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Assessment of p16 immunohistochemistry as a marker of HPV infection in oral cancers

A systematic review

Annabelle Sarah Jacqueline DHERBECOURT

Dissertação conducente ao Grau de Mestre em Medicina Dentária (Ciclo Integrado)

Gandra, 27 de setembro de 2021

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Trabalho realizado sob a Orientação do Professor Doutor Luis Monteiro

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Eu, acima identificado, declaro ter atuado com absoluta integridade na elaboração deste trabalho, confirmo que em todo o trabalho conducente à sua elaboração não recorri a qualquer forma de falsificação de resultados ou à prática de plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria do trabalho intelectual pertencente a outrem, na sua totalidade ou em partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores foram referenciadas ou redigidas com novas palavras, tendo neste caso colocado a citação da fonte bibliográfica.

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RESUMO

Introdução: O papilomavírus humano (HPV) é um fator de risco emergente para o carcinoma de células escamosas da cabeça e pescoço (HNSCC). A cavidade oral é o sítio mais comum de HNSCC não orofaríngeo onde o HPV fica implicado, mesmo se o papel deste vírus no cancro oral ainda esta sobre controvérsia. Um método de diagnóstico barato e fiável para detectar o HPV no carcinoma de células escamosas da cavidade oral (OSCC) está a faltar.

Objetivo: Determinar a fiabilidade da avaliação imuno-histoquímica da p16 (p16-IHC) como método de detecção da infecção por HPV nos cancros orais.

Material e Métodos: Foi realizada uma pesquisa nas bases de dados PubMed e Google Scholar. Uma pesquisa complementar foi feita por meio de bibliografias de outras revisões sistemáticas e metanálises. Os estudos foram incluídos de fevereiro até julho de 2021.

Resultados: 22 artigos foram finalmente incluídos. Um total de 932 pacientes foram testados por ambos p16-IHC e um teste especifico para HPV RNA. A sensibilidade média do p16-IHC nesse grupo foi de 69,11%, e a especificidade média foi de 84,77%. 1119 pacientes foram testados por p16-IHC e para a presença de HPV DNA. Nesse grupo, a sensibilidade média foi de 45,78% e a especificidade, 74,26%.

Discussão: Pode-se constatar alguma heterogeneidade entre os estudos, aquela pude trazer viés nos resultados.

Conclusão: Nossos resultados sugerem que p16-IHC não é um método bastante fiável para ser usado isoladamente. Mais estudos serão necessários para confirmar isso, e para determinar a melhor metodologia para detetar a presença de HPV ativo biologicamente nos cancros da cavidade oral.

Palavras-chave: alfapapilomavírus, papilomavírus humano, HPV, neoplasias bucal, neoplasias da cabeça e pescoço, carcinoma de células escamosas de cabeça e pescoço, cancro oral, inibidor da quinase dependente de ciclina p16, p16, p16INK4a, Reação em Cadeia da Polimerase, PCR, HPV-DNA, diagnóstico, sensibilidade e especificidade

ABSTRACT

Introduction: Human Papillomavirus (HPV) is known as an emerging risk factor for Head and Neck Squamous Cell Carcinoma (HNSCC). Despite an ongoing controversy around the role of HPV in oral cancers, the oral cavity is the most common non-oropharyngeal HNSCC site where this virus is implicated. An inexpensive and trustworthy method for detecting HPV in Oral Squamous Cell Carcinoma (OSCC) is still missing.

Objective: Assess the reliability of p16-IHC as a method for detecting HPV infection in oral cancers.

Material and Methods: A search was performed in PubMed and Google Scholar databases. Complementary research was performed through the bibliographies of other systematic review and meta-analysis works. The whole inclusion of studies was done between February and July 2021.

Results: 22 articles were finally included. A total of 932 patients were tested by both p16-IHC and a specific test for HPV RNA detection. The average sensitivity of p16-IHC in this group was 69.11%, and the average specificity was 84.77%. 1119 patients were tested by p16-IHC and for the presence of HPV DNA. In this group, the average sensitivity was 45.78% and the specificity, 74.26%.

Discussion: Some heterogeneity can be found between studies, which could bring bias to the results.

Conclusion: These results suggest that p16-IHC is not reliable enough to be used alone for the diagnosis of HPV-related oral cavity cancers. More studies, with standardized parameters, will be needed for confirmation, and to determine the best protocol to detect the presence of biologically active HPV in oral cavity cancers.

Keywords: alphapapillomavirus, human papillomavirus, HPV, mouth neoplasms, head and neck neoplasms, squamous cell carcinoma of head and neck, oral cancer, cyclin-dependent kinase inhibitor p16, p16, p16INK4a, Polymerase Chain Reaction, PCR, HPV-DNA, diagnosis, sensitivity and specificity

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INDEX OF ABBREVIATIONS

DNA: Deoxyribonucleic acid

FFPE: formalin- fixed, paraffin-embedded

FNA: Fine-Needle Aspiration

FOM: Floor Of Mouth

HN: Head and neck

HPV: Human Papillomavirus

HR: High-Risk

IHC: Immunohistochemistry

ISH: In Situ Hybridization

LR: Low-Risk

OC: Oral Cavity

OPSCC: Oropharyngeal Squamous Cell Carcinoma

OSCC: Oral Squamous Cell Carcinoma

RT-PCR: Real-time Polymerase Chain Reaction

RNA: Ribonucleic acid

SCC: Squamous cell carcinoma

1 INTRODUCTION

The most common malignancies in the head and neck area (HN) are squamous cell carcinomas (SCCs) [1]. Head and neck squamous cell carcinomas (HNSCC) are the 6th most common malignancies worldwide [2].

Conventionally, tobacco, alcohol, and betel quid chewing[3,4] are the essential risk factors for oral cancer. However, infection with high-risk human papillomavirus (HR-HPV) has been reported as an emerging risk factor for HNSCC[3], especially in young patients who do not have a prolonged history of smoking or drinking [5].

Indeed, recently, Human Papillomavirus (HPV) has been well established as a principal driver for a subset of HNSCC, especially in the oropharynx [6,7]. HR-HPV is a sexually transmitted virus [2]. Sexual behaviors such as regular oral sex practice [8] and having multiple lifetime number of oral or genital sexual partners are associated with a risk of contracting oral or oropharyngeal HPV infection [3].

Despite an ongoing controversy around the role of HPV in oral cancers [6–12], with a reported prevalence that varies greatly among geographic locations [3,13], types of tissue material studied [14], HPV genotypes included [3] or HPV detection methods [3,13–15], the oral cavity is the most common non-oropharyngeal HNSCC site where this virus is implicated[7].

HPV status is being considered as a risk stratification biomarker for patients with HNSCC[6]. Some studies suggest that HPV-related HNSCCs would be more sensitive to chemoradiation therapy [3,15], and associated with better prognosis [2] and improved patient survival [3], in comparison with HPV-unrelated HNSCCs.

Even though many authors do not agree with these statements, especially regarding OSCCs [1,5,6,13,14,16], testing HNSCCs for the presence of HPV is increasingly common in clinical practice [17].

To determine the presence of HPV in a tumor, several methods exist [16].

The most reliable tests to diagnose HPV-associated malignancies aim to detect the expression of HPV oncogenes E6 and E7, by detecting HPV E6/E7 mRNA [1,18]. These methods allow evaluating the presence of transcriptionally active HR-HPV, which is needed to initiate the tumor and to maintain the malignant phenotype [19].

Most authors consider Real-time PCR (RT-PCR) or quantitative PCR as the gold standard to detect transcription of E6/E7 mRNA [2,5,20]. However, the requirement of unfixed fresh frozen tissue makes it technically demanding and expensive for clinical routine practice[20].

On the other hand, RNA ISH is a sensitive and relatively specific method [18]. It can be processed on formalin-fixed, paraffin-embedded (FFPE) samples [9,18], which are more accessible routinely collected materials [14]. But RNA ISH can also be expensive to process[21].

DNA PCR and ISH detection methods are commonly used too but the presence of HPV DNA alone is insufficient to place HPV as the cause of cancer, as it could reflect a transient infection rather than an actual HPV-driven oncogenic process [1,14,16]. Also, DNA PCR lacks specificity [16] and DNA ISH can lack sensitivity [18].

Clearly, a standard methodological approach for HPV testing in OSCC is still missing [9]. An ideal diagnostic protocol would be highly sensitive, specific, technically feasible for routine diagnostic pathology practice [18], and cost-effective [15].

Another quite studied method is immunohistochemical (IHC) examination of P16INK4a (or p16) expression as a surrogate biomarker [12] for HPV infection.

In HPV infected tumors, E6 and E7, oncoproteins of the HR-HPVs, respectively degrade p53 and inhibit the function of pRb (retinoblastoma) tumor suppressor proteins, leading to dysfunctions in apoptosis and DNA damage repair, cell cycle deregulation, and cell immortalization [3,7]. PRb inactivation also induces an upregulation of the cyclin-dependent kinases p16INK4a [3,7].

Recently, it has been found that p16 overexpression can be used as a reliable marker for HPV-induced carcinomas in the oropharynx [2], especially when combined with HPV-

specific testing like HPV-DNA detection [1,4].

However, recent studies have shown that p16 expression would not be as reliable in other locations of the head and neck as in the oropharyngeal area [2,18].

2 OBJECTIVES AND HYPOTHESES

This systematic review aimed to evaluate whether p16 overexpression, determined by immunohistochemistry, is a reliable marker for HPV infection, in cancers from the oral cavity. It will be verified through the determination of the sensitivity and specificity of p16-IHC for HPV detection, in diverse studies.

Null hypothesis: p16-IHC is not reliable for HPV detection in oral cavity cancers.

Alternative hypothesis: p16-IHC is reliable for HPV detection in oral cavity cancers.

3 MATERIAL AND METHODS

Protocol and registration

The guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) checklist were used for the elaboration of this systematic review.

Eligibility criteria

This work aimed to answer the following question: Can p16-IHC be used as a reliable marker of HPV infection in oral cavity cancers?

It was designed according to the PECOS criteria: P (participant), patients with oral cavity cancer: E (exposure), HPV exposure; C (comparators), compare the sensitivity and specificity of p16-IHC with a gold standard test; O (outcomes), whether p16-IHC can be considered a reliable method or not; S (study design), cross-sectional and cohort studies.

Studies which: (i) were not primary sources of information; (ii) were not related to p16-IHC, HPV RNA or DNA assessment or cancers from the oral cavity; (iii) did not allow determining TP, FP, TN, FN values for p16-IHC; (iv) did not have these specific data for the oral cavity isolated; (v) did not use HPV RNA or DNA PCR or ISH as a reference test for HPV detection, (vi) used p16-IHC combined with another test without assessing the results for p16-IHC separately, were excluded.

Information sources and search strategy

A search has been done on Pubmed and Google Scholar databases, targeting articles published from the establishment of the database until the 12th of February 2021, the day the search was initiated, with no language or study design automated restrictions.

The combination of MeSH words and free text words that was entered is the following: ((alphapapillomavirus[MeSH Terms]) OR (human papillomavirus) OR (HPV)) AND ((mouth neoplasms[MeSH Terms]) OR (head and neck neoplasms[MeSH Terms]) OR (squamous cell carcinoma of head and neck[MeSH Terms]) OR (oral cancer)) AND ((cyclin-dependent

kinase inhibitor p16[MeSH Terms]) OR (p16) OR (p16INK4a)) AND ((Polymerase Chain Reaction[MeSH Terms]) OR (PCR) OR (HPV-DNA)) AND (diagnosis[MeSH Terms]) AND (sensitivity and specificity[MeSH Terms]).

This combination of keywords was too long to be used on other databases like ScienceDirect or EBSCO.

In addition, complementary research was performed through the bibliographies of other systematic reviews and meta-analyses works.

The last inclusion of article was done on the 5th of July 2021.

Study selection

First, the title and abstract of each article were screened to pick out relevant articles.

Secondly, the full text of studies that were primary sources and compared the diagnostic results of p16-IHC with a gold standard test for HPV diagnosis (HPV E6/E7 mRNA or HPV DNA detection, either by PCR or ISH) was reviewed.

Studies which: (i) were primary sources; (ii) included patients with OSCC (Oral Squamous Cell Carcinoma); (iii) used HPV E6/E7mRNA detection or HPV DNA detection as a gold standard for the diagnosis of HPV infection of the tumors; (iv) used IHC to detect p16 expression, were included.

The included articles had to provide the number of HPV positive and negative patients, detected by both p16-IHC and the gold standard test, so the values of true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN) could be identified. Thanks to these data, the sensitivity and specificity of p16 IHC could be calculated for each study.

If data lacked in some articles, the author was contacted, in order to obtain the missing information. In case the author did not respond, the study was excluded.

Data extraction

Firstly, basic data were extracted from the studies, namely author, publication year. Then, in each article, study design, tumor location, sample size, age and gender distribution of patients, the gold standard method used for HPV detection and if it detected HPV mRNA or DNA, HPV genotype targeted and threshold value agreed for p16 positivity were collected. Finally, the values of TP, FP, TN, and FN were extracted.

Data analysis

Including articles that did not use the same methods as reference diagnostic tests (PCR or ISH) may bring some bias to the results, as may the inclusion of several studies with reference tests that targeted HPV DNA, and not HPV RNA.

This is why average sensitivity and specificity will be determined for the subgroup of studies that used HPV RNA detection as a gold standard, separately from the subgroup of studies that targeted HPV DNA as a gold standard method.

Test sensitivity is defined as the ability of the test to properly detect patients that have the disease. It is measured by the division of the number of true positive patients by the total number of patients who actually have the disease, as calculated by the following operation: (TP/(TP+FN)) x100.

Test specificity is defined as the ability of this test to properly reject healthy patients, who do not carry the disease. It is measured by the division of the number of true negative patients by the total number of actually healthy people, as calculated by the following operation: (TN/(TN+FP)) x100.

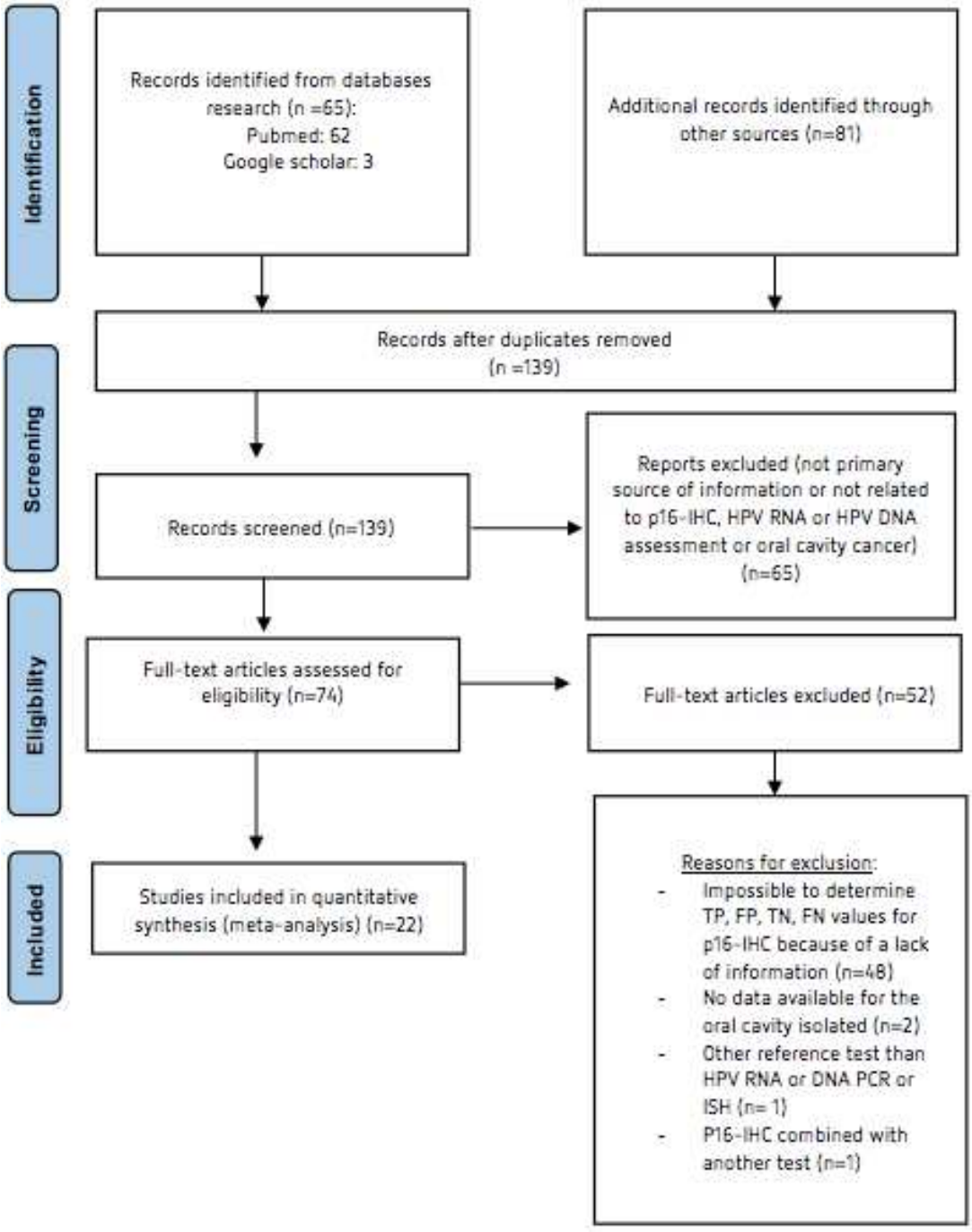

FIGURE 1- PRISMA FLOW CHART OF STUDIES SEARCH AND SELECTION

4 RESULTS

4.1 Search process and features of the included studies

The search initially identified a total of 146 articles, out of which 124 were excluded.
Of these, 7 were excluded because they were duplicates, and 65 were excluded after screening their titles and abstracts. The full text of 74 articles was evaluated, of which 52 did not have sufficient data relative to the subject of interest. In the end, 22 articles were included.
The search and studies inclusion process is shown in a flowchart in Figure 1.

Eighteen studies were cross-sectional studies, and four were cohort studies.

Among the 22 research articles included in the analysis, 4 studies only assessed the presence of HPV RNA as a reference test, whereas 13 articles only assessed the presence of HPV DNA. The remaining 5 articles determined both the presence of HPV RNA and DNA.
In terms of number of patients, 932 patients were tested for the presence of HPV RNA as a reference test; 1119 patients were tested for the presence of HPV DNA as a gold standard test.
Besides Kerr's (RNA n = 7 and DNA n = 6), all studies included in this analysis had a sample size greater than 10 patients.

The majority of studies (n=10) were conducted in Asian countries, six were conducted in North America, five in Europe, and one in Oceania. With regards to the patients tested for HPV RNA, 603 were from North America, 173 were Europeans, 143 were Asians, and 13 were from Oceania. Of the patients that were tested for the presence of HPV DNA, 607 were Asians, 430 were from Europe, 46 from Oceania, and 36 from North America.

The male/female ratio, age ranges and median and/or mean ages were referenced in Table 2 in the annex. Such data were not available in every article. Moreover, many studies did not have information separated for the cases of oral cavity cancers, but only for samples combining oral cancers with cancers from other head and neck areas.
A global male/female ratio was obtained by summing the number of men and women included in each study that mentioned a ratio specific to the sample of oral cavity cancers. The resultant men/women ratio was 1,27:1 (526 men for 413 women).

Global mean and median ages were calculated just as for the previous ratio, by summing, on one hand, the various mean ages of the samples of patients with oral cavity cancer that were evaluated for p16 and HPV positivity, and referred such data, and on the other hand, by summing the various median ages available.

The mean age that could be determined with the available data was 58,17 years old, and the median age was 61,83 years old. The younger referenced patient was 22 years old by the time of the diagnosis [17], and the older was 96 years old [5].

The genotypes of HPV detected in the articles have been specified in Table 1. The term "all" was used when, in a study, the presence of multiple types of HPV, both High-Risk (HR) and Low-Risk (LR) types, was assessed.

For a majority of the cases, the gold-standard method used was PCR: 581 patients were tested by HPV RNA PCR and 841 were tested by HPV DNA PCR. Regarding ISH, 351 individuals were tested by HPV RNA ISH and 278 were tested by HPV DNA ISH.
Concerning the threshold value for p16 positivity, although it varied a lot among the different studies, the most commonly used value remained staining of ≥ 70% of the tumor.

Four of the studies only included cases of tongue carcinomas, while most of the studies (n=18) included cases of tumors located in various other locations of the oral cavity.

Almost all studies assessed both p16 expression and the presence of HPV RNA or DNA from FFPE samples. Frances Wright used Fine Needle Aspiration (FNA) samples. Deng used fresh frozen samples for the detection of HPV DNA and HPV E6/E7 mRNA expression, but FFPE samples for p16 detection.

4.2 Quantitative Analysis

All sensitivities and specificities of p16-IHC for HPV infection in oral cavity tumors, determined from the various studies, are referenced in Table 1.

Average sensitivities and specificities have been calculated separately, on one side for the group of studies that assessed the presence of HPV RNA as a reference test, and on the other side for those who measured the presence of HPV DNA as a gold standard.

Regarding the group of patients tested for HPV RNA presence, the average sensitivity of p16-IHC was 69,11%, and the average specificity was 84,77%.

For the group of patients tested for HPV DNA presence, the average sensitivity was 45,78%, and the average specificity was 74,26%.

TABLE 1 – RESULTS

Author and year	Method (RNA or DNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16+ (% tumor staining)	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)
Smeets et al. 2007	E6I mRNA	HPV 16	PCR	Any positive intensity	OCSCC	6	4	19	0	100,00	82,61
Bishop et al. 2012	E6/E7 mRNA	HR	ISH	>50	OCSCC	1	8	100	0	100,00	92,59
Lingen et al 2013	E6/E7 mRNA	HR	PCR	Tumors with punctuate or diffuse staining specific to tumor cell nuclei were considered positive.	OCSCC	19	27	358	5	79,17	92,99
Poling et al. 2014	E6/E7 mRNA	HR	ISH	>70	Lateral tongue	1	8	69	0	100,00	89,61
Kerr et al. 2015	RNA	HR	ISH Manual	≥70	5 tongue, 1 FOM and 1 retromolar	1	0	6	0	100,00	100,00
Belobrov et al. 2017	E6/E7 mRNA	HR	ISH	>20 [22]	OSCC	1	7	5	0	100,00	41,67
Minami et al. 2017	E6/E7 mRNA	HR	PCR	>70	Mobile tongue cancer	3	15	105	4	42,86	87,5
Palve et al. 2018	E6 or E7 RNA	HPV 16/18	PCR	unspecified ("sections of cervical cancer were used as positive control")	OCSCC	0	2	12	2	0,00	85,71
Soland et al. 2020	E6/E7 mRNA	HR	ISH	>70	Mobile tongue cancer	0	14	130	0	0,00	90,28
Deng et al. 2014	DNA	all	PCR	>40	OCSCC	2	0	16	6	25,00	100,00

Kerr et al. 2015	DNA	HR	PCR	≥70	4 tongue, 1 FOM and 1 retromolar	0	0	4	2	0,00	100,00
Belobrov et al. 2017	DNA	all : HPV 16 E6 and HPV 18 E6 15 HR 8 LR	PCR	>20 [22]	OSCC	3	20	23	0	100,00	53,49
Palve et al. 2018	DNA	all	PCR	unspecified ("sections of cervical cancer were used as positive control")	OCSCC	5	3	14	25	16,67	82,35
Nopmaneepaisarn et al. 2019	DNA	HR	ISH	positive if >70%, equivocal if 30−70%	OSCC	4	5	125	0	100,00	96,15
Vidal Loustau et al. 2019	DNA	all: 19 HR 10 LR 8 others	PCR	≥70	OSCC	1	10	137	4	20,00	93,20
Tachibana et al. 2019	DNA	all: 7 HR 2 LR	PCR	score 2 (moderate to strong nuclear and cytoplasmic staining)	Tongue carcinoma	2	8	69	7	22,22	89,61
Komolmalai et al. 2020	DNA	HPV 16/18 (16 and 18)	PCR	none of the tumor cells are stained (-, negative); positive staining in 1-9% (+/-); 10-49% (1+); 50-89% (2+); and ≥90% (3+) of the tumor cells The specimens with any staining above the background in the invasive parts of tumor were considered p16-positive	OSCC	10	98	60	4	71,43	37,97

Soland et al. 2020	DNA	HR	ISH	>70	Mobile tongue cancer	0	14	130	0	0,00	90,28
Adham et al. 2020	DNA	all	PCR	≥5	OSCC	1	5	13	2	33,33	72,22
Frances Wright et al. 2020	DNA	HPV 16	PCR	>70	OCSCC	1	0	11	1	50,00	100,00
Rooper et al. 2020	DNA	all: 16 HR 6 LR 9 others	PCR	>70	OCSCC	17	0	0	0	100,00	0,00
Tagliabue et al 2020	DNA	all: 8 HR/possibleHR 2 LR	PCR	continuous, diffuse staining	OC cancer	4	3	17	5	44,44	85
Smeets et al. 2007	DNA	HR	PCR	Any positive intensity	OCSCC	6	4	16	3	66,67	80
Kouketsu et al 2015	DNA	HR	PCR	negative (-), weakly to moderately positive (+), and strongly positive (++)	OSCC	13	11	3	0	100	21,43
Nemes et al 2006	DNA	HR	PCR	>10	OSCC	4	9	36	27	12,9	80
Ishibashi et al 2011	DNA	consensus PCR → all = 25 types including HR HPV genotying → all: 13 HR 10 LR/risk-unknown	PCR	≥5	OSCC	2	6	35	7	22,22	85,37
Singh et al 2015	DNA	all	PCR	>10	OSCC	9	7	16	14	39,13	69,57

5 DISCUSSION

5.1 Analysis of the results

The sensitivity and specificity of p16-IHC for HPV infection were higher in the group of patients who were tested for the presence of HPV-RNA than in the group tested for the presence of HPV DNA.

Such results could be explained by the fact that, as it was mentioned earlier, HPV DNA detection does not measure the presence of transcriptionally active HPV. Thus, a tumor tested positive for the presence of HPV DNA, can be tested negative by p16-IHC, as the virus may be present, but biologically inactive in this tumor. The findings of this study are consistent with the literature that affirms that HPV RNA detection is better for diagnosing HPV infection rightly [13,14].

For both groups of patients, the average sensitivity of p16-IHC was lower than its specificity.

In a recent systematic review and meta-analysis by Wang et al. [23], the combined sensitivity of p16-IHC found in the group of non-OPSCC tumors (composed of 79% of OSCC) was also found lower than the combined specificity. However, both values of sensitivity and specificity found by Wang et al. were higher than the values found in this study.

Generally, p16-IHC is considered to have good sensitivity but reduced specificity. This may be due to the fact that, to date, most studies assessed the reliability of p16-IHC in tumors not only restricted to the oral cavity but also from other areas of the head and neck, including the oropharynx. P16-IHC is known to have good sensitivity for detecting HPV infection in OPSCC. As an example, in the study by Wang et al. [23], the group of OPSCC tested for p16-positivity revealed a sensitivity of p16-IHC higher than its specificity.

5.2 P16-IHC limitations

The accuracy of p16 as a surrogate marker for HPV infection in OSCC has some limitations that have to be noted.

Firstly, p16 overexpression is unexclusive for HPV-related cancers [2,5,18]. Lee SY et al. reported that p16 expression has a high false-positive rate in oral cavity SCC [5].
Indeed, p16 overexpression may occur by several non-viral molecular mechanisms, totally independent of HR-HPV infection [9,20].
On the one hand, mutations, deletions, or methylation of the gene coding for p16 (CDKN2A) can increase its secretion [16], just like cellular senescence and/or aging [5].
Furthermore, if p16 overexpression may be related to pRb inactivation, pRb dysfunction is not always related to HPV infection. It could be altered either by a genetic alteration, a functional mutation, or other mechanisms, without resulting in a transformation of the cells [2].
Belobrov even affirmed: "*p16 overexpression is only rarely HPV-related and p16 IHC expression cannot be used as a surrogate marker for the presence of HPV in oral cavity carcinomas*" [7].

Many more shortcomings affect the reliability of p16-IHC as a detection method.

One of them is the fact that there is no universally accepted threshold for p16 positivity, even though strong, diffuse nuclear and cytoplasmic staining in ≥70% of the tumor is the cut-off value most recommended [1] and commonly applied [18]. Deng et al. reported: "*diverse scoring systems may lead to significant discrepancies across studies in the relationship between HPV infection and p16INK4a expression*" [20].

Also, p16-IHC is a quite subjective technique, since it requires the interpretation of a histopathologist [20]. There is a lack of scoring standard [13] as the "strong and diffuse staining" criteria may be unspecific.

Aside from all of this, p16 IHC is considered a simpler method (than PCR) [12], inexpensive [15], and easily applicable on FFPE samples [14]. It can be performed in almost every histopathological laboratory in hospital settings and requires fewer steps in the procedure than does PCR, thus it is less time-consuming [12].

But, given all the shortcomings previously described, p16 IHC should not be used alone for the diagnosis of HPV infection in OSCC [7,20].

In fact, although some diagnostic tests are suggested to have a standalone capacity [15], various studies have shown that no method for HPV detection is sure enough to be used alone [2]. It is recommended to use a combination of at least two different detection methods to avoid false-positive results [2].

Combining tests permit to benefit from the strength of each method, balancing their limitations [15]. In this case, pre-selection with p16-IHC would reduce the workload, and using a second method could improve the specificity of the diagnosis [14]. Thus, the risk of misdiagnosis would be considerably reduced.

Some combinations of techniques have already been proposed, such as p16 immunostaining as a screening step followed by a virus-specific test, which could be either qPCR or ISH targeting HPV RNA or DNA [4]. Have been suggested, among them, P16INK4a IHC followed by HPV-DNA GP5+/6+ PCR on p16-positive cases, or even a triple technique, combining P16INK4a IHC, HPV-DNA PCR, and HPV-DNA ISH [2].

Further studies will be necessary to confirm the reliability of some detection algorithms and find the best combination for HPV detection in OSCC. Indeed, Bishop reported: "*although these algorithms can determine the HPV status for most oropharyngeal carcinomas, there remains a subset of cancers that yield conflicting results*"[15].

5.3 Results limitations

There are some limitations in this work that invite to take some caution when interpreting the results.

The included studies did not all target the same types of HPV, which could have lead to some bias in our results. For example, some focused exclusively on the detection of HPV 16, others only detected HPV 16 and/or 18.

HPV type 16 is generally reported as the most predominant type in OSCC, followed by HPV 18 [3,11,24]. However, in their study, Lingen et al. could observe that the HPV type distribution among OCSCC cases was remarkably more diverse than within the cases of oropharyngeal tumors (94.9% of HPV16 against 5.1% of non-16), with approximately 38% of positive cases attributable to HR-HPV types other than 16.

Furthermore, the relative weight of the different HPV genotypes appears to vary depending on geographic locations [24].

Studies only detecting HPV16, or HPV 16 and 18 could consequently underestimate the number of HPV-positive tumors, leading to falsely negative results for HPV detection [19].

The consistency of the results could also be questioned because of the variety of gold standard tests used among the several studies.

These methods, RNA PCR, RNA ISH, DNA PCR, and DNA ISH, do not have the same level of sensitivity and specificity. Comparing studies that used the same reference test would bring more uniformity to the findings.

Moreover, for many of the included studies, HPV detection has been done on HPV DNA, and not HPV RNA. As is known, detection of HPV DNA alone does not allow detecting the presence of oncologically active virus [17], and might as well suggest the presence of passenger HPV genomes coming from adjacent normal cells, or the existence of biologically inactive or bystander viruses in oral cavity tumors [13].

Here, studies using HPV DNA detection as a gold standard were still included. Thereby, it was possible to compare the two groups: the one with patients tested for HPV RNA presence and the one tested for HPV DNA presence, in terms of sensitivity and specificity of p16-IHC for detecting HPV infection.

Our results would be more reliable if all studies used a reference method based on HPV RNA.

Many different threshold values for p16 positivity were used among studies. They ranged from 5% to more than 70%. Some studies did not even use a numerical cut-off value. For example, Smeets et al. considered p16-positive tumors showing "*any positive intensity*", and Kouketsu et al. classified tumors staining as negative, weakly to moderately positive, or strongly positive for p16.
As mentioned earlier, using studies with the same cut-off value for p16-positivity would increase the accuracy of our results.

Furthermore, in the study by Nopmaneepaisarn et al., all samples have not been assessed with both p16-IHC and an HPV-specific test. P16 was used as a screening test, and then, the presence of HPV was only tested in P16-positives samples. This protocol does not allow verifying properly the efficiency of p16-IHC for detecting HPV, as it is impossible to get true-negative or false-negative results for p16-IHC.

Another matter that needs to be considered is the fact that, anatomically, the oral cavity and the oropharynx are not clearly delineated.
Although the oral cavity is supposed to include the lips, the upper and lower alveolar ridges, the floor of the mouth, the hard palate, the buccal mucosa, the anterior two-thirds of the tongue, and the retromolar triangles, some authors may have mixed oral cavity and base of tongue tumors (normally considered as oropharyngeal tumors). This might lead to falsely higher rates of HPV in oral cancers [7], and thus the rate of p16-positive cases could be affected too.

As a result, a significant number of oropharyngeal tumors might be classified as oral cavity tumors [24]. So, some tumors classified as OSCC-HPV positives could actually be OPSCC-HPV positive tumors, and thus should not be included in this study.

Moreover, some studies focused on specific sites of the oral cavity, and not the oral cavity in its totality. For example, the studies conducted by Poling et al., Minami et al., or even Soland et al. only included tongue cancers samples. As far as is known, the relative importance of HPV in the various specific sites of the oral cavity is not clear yet, but still, including studies that assess tumors of the same site could reduce the risk of bias.

Given all these limitations, some caution might be taken considering our results.

6 CONCLUSION

P16-IHC did not appear to be reliable enough to be used alone for detecting HPV infection in oral cavity cancers. Indeed, several other mechanisms, independent of HPV infection, can lead to p16 overexpression. Furthermore, the p16-IHC protocol itself is still in need of some standardization, especially in terms of interpretation and scoring criteria.

Combining detection methods have already been proposed as an efficient solution to reduce misdiagnosis in oropharyngeal cancers, and further studies are needed to determine the best combination protocol for oral cavity cancers.

The prevalence of HPV in OSCC is not clearly evaluated yet. When it comes to assessing the reliability of p16-IHC for the diagnosis of HPV infection in malignancies of the head and neck area, more assays are available for the oropharynx than for the oral cavity. More studies including samples of OSCC would be necessary to confirm the results of this work. A homogenization of several parameters, such as the threshold value for p16-positivity, the gold standard method used, or even HPV genotypes targeted, will also be useful to reduce the risk of potential bias and achieve better comparability between studies.

BIBLIOGRAPHICAL REFERENCES

1. Tagliabue M, Mena M, Maffini F, Gheit T, Blasco BQ, Holzinger D, et al. Role of human papillomavirus infection in head and neck cancer in Italy: The HPV-AHEAD study. Cancers. 2020 Dec 1;12(12):1–19.
2. Vidal Loustau AC, Dulguerov N, Curvoisier D, McKee T, Lombardi T. Low prevalence of HPV-induced oral squamous cell carcinoma in Geneva, Switzerland. Oral Diseases. 2019 Jul 1;25(5):1283–90.
3. Komolmalai N, Pongsiriwet S, Lertprasertsuke N, Lekwanavijit S, Kintarak S, Phattarataratip E, et al. Human Papillomavirus 16 and 18 Infection in Oral Cancer in Thailand: A Multicenter Study. Asian Pacific Journal of Cancer Prevention. 2020;21(11):3349–55.
4. Nopmaneepaisarn T, Tangjaturonrasme N, Rawangban W, Vinayanuwattikun C, Keelawat S, Bychkov A. Low prevalence of p16-positive HPV-related head-neck cancers in Thailand: Tertiary referral center experience. BMC Cancer. 2019 Nov 6;19(1).
5. Tachibana T, Orita Y, Gion Y, Miki K, Ikegami K, Marunaka H, et al. Young adult patients with squamous cell carcinoma of the tongue strongly express p16 without human papillomavirus infection. Acta Oto-Laryngologica. 2019 Jan 2;139(1):80–4.
6. Rooper LM, Windon MJ, Hernandez T, Miles B, Ha PK, Ryan WR, et al. HPV-positive squamous cell carcinoma of the larynx, oral cavity, and hypopharynx: Clinicopathologic characterization with recognition of a novel warty variant. American Journal of Surgical Pathology. 2020 May 1;44(5):691–702.
7. Belobrov S, Cornall AM, Young RJ, Koo K, Angel C, Wiesenfeld D, et al. The role of human papillomavirus in p16-positive oral cancers. Journal of Oral Pathology and Medicine. 2018 Jan 1;47(1):18–24.
8. Nemes JA, Deli L, Nemes Z, Márton IJ. Expression of p16INK4A, p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 2006 Sep;102(3):344–52.
9. Søland TM, Bjerkli IH, Georgsen JB, Schreurs O, Jebsen P, Laurvik H, et al. High-risk human papilloma virus was not detected in a Norwegian cohort of oral squamous cell carcinoma of the mobile tongue. Clinical and Experimental Dental Research. 2021 Feb 1;7(1):70–7.

10. Kouketsu A, Sato I, Abe S, Oikawa M, Shimizu Y, Takahashi T, et al. Detection of human papillomavirus infection in oral squamous cell carcinoma: a cohort study of Japanese patients. Journal of Oral Pathology and Medicine. 2016 Sep 1;45(8):565–72.

11. Singh V, Husain N, Akhtar N, Kumar V, Tewari S, Mishra S, et al. Do human papilloma viruses play any role in oral squamous cell carcinoma in North Indians. Asian Pacific Journal of Cancer Prevention. 2015;16(16):7077–84.

12. Adham M, Aldino N, Zahra S, Rachmadi L, Bardosono S. Feasibility of p16 surrogate biomarker as adjunct diagnosis of oral and oropharyngeal malignancy in a resource-constrained country. Acta Oto-Laryngologica. 2021;141(1):106–10.

13. Palve V, Bagwan J, Krishnan N, Pareek M, Chandola U, Suresh A, et al. Detection of High-Risk Human Papillomavirus in Oral Cavity Squamous Cell Carcinoma Using Multiple Analytes and Their Role in Patient Survival. 2018.

14. Smeets SJ, Hesselink AT, Speel EJM, Haesevoets A, Snijders PJF, Pawlita M, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. International Journal of Cancer. 2007 Dec 1;121(11):2465–72.

15. Bishop JA, Ma X-J, Wang H, Luo Y, Illei PB, Begum S, et al. Detection of Transcriptionally Active High-risk HPV in Patients With Head and Neck Squamous Cell Carcinoma as Visualized by a Novel E6/E7 mRNA In Situ Hybridization Method [Internet]. 2012. Available from: www.ajsp.com

16. Minami K, Kogashiwa Y, Ebihara Y, Nakahira M, Sugasawa M, Fujino T, et al. Human papillomavirus and p16 protein expression as prognostic biomarkers in mobile tongue cancer. Acta Oto-Laryngologica. 2017 Oct 3;137(10):1121–6.

17. Poling JS, Ma XJ, Bui S, Luo Y, Li R, Koch WM, et al. Human papillomavirus (HPV) status of non-tobacco related squamous cell carcinomas of the lateral tongue. Oral Oncology. 2014;50(4):306–10.

18. Kerr DA, Arora KS, Mahadevan KK, Hornick JL, Krane JF, Rivera MN, et al. Performance of a Branch Chain RNA In Situ Hybridization Assay for the Detection of High-risk Human Papillomavirus in Head and Neck Squamous Cell Carcinoma [Internet]. 2015. Available from: www.ajsp.com

19. Lingen MW, Xiao W, Schmitt A, Jiang B, Pickard R, Kreinbrink P, et al. Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. Oral Oncology. 2013 Jan;49(1):1–8.

20. Deng Z, Hasegawa M, Aoki K, Matayoshi S, Kiyuna A, Yamashita Y, et al. A comprehensive evaluation of human papillomavirus positive status and p16INK4a overexpression as a prognostic biomarker in head and neck squamous cell carcinoma. International Journal of Oncology. 2014;45(1):67–76.

21. Wright MF, Weiss VL, Lewis JS, Schmitz JE, Ely KA. Determination of high-risk HPV status of head and neck squamous cell carcinoma using the Roche cobas HPV test on cytologic specimens and acellular supernatant fluid. Cancer Cytopathology. 2020 Jul 1;128(7):482–90.

22. Belobrov S, Angel C, Wiesenfeld D, McCullough M. Histopathological analysis of oral squamous cell carcinoma in nonsmokers and nondrinkers. Translational Research in Oral Oncology. 2016 Jan 1;1:2057178X1664797.

23. Wang H, Zhang Y, Bai W, Wang B, Wei J, Ji R, et al. Feasibility of Immunohistochemical p16 Staining in the Diagnosis of Human Papillomavirus Infection in Patients With Squamous Cell Carcinoma of the Head and Neck: A Systematic Review and Meta-Analysis. Vol. 10, Frontiers in Oncology. Frontiers Media S.A.; 2020.

24. Ishibashi M, Kishino M, Sato S, Morii E, Ogawa Y, Aozasa K, et al. The prevalence of human papillomavirus in oral premalignant lesions and squamous cell carcinoma in comparison to cervical lesions used as a positive control. International Journal of Clinical Oncology. 2011 Dec;16(6):646–53.

ANNEX

TABLE 2 – ADDITIONAL INFORMATION

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Smeets et al. 2007	NL	/	/	29 (assess-able)	E6I mRNA	HPV 16	RT-PCR	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed inde-pendently by 3 investigators.	OCSCC	6	4	19	0	(6/(6+0) x100 = 100	(19/(4+19) x100 = 82,61	(6/(6+4)) x100 = 60	(19/(19+0)) x100 = 100
Bishop et al. 2012	USA	/	/	109	E6/E7 mRNA	HR	HPV DNA ISH & **HPV E6/E7 mRNA ISH (RNAscope)**	**>50:** "0 = completely negative staining; 1 = focal staining (less than 20% of tumor cells); 2 = patchy staining (20 – 50% of tumor cells); 3 = diffuse staining (greater than 50% of tumor cells). As a surrogate marker of HPV infection, only staining that was diffuse (3) was regarded as positive for p16 overexpression."	use of a mouse monoclonal antibody against p16 - Ultra view polymer detection kit - graded 0–3 proportional to staining intensity	OCSCC	1	8	100	0	(1/(1+0)) x100 = 100	(100/(100+8)) x100 =92,59	(1/(1+8))x100 =11,11	(100/(100+0)) x100 =100

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Lingen et al 2013	USA	median for HPV+: 61 y.o. median for HPV-: 64 y.o.	236/173	409 (assess-able)	E6/E7 mRNA	HR	qRT TaqMan PCR	Tumors with punctuate or diffuse staining specific to tumor cell nuclei were considered positive.	a mouse monoclonal antibody visualized with use of an autostainer and a cone-view secondary detection kit graded 0–3 proportional to staining intensity	OCSCC	19	27	358	5	(19/(19+5)) x100 =79,17	(358/(358+27)) x100 =92,99	(19/(19+27)) x100 = 41,30	(358/(358+5)) x100 =98,62
Poling et al. 2014	USA	median: 55 y.o. [22-84 y.o.]	36/42	78	E6/E7 mRNA	HR	ISH (RNAscope)	**>70:** 0 = completely negative staining; 1 = focal staining (less than 20% of tumor cells); 2 = patchy staining (20–70% of tumor cells); 3 = diffuse staining (nuclear and cytoplasmic staining in greater than 70% of tumor cells). As a surrogate marker of HPV infection, only staining that was diffuse was regarded as positive for p16 overexpression.	a mouse monoclonal antibody against p16, using the Ultra view polymer detection kit	Lateral tongue	1	8	69	0	(1/(1+0)) x100 = 100	(69/(69+8)) x100 = 89.61	(1/(1+8))x100 = 11,11	(69/(69+0)) x100 = 100

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Kerr et al. 2015	USA	mean age: 59,2 median age: 58,5 y.o. [33-99 y.o.]	43/11	7	RNA	HR	Manual RNA ISH assay	**≥70**% of tumor cells demonstrating strong diffuse nuclear and cytoplasmic staining	A mouse monoclonal antibody against p16 (E6H4 clone, CINtec) was utilized with a 1:4 dilution, detected by the Polymer Refine Kit	5 tongue cancers 1 retro-molar cancer 1 floor of mouth cancer	1	0	6	0	(1/(1+0))x100 = 100	(6/(6+0)) x100 = 100	(1/(1+0))x100 = 100	(6/(6+0)) x100 = 100
Kerr et al. 2015	USA	mean age: 59,2 median age: 58,5 y.o. [33-99 y.o.]	43/11	5	RNA	HR (& HPV 16/18)	Automated ISH assay	**≥70%** of tumor cells demonstrating strong diffuse nuclear and cytoplasmic staining	A mouse monoclonal antibody against p16 (E6H4 clone, CINtec) was utilized with a 1:4 dilution, detected by the Polymer Refine Kit	3 tongue cancers 1 retro-molar cancer 1 floor of mouth cancer	0	1	4	0	(0/(0+0)) x100 = 0	(4/(4+1))x100 = 80,00	(0/(0+1)) x100 = 0	(4/(4+0)) x100 = 100
Belobrov et al. 2017	Australia	33<70 y.o. 13>70 y.o.	26/20	13	E6/E7 mRNA	HR	ISH (RNAscope)	**>20:** "For statistical analysis, the average positive pixel count result for each specimen was dichotomized and assigned one of two categories: negative/weak expression for scores of <20% and overexpression for scores >20% for p53, p16, and cyclin D1."	IHC staining using p16 mouse monoclonal antibody	OSCC	1	7	5	0	(1/(1+0)) x100 =100	(5/(5+7))x100 =41.67	(1/(1+7))x100 = 12,50	(5/(5+0)) x100 =100

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Minami et al. 2017	Japan	mean: 63,8 y.o. [20-96]	83/44	127	E6/E7 mRNA	HR	RT-PCR (LightCycler 480)	**>70:** "presence of strong and diffuse nuclear and cytoplasmic staining in greater than 70% of the tumor cells"	a p16 mouse monoclonal primary antibody	Mobile tongue cancer	3	15	105	4	(3/(3+4))x100 =42,86	(105/(105+15)) x100 = 87,50	(3/(3+15)) x100 = 16,67	(105/(105+4)) x100 = 96,33
Palve et al. 2018	India	40≤40 y.o. 72>40 y.o.	114/39	16	E6 or E7 RNA	HPV 16/18	qPCR	**unspecified** ("sections of cervical cancer were used as positive control")	primary antibody from BioGenex (No. AM540-5M; Antip16[NK4], Clone G175-405 in the NordiQC list) and using the PolyHRP detection system	OCSCC	0	2	12	2	(0/(0+2))x100 = 0	(12/(12+2)) x100 = 85,71	(0/(0+2)) x100 = 0	(12/(12+2)) x100 = 85,71
Soland et al. 2020	Norway	primary: median: 65,5 y.o. [25-90] second-ary: median: 72,0 y.o. [42-91]	primary: 77/51 second-ary: 10/8	144 (evaluable)	E6/E7 mRNA	HR	ISH (fully automated RNAscope VS HRP assay)	**>70: scores 2 & 3** "Score 0: no expression, Score 1: positive staining in <70% of the tumor cells, Score 2: positive staining, either nuclear or cytoplasmic in >70% of the tumor cells, Score 3: Strong and uniform p16-staining (both cytoplasmic and nuclear) in >70% of cancer cells"	a mouse monoclonal antibody clone E6H4. Bound antibody was detected by the biotin-free ultraView Universal DAB Detection Kit	Mobile tongue cancer	0	14	130	0	(0/(0+0))x100 = 0	(130/(130+14)) x100 = 90,28	(0/(0+14)) x100 = 0	(130/(130+0)) x100 = 100

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Smeets et al. 2007	NL	/	/	29 (assessable)	DNA GP5+/6+	HR	PCR	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed independently by 3 investigators.	OCSCC	6	4	16	3	(6/(6+3)x100 = 66,67	(16/(4+16)) x100 = 80,00	(6/(6+4)) x100 = 60,00	(16/(16+3)) x100 = 84,21
Smeets et al. 2007	NL	/	/	29 (assessable)	DNA viral load	HPV 16	RT-PCR (LightCycler)	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed independently by 3 investigators.	OCSCC	6	4	18	1	(6/(6+1))x100 = 85,71	(18/(18+4)) x100 = 81,81	(6/(6+4)) x100 = 60,00	(18/(18+1)) x100 = 94,74

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Smeets et al. 2007	NL	/	/	29 (assessable)	DNA	HPV 16/18	FISH	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed independently by 3 investigators.	OCSCC	5	5	19	0	(5/(5+0))x100 = 100	(19/(19+5)) x100 = 79,17	(5/(5+5)) x100 = 50,00	(19/(19+0)) x100 = 100
Smeets et al. 2007	NL	/	/	20 (assessable)	protein L1	HPV 16	Detection of antibodies against the proteins	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed independently by 3 investigators.	OCSCC	2	6	10	2	(2/(2+2))x100 = 50,00	(10/(10+6)) x100 = 62,50	(2/(2+6))x100 =25,00	(10/(10+2)) x100 = 83,33

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Smeets et al. 2007	NL	/	/	20 (assess-able)	protein E6	HPV 16	Detection of antibodies against the proteins	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed inde-pendently by 3 investigators.	OCSCC	4	4	9	3	(4/(4+3))x100 = 57,14	(9/(9+4))x100 = 69,23	(4/(4+4))x100 = 50,00	(9/(9+3)) x100 = 75,00
Smeets et al. 2007	NL	/	/	20 (assess-able)	protein E7	HPV 16	Detection of antibodies against the proteins	**Any positive intensity**: "For every case analyzed, an extra tissue section was stained with a mouse IgG, as a negative control. Staining intensity as a result of this mouse antibody was considered background and all samples with staining intensity above that background were scored as positive."	CINtecTM Histology Kit - Both the staining intensity (graded 0–3 proportional to staining intensity) and the percentage of the tumor cells positively stained per slide were assessed inde-pendently by 3 investigators.	OCSCC	2	6	9	3	(2/(2+3))x100 = 40,00	(9/(9+6)) x100 = 60,00	(2/(2+6))x100 = 25,00	(9/(9+3)) x100 = 75,00

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Kerr et al. 2015	USA	mean age: 59,2 median age: 58,5 y.o. [33-99 y.o.]	43/11	6	DNA	HR	RT-PCR (Cobas 4800 real-time PCR-based system)	**≥70%** of tumor cells demonstrating strong diffuse nuclear and cytoplasmic staining	A mouse monoclonal antibody against p16 (E6H4 clone, CINtec) was utilized with a 1:4 dilution, detected by the Polymer Refine Kit	5 tongue cancers 1 retomolar cancer 1 floor of mouth cancer	0	0	4	2	(0/(0+2))x100 =0	(4/(4+0))x100 =100	(0/(0+0)) x100 =0	(4/(4+2))x100 = 66,67
Kerr et al. 2015	USA	mean age: 59,2 median age: 58,5 y.o. [33-99 y.o.]	43/11	7	DNA	HR	ISH	**≥70%** of tumor cells demonstrating strong diffuse nuclear and cytoplasmic staining	A mouse monoclonal antibody against p16 (E6H4 clone, CINtec) was utilized with a 1:4 dilution, detected by the Polymer Refine Kit	5 tongue cancers 1 retomolar cancer 1 floor of mouth cancer	0	1	6	0	(0/(0+0))x100 =0	(6/(6+1))x100 = 85,71	(0/(0+1)) x100 =0	(6/(0+6)) x100 =0

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Belobrov et al. 2017	Australia	33<70 y.o. 13>70 y.o.	26/20	46	DNA	all: HPV 16 E6 HPV 18 E6 15 HR 8 LR	PCR- includes both SPF10-Lipa PCR and HPV 16/18 E6 qPCR. (LightCycler® 480 Instrument II (Roche))	**>20:** "For statistical analysis, the average positive pixel count result for each specimen was dichotomized and assigned one of two categories: negative/weak expression for scores of <20% and overexpression for scores >20% for p53, p16, and cyclin D1."	IHC staining using p16 mouse monoclonal antibody	OSCC	3	20	23	0	(3/(3+0))x100 = 100	(23/(23+20)) x100 = 53,49	(3/(3+20)) x100 = 13,04	(23/(23+0)) x100 = 100
Palve et al. 2018	India	40≤40 y.o. 72>40 y.o.	114/39	47	DNA	**all:** "PCR results indicate the presence of any HPV subtype with consensus primers or HPV16/18 type-specific primers"	PCR	**unspecified** ("sections of cervical cancer were used as positive control")	primary antibody from BioGenex (No. AM540-5M; Antip16[NK4], Clone G175-405 in the NordiQC list) and using the PolyHRP detection system	OCSCC	5	3	14	25	(5/(5+25)) x100 = 16,67	(14/(14+3)) x100 =82,35	(5/(5+3)) x100 = 62,50	(14/(14+25)) x100 = 35,90

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Palve et al. 2018	India	40≤40 y.o. 72>40 y.o.	114/39	43	DNA	HPV 16: "Quantitati-ve PCR (qPCR) and droplet digital PCR (ddPCR) results are from TaqMan assays with primers and probes for HPV16/HPV 18 and HPV16, respectively"	ddPCR	**unspecified** ("sections of cervical cancer were used as positive control")	primary antibody from BioGenex (No. AM540-5M; Antip16[NK4], Clone G175-405 in the NordiQC list) and using the PolyHRP detection system	OCSCC	6	2	12	23	(6/(6+23)) x100 =20,69	(12/(12+2)) x100 =85,71	(6/(6+2)) x100 =75,00	(12/(12+23)) x100 =34,29
Palve et al. 2018	India	40≤40 y.o. 72>40 y.o.	114/39	43	DNA	HV 16/18	qPCR	**unspecified** ("sections of cervical cancer were used as positive control")	primary antibody from BioGenex (No. AM540-5M; Antip16[NK4], Clone G175-405 in the NordiQC list) and using the PolyHRP detection system	OCSCC	2	4	16	21	(2/(2+21))x100 =8,70	(16/(16+4)) x100 =80,00	(2/(2+4))x100 =33,33	(16/(16+21)) x100 =43,24

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Soland et al. 2020	Norway	primary: median: 65,5 y.o. [25-90] second-ary: median: 72,0 y.o. [42-91]	primary: 77/51 second-ary: 10/8	144 (evaluable)	DNA	HR	ISH (Research ISH UltraMap XT procedure)	**>70: scores 2 & 3** "Score 0: no expression, Score 1: positive staining in <70% of the tumor cells, Score 2: positive staining, either nuclear or cytoplasmic in >70% of the tumor cells, Score 3: Strong and uniform p16-staining (both cytoplasmic and nuclear) in >70% of cancer cells"	a mouse monoclonal antibody clone E6H4. Bound antibody was detected by the biotin-free ultraView Universal DAB Detection Kit	Mobile tongue cancer	0	14	130	0	(0/(0+0))x100 = 0	(130/(130+14)) x100 = 90,28	(0/(0+14)) x100 = 0	(130/(130+0)) x100 = 100
Deng et al. 2014	China	mean: 64,1 y.o. [28-89]	127/23	24 8 DNA+ e 2 p16+	DNA, then E6/E7 mRNA on the DNA positive samples.	all	HPV DNA PCR (general consensus primer sets GP5+/GP6+ and MY09/11), then HPV E6/E7 mRNA PCR on the HPV DNA positive samples	**>40**: "0 (no staining), 1 (1-10% of tumor cells positive), 2 (11-40% positive), 3 (40-70% positive) and 4 (>70% positive). The term 'p16INK4a overexpression' is defined as a score of 3 or 4."	A monoclonal mouse anti-p16 antibody - Percentage scored was divided into a quartile scale of 1 to 4	OCSCC	2	0	16	6	(2/(2+6))x100 =25,00	(16/(16+0)) x100 = 100	(2/(2+0)) x100 =100	(16/(16+6)) x100 = 72,73
Nopman-eepai-sarn et al. 2019	Thailand	mean: 61,3 y.o. [29-95]	151/109	134 (with informa-tion)	DNA	HR	p16 IHC followed by HPV DNA ISH for the positive and equivocal cases found p16-positive.	positive if >70%, equivocal if 30–70%	using a monoclonal antibody to p16	OSCC	4	5	125	0	(4/(4+0))x100 = 100	(125/(125+5)) x100 =96,15	(4/(4+5))x100 = 44,44	(125/(125+0)) x100 = 100

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Vidal Loustau et al. 2019	Switzerland	mean for p16+: 69,2 y.o. Mean for p16-: 66,9 y.o. [34-95]	107/48	152 ("152 only with quantity of DNA suficient for analysis")	DNA	all: 19 HR 10 LR 8 others	qualitative DNA PCR (ProFlexTM thermocycler) followed by DNA ISH (Roche Linear Array protocol)	**≥70:** "staining of the cell nuclei and cytoplasm present in 70% or more of malignant cells"	the automated Ventana Medical System on BenchMark XT with the histological test kit VENTANA anti-CINtec p16	OSCC	1	10	137	4	(1/(1+4))x100 = 20,00	(137/(137+10))x100 = 93,20	(1/(1+10)) x100 = 9,09	(137/(137+4)) x100 = 97,16
Tachibana et al. 2019	Japan	mean: 60,2 y.o. [23-96]	57/29	86	DNA	all: 7 HR 2 LR	PCR (TaKaRa PCR Human Papillomavirus Typing Set)	score 2 (moderate to strong nuclear and cytoplasmic staining)	the CDKN2A/p16I NK4a antibody (EPR1473) and an automated Bond Max stainer	Tongue carcinoma	2	8	69	7	(2/(2+7))x100 =22,22	(69/(69+8)) x100 = 89,61	(2/(2+8)) x100 = 20,00	(69/(69+7)) x100 = 90,79
Komolmalai et al. 2020	Thailand	median: 66 y.o. 24 <50 y.o. 90 [50-69] 58 ≥70 y.o.	78/94 (1:1,2)	172 (amplifiable extracted DNA)	DNA	HPV 16/18 (16 and 18)	PCR	none of the tumor cells are stained (-, negative); positive staining in 1-9% (+/-); 10-49% (1+); 50-89% (2+); and ≥90% (3+) of the tumor cells The specimens with any staining above the background in the invasive parts of tumor were considered p16-positive	p16 IHC was performed with the Ventana Benchmark ULTRA autostainer using the CINtec p16 Histology Kit	OSCC	10	98	60	4	(10/(10+4)) x100 = 71,43	(60/(60+98)) x100 = 37,97	(10/(10+98)) x100 = 9,26	(60/(60+4)) x100 = 93,75

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Adham et al. 2020	Indonesia	mean: 50,5 y.o. [38-77]	10:11	21	DNA	all	Conventional DNA PCR (targeting the MY09/11 primers first, and then GP5þ/6þ primers)	≥5	GeneTex CCKN2A/p16INK4a antibody in 1:200 dilution overnight	OSCC	1	5	13	2	(1/(1+2))x100 = 33, 33	(13/(13+5)) x100 = 72,22	(1/(1+5))x100 = 16,67	(13/(13+2)) x100 = 86,67
Frances Wright et al. 2020	USA	mean: 63,6 y.o. [38-82]	23:11	13	DNA & E6/E7 mRNA only in discrepant cases	HPV 16	Multiplex RT-PCR & on discrepant cases: ISH for HR-HPV E6 and/or E7 mRNA (RNAscope)	**>70:** "strong, diffuse nuclear and cytoplasmic staining present in >70% of tumor cells on both histologic samples and cell blocks."	Expression was assessed using the p16 mouse monoclonal antibody (E6H4 clone)	OCSCC	1	0	11	1	(1/(1+1))x100 = 50,00	(11/(11+0)) x100 = 100,00	(1/(1+0))x100 = 100	(11/(11+1)) x100 = 91,67
Rooper et al. 2020	USA	median: 59 y.o. [29-85]	35/16	17 (assess-able)	DNA	all: 16 HR 6 LR 9 others	RT-PCR (LightCycler 480 instrument (Roche))	**>70:** "strong nuclear and cytoplasmic staining in > 70% of tumor cells"	A mouse monoclonal antibody for p16 (clone E6H4) and visualized using the ultraView polymer detection kit	OCSCC	17	0	0	0	(17/(17+0)) x100 = 100	(0/(0+0)) x100 = 0	(17/(17+0)) x100 = 100	(0/(0+0)) x100 = 0

Author and Year *	Geogra-fic localiza-tion	Age (in years) (mean or median and range) **	Ratio men/ wo-men**	Samples (n)	Me-thod (DNA or RNA)	HPV geno-types (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Rooper et al. 2020	USA	median: 59 y.o. [29-85]	35/16	16 (assess-able)	DNA	HR (or HPV 16)	ISH (GenPoint HPV16 Probe, Vcntana Inform HPV III Family 16 Probe)	**>70:** "strong nuclear and cytoplasmic staining in > 70% of tumor cells"	A mouse monoclonal antibody for p16 (clone E6H4) and visualized using the ultraView polymer detection kit	OCSCC	16	0	0	0	(16/(16+0)) x100 = 100	(0/(0+0)) x100 = 0	(16/(16+0)) x100 = 100	(0/(0+0)) x100 = 0
Taglia-bue et al 2020	Italy	[17-94]	99/65	29	DNA	all: 8 HR / possible HR 2 LR	type specific PCR bead-based multiplex genotyping (E7-MPG) assay that combines multiplex PCR and bead-based Luminex technology (Luminex Corps., Austin, TX, USA)	continuous, diffuse staining	CINtec p16 Histology Kit (Roche mtm laboratories AG, Mannheim, Germany)	Oral Cavity cancer	4	3	17	5	(4/(4+5))x100 = 44,44	(17/(17+3)) x100 = 85,00	(4/(4+3))x100 = 57,14	(17/(17+5)) x100 = 77,27

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Kouketsu et al 2015	Japan	mean: 67,6 y.o. [32-93]	76/98	27 ISH and RT-PCR analyses were performed in 24 p16-positive and 3 p16-negative OSCC specimens	DNA	HR	RT-PCR (Cobas 4800 HPV Amplification /detection Kit and Cobas 4800 System (Roche))	negative (-), weakly to moderately positive (+), and strongly positive (++)	CINtec p16 Histology Kit (Roche, Heidelberg, Germany) and the automated IHC/ISH slide staining system (Ventana Benchmark ULTRA; Roche)	OSCC	13	11	3	0	(13/(13+0)) x100 = 100	(3/(3+11)) x100 = 21,43	(13/(13+11)) x100 = 54,17	(3/(3+0)) x100 = 100
Kouketsu et al 2015	Japan	mean: 67,6 y.o. [32-93]	76/98	27 ISH and RT-PCR analyses were performed in 24 p16-positive and 3 p16-negative OSCC specimens	DNA	HR	ISH (Ventana INFORM HPV III Family 16 probe (Roche))	negative (-), weakly to moderately positive (+), and strongly positive (++)	CINtec p16 Histology Kit (Roche, Heidelberg, Germany) and the automated IHC/ISH slide staining system (Ventana Benchmark ULTRA; Roche)	OSCC	0	24	3	0	(0/(0+0))x100 = 0	(3/(3+24)) x100 = 11,11	(0/(0+24)) x100 = 0	(3/(3+0)) x100 = 100
Nemes et al 2006	Hungary	mean: 55,8 y.o. [32-84]	67/12	76	DNA	HR	PCR (Biometra T1 thermocycler)	**>10:** "positive if more than 10% of the tumor cells showed immunoreactivity"	DAKO LSAB2 alkaline phosphatase system	OSCC	4	9	36	27	(4/(4+27))x100 = 12,90	(36/(36+9)) x100 = 80,00	(4/(4+9))x100 = 30,77	(36/(36+27)) x100 = 57,14

Author and Year *	Geografic localization	Age (in years) (mean or median and range) **	Ratio men/ women**	Samples (n)	Method (DNA or RNA)	HPV genotypes (all/HR/16/18)	Method	Threshold for p16 positivity (in% of tumor cells stained)	IHC of p16	Cancer type	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ishibashi et al 2011	Japan	mean: 59,2 y.o. [12-81]	57/50	50	DNA	consensus PCR → all : 25 types including HR HPV genotying → all: 13 HR 10 LR/risk-unknown	consensus PCR method (using consensus primers (My09/My11, GP5+/GP6+)) or genotyping method (PCR-based micro-array system)	**≥5:** "Diffuse (80%) or focal (5–80%) strong staining was scored as positive"	CINtec Histology kit (monoclonal antibody: clone E6H4, prediluted, mtm laboratories, Hei- delberg, Germany)	OSCC	2	6	35	7	(2/(2+7))x100 = 22,22	(35/(35+6)) x100 = 85,37	(2/(2+35)) x100 = 5,41	(35/(35+7)) x100 = 83,33
Singh et al 2015	India	mean for HPV+: 47,17 y.o. mean for HPV-:47,69 y.o. [<30 - >60]	200/50	46	DNA	all	RT-PCR (Biorad CFX 96TM) using 13 HIGH RISK HPV REAL TIME PCR KIT confirmed by conventional PCR with PGMY09/ PGMY11 primers.	**>10:** "positive if more than 10% of the tumor cells showed immunoreactivity"	p16 primary antibody (Biogenex) at RT for one hour, followed by treatment with polymer based secondary antibody kit with DAB (DAKO, Denmark)	OSCC	9	7	16	14	(9/(9+14))x100 = 39,13	(16/(16+7)) x100 = 69,57	(9/(9+7))x100 = 56,25	(16/(16+14)) x100 = 53,33

*Name of autor & publication year were put in bold and underlined when the corresponding analysis was included in the results table.

**Mean/median ages, age ranges and gender ratios were underlined when they corresponded specifically to the sample of oral cavity cancers assessed (isolated from other areas of the head and neck).