2 + anti-E6 antibodies are prognostic of OPC. Besides, the

immunoglobulin isotype also seems to play an important role in differentiating the site where the HPV infection develops, as it was shown in CC. It is clear that the presence of anti-HPV serum antibodies can vary according to the anatomical site where the cancer is generated by the HPV infection. These results are very promising, however, more studies with larger populations, different anatomical sites, evaluation in precancerous lesions, and the development of new and more sensitive methodologies are required to better characterize the humoral immune response against HPV and to validate these anti-HPV antibodies as serological markers of different HPV-associated cancers.

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Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish.

Author details

Lourdes Gutierrez-Xicotencatl[1]*, Azucena Salazar-Piña[2], Lilia Chihu-Amparan[1] and Adolfo Pedroza-Saavedra[1]

*Address all correspondence to: mlxico@insp.mx

1 Department of Epidemiological Interactions, Center of Research on Infectious Diseases, National Institute of Public Health, Cuernavaca, Morelos, México

2 Faculty of Nutrition, Autonomous University of Morelos State, Cuernavaca, Morelos, México

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