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Daniela Russo[1]#, Francesco Merolla[2]#, Gennaro Ilardi[1]*, Silvia Varricchio[1], Danila Caroppo[1], Virginia Napolitano[1], Massimo Mascolo[1] and Stefania Staibano[1]^

[1]Department of Advanced Biomedical Sciences, University of Naples "Federico II". Naples, Italy
[2]Department of Medicine and Health Sciences "V. Tiberio", University of Molise, Campobasso, Italy
#These authors contributed equally to this work
^Senior co-authorship

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***Corresponding author:** Gennaro Ilardi, Department of Advanced Biomedical Sciences, University of Naples "Federico II". Naples, Italy, E-mail: gennaro.ilardi@unina.it

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Short Communication

Diagnosing HPV-Related Oropharyngeal Cancers: The Need to Speak a Common Language

Short Communication

Oral cavity squamous cell cancer (OSCC) and Oropharynx squamous cell carcinoma (OPSCC) are the most frequent forms of Head and Neck Cancers (HNCs) [1]. About 300,000 new cases of oral cancers are being counted yearly worldwide, having registered an increase of incidence of 225% in U.S.A. in the last 20 years, with about 50% of related deaths [2,3]. The relevant advances in treatments of OSCC during the last decades, allowed updated surgery techniques, robotic surgery, intensive induction chemotherapy and hyperfractionated radiotherapy to be currently applied to the advanced cases. Unfortunately, most of these therapies often carry severe acute and chronic side effects, heavily impacting on patients' quality of life.

Very interestingly, OSCC is generally associated with a history of alcohol and tobacco abuse, whereas OPSCC is frequently related to a persistent infection by high-risk Human Papilloma Viruses (HR-HPV), mainly HPV16, and frequently arises in non-smokers, younger patients than OSCC.

The striking expansion of the incidence of the OPSCC HPV-related subgroup, in the last 30 years, has led to a unprecedented change in the epidemiology of HNCs, to the point that HPV-positive OPSCCs are now regarded as "the New Face of HNCs" [4], and are considered responsible of the peak of HNCs incidence currently observed in Western Countries, where tobacco abuse started to decline years ago (i.e., U.S.A.), due to the success of decades of anti-smoking campaigns [5].

The difference between HPV-positive and HPV-negative cancers bears a significance that goes beyond the epidemiology. An increasing amount of data indicates, in fact, that the HPV-related OPSCC subgroup shows a lesser aggressive behavior and a more radio- and chemo-responsivity than alcohol and/or smoking related OSCCs [6].

Several reasons may underlie the important differences in biological aggressiveness among HPV-positive and HPV-negative cancers.

Changes in the tumor microenvironment and immune surveillance are emerging as one of the major determinants of clinical behavior of these tumors, through the crosstalk of cancer-directed immune cells, hypoxia regulating genes, and chemokines production [7].

Hypoxia has been reported as a common event in OSCC, that experience important adaptive changes (i.e.anaerobic glycolysis, pH stabilization) mainly directed by the hypoxia inducible factor (HIF)-1, which acts early in tumor progression, contributing to the generation of an immune escape phenotype and poor clinical outcome of patients with advanced disease [8,9].

By converse, a heavy lymphocytic infiltration (*aka*, a T-cell inflamed tumor phenotype) both of the stroma and tumor nests, is a frequent finding in HPV-positive OPSCC, and often may be regarded as the epiphenomenon of a host strong immune response toward the invading viral-driven cancer, at least in part responsible for the better clinical behavior of these tumors.

However, this finding sometimes represents a confounding feature, as OPSCC can escape immune surveillance recruiting regulatory T lymphocytes and myeloid-derived suppressor cells, through the local secretion of inhibitory cytokines and the expression of immune inhibitory ligands by cancer cells and inhibitory co-receptors in tumor infiltrating lymphocytes (TILs). In particular, it has been recently shown that the PD-1 immune checkpoint receptor expressed by tumor infiltrating lymphocytes and its ligand, PDL-1, mainly present on the

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membrane of tumor cells, may create an immune-privileged site during development and progression of HPV-related OPSCC [10].

The understanding of the differences between HPV infection and tobacco/alcohol exposure-mediated development of an immunosuppressive tumor microenvironment could probably provide us with a better comprehension of one of the causative background underlying the divergent clinical behavior of these two subset of tumors, both in terms of response to therapy and overall survival of patients. There's still a long way to the end of the story.

As anticipated by zur Hausen in at the beginning of this century [11], HPV leads to the evasion from host-cell control in early events in carcinogenesis, and actually we know that it actively contributes to the impairment of the immune response, negatively modulating the IFNgamma secretion and the HLA-mediated antigen presentation, allowing early immune escaping, during the first steps of infection [12].

As well, many other factors and molecular pathway need to be fully investigated, before we could exactly know how and why HPV-positive OPSCC behaves in a more favorable way, and how we can maximize our efforts to completely eradicate these tumors, even when in advanced stage, without significant side-effects for patients.

In addition, several authors recently questioned the existence of a clear-cut distinction between HPV+ and smoking/alcohol-related cancers, outlining the existence of crosstalk between their pathogenic mechanisms, with possible variable fallout on clinical behavior and outcome of tumors.

In other terms, although most HPV-OPSCC show an innate better outcome and a good response to current treatment strategies, sometimes they can be associated with other major risk factors, this HPV-positive OPSCC with distinct, individual risk profiles [13].

Moreover, several reports signal that HPV DNA-positive OPSCCs may be heterogeneous for both biological and clinical behavior, possibly due to differences in viral load and viral oncogene expression [14–25].

More data are needed, on even greater series of cases, worldwide, before we reach definitive results.

In the meantime, we still lack a definite panel of prognostic markers able to unequivocally detect patients with poorer outcome to be treated with intensive therapeutic regimens [26], and to date, the only discriminant parameter available in the clinical-pathological practice, is the active presence of an HR-HPV (HPV16, in the major part of cases) on formalin-fixed and paraffin-embedded tissue (FFPE) OPSCC tissue, which is actually emerging as a real prognostic and predictive biomarker.

This constitutes a real conundrum, in the real setting.

In fact, differently from cervical cancer, there are currently no definitive reliable and cost-effective diagnostic tests approved by the FDA for the unequivocal determination of HPV in Head and Neck Cancers. The various techniques currently in use for HPV detection, range from consensus and type-specific PCR methods, real-time PCR assays, DNA in-situ hybridization (ISH), and immunohistochemical detection of surrogate biomarkers (e.g., $P16^{INK4a}$ protein) [27]. None of these methods offers optimal sensitivity and specificity levels.

P16 immunohistochemistry has been judged as the best surrogate test to use for risk stratification of OPSCC in a routine pathology laboratory setting [27]. However, this test may produce false positive and false negative results. The gold standard of the testing methods is qRT-PCR [28]. that requires fresh frozen tissue for optimal results and is technically complex, factors that restrict its use only to research laboratories. Therefore, stepwise algorithms that combine different HPV tests have been proposed as a strategy to compensate for the limitations of individual tests.

As confirmatory tests after a positive IHC for $P16^{INK4a}$ protein, fluorescence in situ hybridization (FISH) on DNA and/or PCR are used to detect HPV in OPSCC [29].

These tests certainly add significant information to IHC, as viral DNA is found in squamous cell carcinomas of the head and neck region, and particularly the oropharyngeal region, in a proportion of cases in continuous and progressive growth. This is fully in agreement with the knowledge that persistent infection with high risk HPV (HR-HPV) causes cellular immortalization and tumorigenesis of epithelial cells involved. However, to have biological and clinical relevance, HPV should be transcriptionally active, and all the above mentioned tests do not assess the viral transcript (i.e. the E6/E7messenger RiboNucleic Acid, mRNA), which currently represents the best effective indicator of HPV status of OPSCC [30].

The presence of a transcriptionally active HPV in cancer cells is considered the direct evidence of HPV-related oncogenesis: for this reason, a ISH RNA based HPV E6/E7 for transcripts is definitely the preferred method to identify the "actual" HPV-related OPSCC [31].

Therefore, the gold standard for detecting oncogenic HPV is the demonstration of transcriptionally active high-risk HPV in tumor tissue. Whereas quantitative reverse transcription and polymerase chain reaction (qRT-PCR) requires the extraction of RNA, which destroys the tumor, making impossible the morphological diagnostic correlation, new techniques, as the RNAscope® assay, allow the direct, in situ visualization of RNA on FFPE, with sensitivity at single molecule and single cell resolution. The essay for HPV RNAscope® is designed to detect the mRNA of E6/E7 of high-risk HPV genotypes (e.g. HPV 16, 18, 31, 33, 35, 52 and 58) using a pool of probes specific for these genotypes [32–34].

RNAscope® can be applied also on tissue microarrays (TMA), and shows a highly simplified workflow, similar to the IHC standard protocols, preserving the morphology of the tissues and allowing the pathologist to perform histopathological correlations [34].

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HPV testing by RNAscope® demonstrated 97% sensitivity and specificity 93%, taking as reference the qRT-PCR method. The conventional method chromogenic medium for HR-HPV DNA ISH is highly specific, but has a sensitivity of 80%. Immunohistochemical staining (IHC) for cell marker surrogate p16 shows, instead, excellent sensitivity but it can produce false positives.

In our laboratory, the RNA ISH assay, currently used to resolve the equivocal OPSCC cases (primary tumors and/or metastatic neck lymph-nodes from occult primary tumors) characterized by divergent IHC for $P16^{INK4a}$ protein and HR-HPV DNA testing results, has identified several false $P16^{INK4a}$ protein-positive tumors.

To date, it is believed that the prevalence of HPV in OPSCC ranges from 50% to 80% at the world level, with sharp variations depending on the study population and the geographical location. However, these estimates could be inaccurate, since the methods of detection are still extremely variable among different laboratories and most of those currently used for screening purposes does not say whether the virus is transcriptionally active in tumors [35].

There is a strong need for a consensus on tests to use when determining whether a case of OPSCC is a "real" HPV-related neoplasm, in view of the new strategies for treatment deintensification for these patients that are already under clinical evaluation.

We strongly outline the urgency to move jointly, worldwide, at the pathology laboratory level, to define and standardize the best molecular algorithm able to accurately evaluate the HPV status of OPSCC. Only by speaking all the same language, it will be possible to give the right information to every patient, allowing also the scientific community to share precious information, constituting open, common databases useful to the further comprehension of the molecular differences among HPV-positive and negative OPSCC.

A great amount of results has been collected in the last decade, for that concerning our knowledge of the oral HPV-related cancerogenesis. Further investigations are undoubtedly needed, but we could be very close to make a snapshot of the real face of HPV-positive OPSCC.

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