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HPV in Oropharyngeal Cancer

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Over the last few years Oropharyngeal Squamous Cell Cancer has increased its incidence worldwide [1]. Approximately 60-70% of oropharyngeal cancers **(OPC)** are HPV positive and only 10-19% of those cases correspond to larynx and hypopharynx [2]. This data suggest that regardless alcohol and tobacco or alcohol consumption, Human papilloma virus **(HPV)** infection is one of the major risk factors and recent data indicates that HPV-positive patients are usually young and non-smokers [3,4]. How then we acquire HPV in the oropharyngeal tissue? Several studies suggest [2-5] that HPV-infection is sexually transmitted and that patients with more than 26 lifetime sexual partners o more than six oral sex partners are more susceptible to developed OPC. Hence, patients with HPV-related anogenital cancer or immunosuppressed are at high risk of developing tonsillar carcinomas [6].

HPV-negative related OPCs are epidemiologically similar to squamous cell carcinoma of the upper airways, where long-term exposure to tobacco and alcohol induces the onset of the disease. Usually mutations in p53 are present resulting in malignant transformation. In contrast, in HPV-positive OPC the disease starts with the exposure to high-risk HPV (generally HPV-16) and, as we mentioned above, its development is independent of tobacco or o alcohol consumption. [7].

Although HNSCC present high mortality rates, due to the limitations and associated morbidity of current therapies, patients with HPV-positive tumours have a better prognosis and overall survival than patients with HPV-negative tumours [8,9]. The higher response rate to chemotherapy and radiation of these HPV-positive tumours partially explains this result [8]. This finding is crucial for treatment design, considering that about 50% HNSCC cases have an estimated 5-year survival rate with a general age at diagnosis over 45 years [10].

PAPILLOMAVIRIDAE FAMILY

Papilloma viruses **(PV)** are epitheliotropic DNA viruses present in the skin and mucosa of some animals-including mammals and birds [11]. One hundred-eighteen PV types have been completely described in different zoological species, and an even larger number of probable new types have been detected via subgenomic amplification [12]. The only extensive study in human hosts described approximately 100 HPV types through complete genome isolation. A large number of these PVs proved to be ubiquitous and globally distributed [12]. About 30 HPV types have been associated with genital and mucosa lesions and further divided into high and low risk groups [11]. The high-risk types are present in over 95% of squamous-cell cervical carcinomas, whereas Low-risk HPVs are commonly found on mucosas and can cause benign lesions, such as papilloma warts and acuminated condylomas (HPV-6 y HPV-11), or focal epithelial hyperplasia (HPV-13 y HPV 32) [13].

Domenico Antonio Rigoni Stern was the first to show an association between an infective agent and the onset of cervical cancer, back in 1842. He studied the death certificates of hundreds of women and suggested that cervix cancer was associated to sexual activity, since it was predominant in married women and prostitutes and practically non-existent in virgins and nuns [14]. Later, ZurHausen [15] demonstrated the role of HPV in carcinogenesis and this virus has been linked ever since to hyperplasia, papilloma and warty lesions to squamous epithelia of the skin and mucosas, including the anogenital area, urethra, larynx, tracheo-bronchial mucosa and nasal and oral cavities [15,16].

In oral lesion 16 HPV DNA genotypes have been isolated (HPV-1, 2, 3, 4, 6, 7, 10, 11, 13, 16, 18, 31, 32, 33, 35 and 57), from which HPV-6, 11, 13 and 32 are associated with benign papillomatous lesions with low malignant potential such as squamous papilloma, acuminated condyloma, vulgar wart and focal epithelial hyperplasia, while HPV-16, 18, 31, 33 and 35 are usually related to lesions with high malignant potential [17].

HPV FEATURES

Human Papilloma Viruses are small, 55-nm viruses, with non-enveloped icosahedral capsid. The genome of this virus is a circular bicatenary DNA of about 8000 bps [17]. Despite its small size, its molecular biology is complex [18].

Three genomic regions have been identified: late region **(L)**, early region **(E)** and long control region **(LCR).** The early region contains genes that encode early proteins (E1, E2, E3, E4, E5, E6 and E7), among which only E5, E6, and E7 are considered oncogenic [19]. E1 and E2 proteins are involved in viral DNA replication and transcription, respectively. Gene E6 encodes a 160-aminoacid protein, whereas E7 produces a small 100-aminoacid polypeptide, both essential for cellular induction, immortalization and transformation. The late region contains genes L1 and L2,which encode the proteins for the viral capsid [13,16,18].

Genes E1, E2, L1 and L2 are particularly well preserved among all members of the family [18]. There is also strong evidence that HPV genome is quite conservative and sequencing changes by mutation or recombination are very rare events [18].

HPV AND HEAD AND NECK SQUAMOUS-CELL CARCINOMA (HNSCC)

HPV-16, 18, 31, 33 and 35 correspond to the oncogenic subtypes of HPV with subtypes HPV-16 responsible of 87% of the OPC [9,20]. Thus, to design effective vaccination programs and therapies we need to understand the virus biology and how the virus is able to lead to cancer.

The virus infects undifferentiated epithelial cells from the basal layer integrating its genome within the host-cell DNA. The integration of the viral genome into the cell DNA is one of the most common factor contributing to malignancy and several studies in carcinomas and cell lines have demonstrated that chromosomal localization of HPV-16 genome is higher in chromosomes 1, 2, 8, 9, 3, 12, 13 and 20 [21].

Upon infection the virus starts to express E1 and E2 proteins. In turn, these proteins regulate its replication and trigger the expression of other early viral genes. Once the infected cells migrate towards the superficial layers of the squamous-epithelia, the oncogenes E6 and E7 are expressed, and as a consequence the cell cycle is modified. E6 is able to remove p53 by ubiquitination, while E7 protein can disrupt the E2F-retinoblastoma-tumour-suppressor protein complex liberating an active E2F transcription factor, which in turn, facilitates cell cycle progression [22]. Similarly, E5 can promote the activation of the cell cycle by over expressing the Epidermal Growth Factor Receptor **(EGFR)** within the cell and the subsequent up regulation of p21, which is involved in apoptosis and differentiation control [19]. These situations can create favorable scenarios for developing cancer (defects in DNA repair, cell cycle control, apoptosis and differentiation). Moreover, upon viral integration some of the late (L1 and L2) and early genes (E1 and E2) are deleted and the disruption of E2 leads to an up regulation of E6 and E7 [23].

Nevertheless, it is important to point out that only a fraction (about 11-50%) of HPV-positive HNSCC present active transcription of E6 and E7 [24,25,26]. This finding suggests the existence a subgroup of HPV-positive HNSCC that may behave differently. For example, some studies attest that HPV-positive HNSCC showing active transcription of E6 and E7 mRNA, are not always associated with TP53 mutations, whereas the opposite is observed in HPV-positive HNSCC lacking E6 and E7 active transcription [19,24,25,26].

METHODS OF HPV DETECTION

Determination of HPV-infection in HNSCC is essential to define the therapeutic strategy and the diseases prognosis. Accordingly, expert groups such as the American College of Pathology recommend HPV screening in all patients squamous-cell OPC [27-29].

Despite the relevance of HPV identification in patients with OPC, there is no consent regarding the most adequate techniques for viral determination. Currently a large variety of methods are used, including viral DNA detection by Polymerase Chain Reaction **(PCR)** and p16 detection by immunohistochemistry. Moreover, other methods such as HPV study by RNAscopeTMare under development [29,30]. Thus, HPV detection methods can be divided into 2 groups: direct and indirect HPV detection tests.

PCR, PCR *in situ* Hybridization, Southern Blotting Assay and Hybrid Capture II **(HCII)** Test are considered direct HPV detection tests The molecular detection of viral DNA sequencing is the Gold Standard to determine the presence of HPV infection [13]. PCR is a sensitive, highly available, quick and cost-effective method [29] and can be easily applied for epidemiological studies.

By using PCR, HPV-DNA can be amplified and hybridized with specific probes allowing detection and genotyping. Moreover, frozen fresh tissue, paraffin-soaked, formalin-fixed specimens, brushing of the oral cavity or any bodily fluid can be used [27]. Diagnostics of HPV-related HNSCC based on PCR usually reveals the presence of high risks virus types 16,18,31,33 and 35 in high-copy numbers within the malignant cell nuclei either integrated or in episomal form (primary or metastatic tumours) [6,31,32,8].

However, even when PCR is considered highly reproducible and effective, there are some discrepancies among obtained results, mainly due to differences in the use of primers sets, protocols and types of tissue (frozen fresh tissue or paraffin-soaked, formalin-fixed specimen); for example PCR amplification is more effective when frozen tissue is used [27,29]. Moreover, the low specificity and high sensitivity, the specimen contamination (false positives); and the impossibility to identify whether the detected DNA comes from tumor or adjacent tissues may complicate interpretation and confuse the clinical significance of results [19,29,30].

Southern blotting assay, on the other hand, was considered the standard technique for a long time, but it is currently disused. By Southern is possible to detect the presence of a DNA sequence within a complex mix of this nucleic acid, with higher sensitivity than specificity. This test offers the advantage of differentiating episomal from integrated DNA and can even detect copies 0.1 copies of viral DNA. However, it cannot be performed on paraffin-soaked, formalin-fixed tissue [27].

Hybrid Capture II **(HCII)** Test has been widely used over the last decades and is still used for screening programs in cervical cancer [33]. It is a signaling-amplification method combining initial probe hybridization, DNA viral amplification and its later visualization. The HCII test increases

sensitivity by forming multimeric layers of informing molecules over DNA probes. RNA-specific probes are used for individual DNA sequences containing the HPV genotype to be detected. An antibody anti- DNA-RNA hybrids (capture) is used, and for later detection chemo-luminescence visualization is used. Cervista HR HPV test, is another example that can be performed in liquid-phase and, like the previous one, is used to detect HPV in cervical cancer [34].

Finally, PCR in-situ Hybridization uses a combination between HPV-PCR on intact specimens of squamous cell carcinoma, followed by detection by *in situ* hybridization. It is a technique studied for HPV detection in cervical cancer, but it is little used in OPC. Its technical difficulties decrease the probability of its use as routine test [27].

As part of indirect HPV detection tests immunohistochemistry can detect the presence of proteins, particularly p16, through marked antibodies. A result is considered positive when a cellular nucleus is stained, regardless of cytoplasmic staining. Saliva samples can be used with this technique with a sensitivity of 77% (95% CI 54-91%) and a specificity of 94% (95% CI 77-99%), respectively, whatever the site of the positive tumour [35]. However, some types of HPV-negative tumors can over express p16 and, therefore, clinicians have to take this into consideration. Nevertheless, it is a useful prognostic marker, specifically for oral cancers, where an HPV^-, $p16^+$ result yields favorable prognosis [27,36].

FACING HPV-RELATED HEAD AND NECK CANCERS

Over the past decade, several countries started vaccinations programs against HPV to prevent HPV-related anogenital diseases. The vaccines included hollow viral-like proteins **(VLPs)** to trigger antibody production for subsequent protection. Quadrivalent (HPV types 6/11/16/18) and bivalent (HPV types16/18) VLP vaccines were developed for girls and women (ranging from 9 to 26 and from 10 -25 years old respectively) and implemented in school and non-school based programs. The trials showed that quadrivalent vaccines could prevent HPV-related anogenital diseases, including cervical and intraepithelial neoplasias in 100% of the studied cases [37,38]. The bivalent vaccine (approved in October of 2009), showed even higher antibody titers against the high risk HPV-types than the quadrivalent vaccine. Moreover, in October 2009, a quadrivalent vaccine was approved for boys and men (9 to 26 years of age), which a 90.4% efficacy against developing anogenital lesions, decreasing to 65.5% in patients with an unknown HPV exposure history [39].

But can the current vaccines strategies protect against non-cervical HPV infection and, therefore, pre-cancerous lesions in areas such as the oropharyngeal cavity? Recent modeling data analysis indicates so [40].

Radiotherapy is usually the first line of therapy for HNSCC patients and probably the lower p53 mutation burden of HPV-positive tumours may contribute to the increased radio sensitivity of these malignant cells, although more studies are needed to conclude this [41-43]. Radiotherapy

may also increase EGFR expression in the irradiated cells, a factor associated with poor prognosis [44]. However, HPV-infected cells have lower EGFR expression suggesting another explanation for the better prognosis in HPV-HNSCC patients [45].

Radiotherapy alters the tumour microenvironment providing a favorable scenario for immune-based therapies [8]. Radiotherapy helps to 1) increase the viral-antigen uptake, 2) provide factors such as high-mobility-group **(HMGB1)** from dying irradiated cells, which in turns results in a activation of the toll-like-receptor-4 pathway on dendritic cells 3) enhance vascularization and permeability of the tumour tissue, facilitating trafficking of T lymphocytes and dendritic cells 4) activate T cells cytotoxic response by suppressing the inhibitory signalling from regulatory T cells and immature myeloid cells 5) reduce pro-inflammatory molecules such as TNF-α and 6) increase the expression of MCH class-I molecules, which facilitates the antigen presentation to $CD8^+$-T cells (46).

HPV INFECTION AND IMMUNE RESPONSE

About 10% of infected individuals are at risk of developing HPV-related lesions. Histological examinations indicate that upon infection an adaptive immune response is delivered. $CD4^+$ and $CD8^+$ T-cells infiltrate the lesion to clear the HPV-infected cells. HPV can also trigger a humoral immune response producing antibodies against L1 protein and also early genes such as E7, but this response is weak and inconsistent. It is the cell-mediated immune response or the specific $CD4^+$ and $CD8^+$ T cells against the virus that clear the infection. However, antibody production is useful as a screening tool for HPV-associated OPCs and since antibodies against the virus last for many years, they can be used as markers for past infections [22].

The importance of the immune system to clear HPV infection is demonstrated in immune-deficient patients, for example after organ transplant or patients infected with immunodeficiency virus **(HIV),** which are at high risk for HPV-infections and HPV-related diseases. However, these patients are able to recover and eliminate the virus once their immune competence is resumed. Lack of T-cell response and its relationship with HPV infection have been well documented in patients with cervical cancers and animal models [22].

In order to escape from immune surveillance, the virus coordinates its replications according to the cell differentiation process. In uterine cervix, for example, HPV infects basal-layer keratinocytes expressing low levels of viral proteins. As soon as the infected keratinocytes differentiates, they move upward to more superficial layers, where the immune-surveillance is reduced and antigen-presenting cells have limited access to antigens such as HPV-E7 peptides, [47]. There, HPV upregulates the expression o viral genes, allowing viral replication and virions release by breaking the epithelium surface but avoiding the complete keratinocytes lysis. With these strategies the virus achieve two important goals: 1) moving away from active immune-surveillance regions and 2) avoid inflammatory response, thus, the virus can replicate unnoticed.

Infections in lymphoid organs such tonsils suggests that in the deep crypts, where HPV infects, others mechanism may be at play to inhibit T-cell specific immune response [48]. HPV dismantles both the innate and the adaptive immune responses using different strategies. For example, HPV proteins can inhibit cytokines such as IL-8 and IL-18 and the expression of toll – like receptor 9 [22]. E6 and E7 expression is also implicated in the down-regulation of the Major Histocompatibility Complex **(MHC)** class I, thereby, limiting the $CD8^+$ T-cell response against the virus [22]. The expression of the interferon regulatory factor 1 **(IRF-1)** is also affected upon virus infection, leading to a poor interferon (IFN-α and IFN-γ) response [49]. Thus the virus escapes from the immune system surveillance and at the same time is avoids anti-proliferative agents such as interferons.

IMMUNOTHERAPEUTIC APPROACHES

Several advances have been made in surgery, radiation and chemotherapy, but these procedures are still insufficient to treat advanced HNSCC. Treatment-associated morbidity is high and HNSCC-tumor microenvironment prevents a proper immune response [50]. Moreover, current HPV vaccines are preventive rather than therapeutic (when the disease has already appeared).

DNA-vaccines are examples of therapeutic vaccines, seeking to elicit a strong T-cell-mediated immune response ($CD4^+$ and $CD8^+$ T cells) against an antigen of interest. In this approach, DNA-encoding tumor-associated antigens **(TAA)** can be cloned into plasmid DNA and delivered to patients by shotgun or electroporation in combination with an immune-stimulatory adjuvant. These two ways of administration are more effective than intramuscular needle injections for antigen expression and/or antigen-presenting cells transfection. Once inside the cell, these antigens are expressed and processed in the proteasome to be presented at the cell surface on MHC-class-I molecules, although it is also possible to induce MHC-class-II antigen presentation. DNA vaccines can stimulate B-cell response and are more stable than traditional vaccines. Since they only include the antigen or antigens of interest, they are safe and relatively easy to produce in large-scale quantities [51].

Peptide vaccines use antigenic peptides that are highly expressed in tumour cells, or produced during embryogenesis, but absent in adult tissues such as testis and placenta. These peptides can be expressed *in vitro* and purified for the vaccine. The amino-acid sequence is selected according to its antigenic properties and can be modified to enhance immune response and stability as well as to avoid toxicity (for example, unwanted side effects present in the naive sequence). A disadvantage is that antigenic epitopes are usually not composed by a single amino but rather by a complex three-dimensional structure composed by several acid sequences [52].

Another option is to use Biological vaccines, which are built using live, attenuated bacterial strains such as Listeria monocytogenes. This intracellular bacterium is engineered to secrete antigen peptides (for example HPV-E7) fused to listeriolysin O protein. The aim is to infect

antigen-presenting cells in order to trigger a $CD4^+$ and $CD8^+$ T-cell immune response against the HPV-infected tumor cells [53].

Semi-Allogenic Fibroblast vaccines, which are based on the "semi-allogenic cell-transfer theory" have also been suggested. The idea is that weak tumor antigens can become highly immunogenic when they are presented by allogenic fibroblast. Thus, allogenic fibroblasts is transfected with patient derived malignant-cell DNA and grown in culture. Before this transfected allogenic fibroblast is injected back to the patient islethally irradiated [50].

ANTIBODIES AND CANCER THERAPY

Monoclonal antibodies are specific and can be used to block deregulated signalling pathways leading to cancer growth, or against tumour cells and trigger antibody-dependent cell-mediated cytotoxic **(ADCC)** response. To this end, three humanized monoclonal antibodies (Cetuximab, Nimotuzumab and Imgatuzumab (GA201) specifically targeting the tyrosine kinase receptor EGFR have been developedbut only Cetuximab and Nimotuzumab have been tested for HNSCC. These antibodies block the binding of the ligand to its receptor and thus the whole signalling cascade [54]. Cetuximab, is effective for wild-type and mutant K-RAS tumors [50] and have been approved for HNSCC since 2006 [50]. Nimotuzumab, have been tested in a phase IIb trial for advanced HNSCC (stages III or IV), exhibiting fewer side effects (severe skin, mucosal, renal, and gastrointestinal toxicity) than Cetuximab. Since these results are promising, a Phase III trial of Nimotuzumab is currently recruiting HNSCC patients; results will be available in 2021 [50].

Another interesting target is the Hepatocyte growth factor **(HGF),** which is upregulated in HNSCC tumor cells [55,56]. This factor is the ligand of the MET tyrosine kinase receptor; upon binding it activates both PI3K/Akt, Ras/Rac/Rho and Ras/MAPK signalling pathways leading to cell growth, angiogenesis, metastasis and drug resistance [55]. In order to target this factor, a humanized monoclonal antibody called Ficlatuzumab has been developed. Preclinical trials for non-small cell lung cancer **(NSCLC),** showed reduction in tumor growth andlower levels of several molecules downstream in both pathways. Although NSCLC has a similar genetic profile to HNSCC, is unclear how HNSCC patients will respond to this therapy. Ongoing studies suggest that ficlatuzumab may benefit only a subgroup of patients with specific mutational and gene-expression profiles [50].

The above strategies represent a great advance in cancer therapy, but most of HNSCCs have only a modestresponse. The nature of tumor microenvironment may provide clues to this problem.

HPV-related HNSCC arises in the lymphoid tissue of the tonsils and the tongue base, where an effective immune response is expected. The scenario in HPV-HNSCC tumors is quite different from what is found in the tobacco-related neoplasms microenvironment [57]. Although in HSNSCC, HPV-infected cells and tumors express the highly immunogenic E6 and E7 viral proteins and lymphocytes are present, the immune-response against these foreign proteins is weak [58].

To understand the mechanisms behind this immune-resistance, we need to learn how the immune system works. When a molecule is recognised as foreign the immune response is activated and the molecule is set for elimination. However, to avoid an uncontrolled cell-mediated cytotoxic response, the immune system has immunological checkpoints and regulatory mechanisms. For example, regulatory T-cells (T-regs) are essential to maintain immunological tolerance. Thus, T-regs inactivate effectors T-cells when the pathogens are cleared or they suppress autoreactive T cells avoiding autoimmune diseases. T regs can be recognized as $CD4^{+}FOXP3^{+}$ T and $CD4^{+}FOXP3^{-}$ T-reg cells [59].

Besides T-regs, the immune system has several checkpoints to control the over activation of a T-cell-mediated immune reaction and prevent autoimmunity. When the programmed cell-death-1 **(PD-1)** receptor at the surface of activated T and B cells is expressed and binds its ligand (PD-1 ligand-1, PD-L1), T-cells become anergic and fail to produce sufficient effect or cytokines. Another example is CTLA-4, which is expressed on activated T-cells and binds to B7 expressed on the surface of antigen-presenting cells, thus preventing its interaction with CD28 (an essential co-stimulatory molecule for T-cell activation and IL-2 production) [60,61].

The expression of both PD-L1 and CTLA-4 represents important immune-checkpoint blockades and are present in HPV-associated tumors. Both HPV-positive and negative tumors equally express PD-1 receptor (at the surface of activated T- and B-cells) and PD-L1 (at the surface of stroma cells), but the inhibitory immune receptor CTLA-4 expressed by T-cells is significantly over expressed in HPV-positive in comparison to HPV-negative tumors [62]. HPV-positive-tumors also contain higher numbers of infiltrating T-regs and higher levels of T-reg/$CD8^{+}$-T lymphocytes ratios, implying that HPV-positive and HPV-negative HNSCCs are different pathologies [62].

The high levels of T-reg and CTLA-4 may explain the immune-response suppression in HPV-positive HNSCCs. Thus HPV-HNSCC patients can be treated using monoclonal antibodies specific for the mentioned checkpoints. Hence, five checkpoint inhibitors have been developed: Pembrolizumab and Nivolumab both target PD-1 [63]. Avelumabtargeting PD-L1 (currently being tested in HNSCC Phase I, II, and III trials) [64,65] and Ipilimumab which target is CTLA-4 (approved in 2011 for melanoma and currently in a Phase I/II trial for virus-associated tumors, including HNSCC) [66] and finally, AMG 228 which aim for the glucocorticoid-induced tumor-necrosis-factor receptor **(GITR),** expressed in $CD25^{+}$ $CD4^{+}$ regulatory T-cells (so far there are promising preclinical results from a Phase I safety trial using this antibody in several solid tumors including HNSCC) [67,50].

Immunotherapy has open new frontier sin treating different tumors including HPV-HNSCCs. These new therapies have the potential to improve patient survival and to reduce HNSCC-associated morbidity, especially if they are used in combination with classic therapeutic approaches. However, there is yet a long way to go and learn; for example not all patients show the same clinical response to immunotherapy and although the immunotherapy response seems to endure, usually takes a long time in comparison to traditional chemotherapeutic approaches.

If we want to improve current protocols, we need to face pending challenges such as therapeutic-vaccine design - according to tumor biology-, better social-economic implementation plans and understanding of the relationship between immunotherapy and virus-related cancers.

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