THE ISOTHERMAL AMPLIFICATION AMPFIRE ASSAY FOR HPV DETECTION AND GENOTYPING IN FORMALIN-FIXED PARAFFIN-EMBEDDED OROPHARYNGEAL CANCER SAMPLES

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ABSTRACT

HPV-related oropharyngeal squamous cell carcinomas (OPSCC) represent a distinct clinical entity compared to HPV-negative tumours with particular regard to treatment-response and survival outcome. The aim of this study was to assess the AmpFire Multiplex HR-HPV tests, for the detection and genotyping of 15 HR-HPV types in formalin-fixed paraffin-embedded (FFPE) samples and identify HPV-driven OPSCC. DNA from 160 OPSCC FFPE specimens, plus 23 samples from other head and neck primary sites were tested. Results were compared with those obtained using Linear Array HPV-DNA Genotyping Test. LA and AmpFire Multiplex HR-HPV tests showed, for all samples and specifically for OPSCCs, an overall concordance agreement of 98.9% and 99.4% and a Cohen's Kappa coefficient of 0.972 and 0.984, respectively. A concordance of 100% for HPV16 and HPV18 was observed. The overall agreement between $p16^{INK4a}$ overexpression and HPV detection by the Ampfire Multiplex HR-HPV assay in 145 OPSCC samples was 93.8% with a Cohen's Kappa coefficient of 0.848. The AmpFire HPV Tests are simple assays for detection and genotyping of HPV-DNA in OPSCC FFPE samples and can be easily implemented in the clinical practice setting for HPV-DNA detection.

1. INTRODUCTION

About a decade ago the International Agency for Research on Cancer (IARC) established high-risk Human papillomavirus as the main cause of HPV-driven OPSCC [1]. Since then, it has been estimated that approximately 42,000 new HPV-related OPSCC cases occur every year, corresponding to 30% of the worldwide number of the overall incident OPSCC cases [2], with a clear geographical variation in HPV-attributable fractions of OPSCC ranging from <20% to more than 60%, depending on the world region (reviewed in [3]).

The detection of the attributable fraction of OPSCC associated with HPV is a powerful prognostic marker in OPSCC and despite is not used for treatment selection is described in the clinical guidelines as a useful diagnostic tool to anticipate the clinical outcome. The E6/E7 HPV mRNA detection on fresh frozen samples is the reference standard for the identification of the HPV-driven cases [4–6]. However, its application on the clinical settings is still challenging and, consequently, the lack of a gold standard testing method hampers the judgment of the real HPV-driven cases.

Currently, $p16^{INK4a}$-IHC evaluation is the most recommended technique for OPSCC HPV diagnostic (NCCN Guidelines for Head and Neck Cancers, Version 3.2021 available at https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1437, last accessed date july 10, 2021). $p16^{INK4a}$ is considered positive when more than 70% of the tumour tissue shows moderate to strong diffuse nuclear and cytoplasmic immunoreactivity [7]. However, previous studies have demonstrated a proportion (up to 20%) of patients who have tumours that are $p16^{INK4a}$ positive, but negative on testing for HPV-DNA [8,9]. Importantly, the outcome of these patients seems to be significantly worse than the outcomes of patients who are double positive for HPV and $p16^{INK4a}$ positive [10,11].

In view of these considerations, the College of American Pathologists and the American Joint Committee on Cancer produced an evidence-based guideline on testing, application, interpretation, and reporting of human papillomavirus (HPV) and surrogate marker tests in head and neck carcinomas [7]. For OPSCC tissue specimens, pathologists should perform HR-HPV testing by surrogate marker $p16^{INK4a}$ immunohistochemistry (IHC). Additional HPV-specific testing may be done at the discretion of the pathologist and/or treating clinician, or in the context of a clinical trial [7,12,13]. However, double positivity for HPV-DNA/$p16^{INK4a}$ is the diagnostic strategy showing the best prognostic value for HPV-driven OPSCC patients as also reported in a recent work [11].

Nevertheless, the assays for HPV-DNA detection in OPSCC have not yet been entirely defined (reviewed in [14]). The HPV tests currently used for OPSCC diagnosis are those intended for use

in cervical carcinoma. Additionally, HPV-DNA automatic platforms developed for cervical cancer screening, which frequently work with liquid-based cytology, are usually expensive, require well trained staff and have been designed for a high-throughput workflow, hampering its implementation for OPSCC diagnosis and raising the need for cheaper molecular methods. Different PCR assays will vary in their performance based on the choice of primer sets, PCR protocols and the type of tissue analysed.

Identifying molecular diagnostic tests that are able to discriminate those cancers etiologically associated with HPV is a priority for researchers and clinicians. Our objective was to evaluate the suitability and performance of the Ampfire HPV tests (Atila Biosystems) for the detection and genotyping of HPV in OPSCC. The tests were compared to Linear Array assay, one of the most extensively used assays for the detection and genotyping of FFPE samples (reviewed in [15]).

2. MATERIALS AND METHODS

2.1 Study samples

A retrospective series of 183 FFPE cases, diagnosed between 2014 and 2019, were retrieved from the archives of the Hospital Universitari de Bellvitge, Spain. Among the 183 samples of head and neck origin (HNSCC), 160 were of oropharyngeal origin (OPSCC) and 23 from other head and neck sites (non-OPSCC) (11 Oral Cavity, 6 Nasopharynx, 3 Larynx and 3 Hypopharynx). All the samples had previously been genotyped for the presence of HPV-DNA by Linear Array assay and 165 samples were evaluated for the expression of $p16^{INK4a}$ by IHC. Pathological classification was based on the World Health Organization pathological criteria for head and neck cancer [16].

The study was approved by the Ethics Committee of Hospital Universitari de Bellvitge (Protocol code: PR342/20).

2.2 FFPE blocks processing

Four paraffin sections were obtained for each block. First and last sections of 3 µm were used for histopathological evaluation after hematoxylin and eosin (HE) staining to perform tumour confirmation, and the second and third in-between sections of 5 µm were used for HPV testing and genotyping (sandwich method) [11]. FFPE blocks were processed under strict pre/post polymerase chain reaction (PCR) physical separation, and blank paraffin blocks were systematically tested in parallel to serve as sentinels for contamination as previously published.

144

145 **2.3 DNA extraction**

146 Total nucleic acids were extracted from one FFPE section using the Maxwell 16 FFPE Plus LEV
147 DNA Purification kit (Promega Corp., Madison, WI, USA). The DNA was eluted with 100 µl of
148 nuclease free water and the isolated DNA was diluted 10 times. A given volume of the 1/10
149 dilution of the extracted DNA, stated in each assay section, was used as a template for the
150 amplification reaction. Extracted DNA and their corresponding 1/10 dilutions were stored at -
151 80ºC immediately after isolation. Mean storage time was 786 days (standard deviation = 511
152 days).

153

154 **2.4 Linear Array genotyping**

155 For all the samples collected, the Roche Linear Array HPV Genotyping test (Roche Molecular
156 Systems Inc., Alameda, CA, USA) was used to detect the 37 HPV-types. This assay involves a
157 PCR amplification of target DNA followed by nucleic acid hybridization on a nylon strip and
158 detection of up to 37 high- and low-risk HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42,
159 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39
160 and CP6108). PCR was performed in a 50 µl reaction volume composed of 25 µl Linear Array
161 master mix and 25 µl of the 10-fold diluted DNA mentioned above. Amplification was
162 performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using
163 the recommended cycle parameters: 2 min at 50 °C and 9 min at 95 °C, followed by 40 cycles
164 of 30 s at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a final extension at 72 °C for 5
165 min. 50 µl of PCR amplicons were denatured with 50 µl of denaturing solution and detected by
166 nucleic acid hybridization and colorimetric detection. All washes and hybridization steps were
167 undertaken in a 48-well tray and the robot Auto-LiPA48 (Innogenetics N.V. (now Fujirebio
168 Europe N.V.), Ghent, Belgium) was used for this purpose. The denatured amplicons were
169 hybridized on to the strip containing specific probes for 37 HPV genotypes and β-globin
170 reference lines before undergoing stringent washes. Colorimetric determination was
171 performed with the Linear Array Detection Kit. The colour change reaction was from
172 Streptavidin-horseradish peroxidase mediated precipitation of working substrate. Positive
173 reactions appeared as blue lines on the strip. The strips were interpreted using the HPV
174 reference guide provided to visually match the vertical locations of the horizontal bars in the
175 LA strip to specific types of HPV. The interpretation of the results was done by two evaluators
176 that determined the positivity and the intensity of the band as strong or weak.
177 The genotyping strip contains probes that specifically hybridize with HPV6, 11, 16, 18, 26, 31,
178 33, 35, 39, 40, 42, 45, 51, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82,

83, 84, IS39 and CP6108. Also, the strip includes a cross reactive probe that detects HPV33, 35, 52 and 58 as a pool; therefore the probe cannot exclude an HPV52 co-infection. Those samples with a positive result for this probe were re-tested using a specific HPV52 PCR system [17].

2.5 Ampfire Assays (Multiplex HR-HPV Real Time Fluorescent Detection with HPV16/18 Genotyping test and Genotyping HR-HPV Real Time Fluorescent Detection test)

The Multiplex HR-HPV Real Time Fluorescent Detection with HPV16/18 Genotyping (developed by Atila Biosystems, Inc, Mountain View, CA, USA) (from now on referred to as Ampfire Multiplex HR-HPV) performs isothermal nucleic acid amplification of around 100 bp targets of E1 and/or L1 and uses real-time fluorescence detection for the qualitative detection of high-risk HPV (HR-HPV) types. The detection assay allows pooled real time fluorescent detection of 15 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66 and 68) and individual genotyping of HPV16 and 18 in a single assay tube. The assay uses specific probes labelled with CY5, ROX, FAM, HEX to detect HV16, HPV18, HR-HPV non-HPV16/18 and also an internal control, respectively. The presence and integrity of DNA in all samples is verified by β-globin gene amplification tested in HEX channel (internal control, IC). The lack of an exponential amplification curve in that channel is interpreted as an invalid result. According to the manufacturer, the threshold per reaction is 80 copies of the 13 non-HPV16/18 types, and 20 copies of HPV16 or HPV18. However, other studies have shown a limit of detection ranging from 2 to 20 copies/reaction for the 15 HPV genotypes [18].

A second kit, Genotyping HR-HPV Real Time Fluorescent Detection test (from now on referred to as Ampfire Genotyping HR-HPV), to individually genotype 15 high-risk HPV types was used. In this case, the specific genotyping of the 15 high-risk HPV types is performed in 4 wells. The AmpFire Genotyping HR-HPV kit includes a reaction mix and primer sets 1, 2, 3, and 4, which cover the 31/51/39/16, 35/68/18/59, 33/66/IC/45, and 58/56/53/52 HPV genotypes, respectively. Probes for the 16/59/45/52, 39/18/66/53, 31/35/33/58, and 51/68/IC/56 HPV genotypes are labelled with CY5, ROX, FAM, and HEX, respectively.

Both kits also provide positive (containing synthetic plasmids) and negative controls to ensure the results are trustworthy.

First of all, 9.5 µl of the 10-fold diluted DNA sample was mixed with 0.5 µl of 20x lysis buffer included in the kit and incubated at 95 °C for 15 min in a microcentrifuge tube. Next, the sample was briefly spun and 2 µl of the sample was added to a reaction mix and used for the isothermal amplification following manufacturer's instructions.

Viral DNA was amplified and detected in a CFX96 real time qPCR instrument (Bio-Rad, Hercules, CA, USA) using a 60°C isothermal reaction for 74 min with fluorescence being recorded once every minute (74 cycles) from the according channels. The results of the cycle thereshold (Ct) values for each amplification curve in all fluorescence channels are automatically reported by the CFX Manager Software IVD Edition 1.6 (Bio-Rad). A value of 55 Ct was chosen as a cutoff value.

2.6 HPV52 genotyping

In order to retest positive samples detected by Linear Array using the probe that combines HPV33, HPV35, HPV52 and HPV58 detection, a HPV52 Real-Time PCR single assaywas performed [17]. FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) was used for this purpose and according to manufacturer instructions, in combination with 5 µl of 1/10 diluted DNA. The primers used were (5'-3') E6-52F (GAACACAGTGTAGCTAACGCACG) and E6-52R (GCATGACGTTACACTTGGGTCA). HPV52 DNA was amplified and detected in a LightCycler 96 real time qPCR instrument (Roche Diagnostics, Mannheim, Germany), using the following cycle parameters: 10 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 55 °C, and 10 s at 72 °C, followed by a melting cycle of 10 s at 95 °C, 1 min at 65 °C and 1 s at 97 °C. The results of the Ct values for each amplification curve and the melting curve were automatically reported by the LightCycler 96 v1.1 software (Roche Diagnostics GmbH, Mannheim, Germany). Samples with a negative result were retested using 5 µl of DNA sample directly extracted from the FFPE specimens.

2.7 p16 ^{INK4a} immunohistochemistry

When needed additional slides were processed to assess expression of cellular proteins by immunohistochemistry (IHC). The expression of $p16^{INK4a}$ was analysed through IHC under the manufacturer's standards. Commercially available $p16^{INK4a}$-specific monoclonal antibody clone (Roche MTM Laboratories AG, Heidelberg, Germany), were used. For $p16^{INK4a}$, the intensity of nuclear and cytoplasmic staining within the tumours was scored and those with a strong staining of > 70% were considered $p16^{INK4a}$ positive [7].

2.8 Statistical Analysis

The raw agreement proportions and Cohen's Kappa coefficient were calculated to measure the inter-rater agreement between the AmpFire and Linear Array HPV test techniques (HPV negative vs. positive irrespective of the specific genotype/s identified).

A Kappa value of 0 indicates no agreement and a value of 1 indicates perfect agreement. Values from 0.00–0.20 indicate poor agreement, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 good, and 0.81–0.99 very good agreement. The raw agreement proportions and Cohen's Kappa were also used to assess the concordance between the HPV test results and $p16^{INK4a}$ outcomes. Statistical significance for all analyses was set at the 2-sided 0.05 level. Data analyses were performed with STATA software v.16 (Stata Corp., College Station, TX, USA).

2.9 Informed Consent

Samples were received in encrypted form (with a local identifier) and once received, the DNA was extracted and a new identifier was assigned to ensure its traceability. The remaining sample DNA, initially obtained and used for diagnostic purposes, was analysed retrospectively. Given the characteristics of the study, patient consent was waived because of the vital status of the patients and the retrospective nature of this study which represented an unreasonable effort (disproportionate time, work and expenses).

2.10 Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hospital Universitari de Bellvitge (Protocol code: PR342/20).

3. RESULTS

3.1 Ampfire Multiplex HR-HPV test versus Linear Array

DNAs obtained from 183 Head and Neck (160 OPSCC and 23 non-OPSCC) carcinomas were analysed using the Ampfire Multiplex HR-HPV test and the Linear Array assay. Amongst the 183 FFPE samples, three samples were found invalid, as the β-GLobin gene was not detected. One was invalid for both techniques and two of them only for the Ampfire Multiplex HR-HPV test, which means an invalid rate of 1.63% (3/183). According to the Ampfire Multiplex HR-HPV test, HPV-DNA was found in 47 samples (26.1%) (Table 1). Comparing the positive and negative agreement with Linear Array HPV test, a 95.9% (47/49) and a 100% (133/133) was observed, respectively (Table 1). This represents an overall agreement between the Ampfire Multiplex HR-HPV test and the Linear Array test of 98.9% (178/180; CI: 97.3%-100.0%) and a very good agreement according to Cohen's Kappa coefficient index (0.972; p-value<0.001; 95% CI: 0.932-1.000).

Comparing samples from different head and neck primary sites, the concordance of both techniques did not show statistically differences (p-value = 0.361). That is, either on OPSCC and non-OPSCC samples (Table 1), the performance of both tests showed a very good agreement (OPSCC samples, 99.4% concordance; Cohen's Kappa = 0.984; p-value<0.001; 95% CI: 0.953-1.000 and non-OPSCC samples, 95.7% concordance; Cohen's Kappa = 0.832; 95% CI: 0.489-1.000).

A concordance of 100% (40/40) for the presence of types HPV16 (39/39) and 18 (1/1) between Ampfire Multiplex HR-HPV assay and Linear Array was observed. In terms of "Other HR-HPV non-HPV16/18" concordance, the agreement between both assays was 70% (7/10) (Table 2). Two of the discordant samples were positive for HPV45 type, according to Linear Array, however the Ampfire Multiplex HR-HPV assay yielded a negative result. Similarly, Linear Array detected HPV16 and HPV52 in a sample where the Ampfire test was only able to genotype HPV16.

3.2 Concordance between Linear Array and Ampfire Genotyping HR-HPV test

Samples positive for high-risk types other than HPV16 or HPV18 were tested with the Ampfire Genotyping HR-HPV test to elucidate the HR-HPV types present. Agreements and discrepancies between both techniques and types are shown in Table 3. HPV33 type was detected by the Ampfire Genotyping HR-HPV assay in 5 out of the 6 samples identified by Linear Array. HPV35 type was present in only one sample and it was equally detected by both techniques. In addition, those samples discordant between Linear Array and the Ampfire Multiplex HR-HPV test were also tested with the Ampfire Genotyping HR-HPV to assess whether the discrepancies were dragged to this assay. This is the case of HPV45 that was identified in two samples by Linear Array, but not by the Ampfire Multiplex HR-HPV assay. When the Ampfire Genotyping HR-HPV assay was used, HPV45 was only amplified in one of the two samples.

In all the samples tested, HPV52 detected by Linear Array, was always in combination with genotypes HPV33 or HPV35, with the exception of one sample where HPV52 was found with HPV16. The Ampfire Genotyping HR-HPV test did not detect HPV52 in any of the 6 samples. To rule out the genuine presence of HPV52 in the genotype-discordant samples, real-time PCR was performed using specific primers to detect HPV52. Indeed, the results confirmed that HPV52 was not present in those samples. Thus, a 100% agreement between the Ampfire assays and the specific HPV52 testing was observed. Also, in agreement with the PCR result, in the particular case of that sample were HPV16 co-exists with a low HPV52 load, the Ampfire Multiplex HR-HPV and Genotyping HR-HPV tests classified this sample as positive for a single infection with HPV16.

3.3 Ampfire HPV detection test versus p16^{INK4a} IHC in OPSCC cases

Out of the 157 HPV-valid oropharyngeal samples, 145 had the p16^{INK4A} immunostaining completed. As shown in Table 4, almost all the cases that tested positive for Linear Array were also p16^{INK4A}-positive (38/42). Similarly, 37 out of 41 of the AmpFire Multiplex HR-HPV-positive cases displayed a p16^{INK4A} positive staining (Table 4). The concordance between positives and negatives for HPV and p16^{INK4A} was not statistically significant if we compare the performance of both dual tests as the p-value comparing both Cohen's Kappa index was 0.791.

4. DISCUSION

Several studies from different cohorts of OPSCC have shown that double positivity for HPV-DNA and p16^{INK4A}-IHC provide strongest diagnostic accuracy and prognostic value for HPV-driven OPSCC than any of both techniques alone, thus allowing a more accurate patient classification [11,19]. Importantly, these tests can both be straightforwardly implemented and used in the clinical setting [11]. Considerable HPV-DNA testing options are currently available on the market [20] and a few have been clinically validated for the detection and genotyping of HPV in liquid-based cytology samples for detecting cervical lesions [21]. All commercial HPV tests have been developed mainly to achieve optimal performance for cervical cancer screening although have not been validated using formalin-fixed, paraffin-embedded biopsies. Conversely, formalin-fixed, paraffin-embedded (FFPE) biopsies, which require specific processing before PCR amplification, are one of the most widely practiced methods for clinical sample preservation and are frequently the only existing samples for molecular testing. Currently there is no agreement on the finest detection method that should be used to classify HPV positive OPSCC and serve as a standard test for routine diagnostic use.

The present study compares two commercial HPV tests in a routine diagnostic setting. We have analysed the performance of the AmpFire Multiplex HR-HPV assay on 183 DNA samples extracted from primary tumour FFPE samples from patients with HNSCC, and compared the results with those obtained with the HPV Linear Array assay, one of the most widely used methods for testing and genotyping of FFPE samples (reviewed in [15]). Moreover, the results of HPV obtained by the Ampfire test in OPSCC samples were compared with the immunohistochemical expression of p16^{INK4A}, the last being available for 145 cases. To our knowledge, this is the largest study that tests the AmpFire assay for a set of OPSCC FFPE samples. Although most of the samples analysed were small diagnostic biopsies and some authors claim that unremoved paraffin can lead to PCR inhibition and thus to invalid results [22],

this was not a main issue in this study. Indeed, the rate of invalid test results was low (3/183, 1.63%) compared to other previously published studies showing invalid rates around 7-9% [23,24]. Although in the present study, extracted DNA was used and showed a good performance; the Ampfire test, contrarily to Linear Array, has the capacity to be used with or without DNA extraction. As an example, a recent work that detected HPV from FFPE specimens but did not perform DNA extraction [18] have shown a 0.5% rate of invalid samples. This is an important point because DNA extraction step is time-consuming, increase the costs of HPV-DNA testing, could increase the rate of invalid samples [25] and inevitably introduces a source of variability.

When comparing both tests -Linear Array and Ampfire Multiplex HR-HPV assays- an excellent agreement was achieved (Cohen's Kappa = 0.972, 95% CI: 0.932-1.000). Prominently, this large agreement between assays was not only observed for the HPV status (positive or negative) but also for the genotyping results. The AmpFire Multiplex HR-HPV assay allows pooled real time fluorescent detection of 15 HR-HPV types in addition to individual genotyping of HPV16 and HPV18 in a single assay tube. This represents an advantage of the test since HPV16 is by far the most common of the types found amongst HPV-driven OPSCCs (prevalence over the 80%) [26]. The Ampfire test identified HPV16 (39/39) and HPV18 (1/1) in all the samples that tested HPV16/18-positive with Linear Array. A 70% agreement between both assays was also observed for the detection of other HR-HPV non-HPV16/18 genotypes in 10 samples including HPV33 and HPV35 being beyond the HPV16, the most prevalent types in oropharyngeal tumours [26,27].

The full set of samples that tested positive for other HR-HPV non-HPV16/18, were specifically genotyped with the Ampfire Genotyping HR-HPV test (Linear array-blinded). Despite the high overall agreement rate between both techniques, discordant results for HPV52 in 6 specimens were detected. Five out of six samples that showed a HPV52-negative result by the Ampfire Genotyping HR-HPV test but positive by Linear Array showed a coinfection for HPV33 and HPV35. It is worth to mention a reported limitation of Linear Array is that additional testing is necessary to detect the high-risk HPV52 genotype when HPV33, HPV35 and/or HPV58 are present in the same sample [28]. Real-Time PCR using specific HPV52 probes confirmed the negativity of HPV52 in the 5 samples co-infected with HPV33 or HPV35 and suggested a probable false-positive result of the Linear Array test.

Additionally, the Ampfire Genotyping HR-HPV Real Time Fluorescent Detection test identified five out of the six HPV33 genotypes detected by Linear Array and also, a single HPV35 was identified by both techniques.

All the experiments were carried out using the same DNA sample. However, it is worth to mention that both assays were not performed contemporaneously, and thus, the invalidity or

384 negativization of some samples could be explained for this reason. Discordant results could
385 also be associated with the DNA quantity recommended by the manufacturer used by each
386 test, as the amount of DNA added to the Ampfire tests was much lower than the amount
387 added to Linear Array.
388 To the best of our knowledge, only 2 studies have previously evaluated the Ampfire tests on
389 Head and Neck specimens, however, both assays included a small number of OPSCC samples.
390 Jang and colleagues analysed 81 samples directly from saliva by Ampfire Multiplex HR-HPV test
391 and compared the results to a well-validated technique, Cobas4800. The percentage of overall
392 agreement between both assays was 65.4% (Cohen's Kappa=0.34) due to a poor positivity
393 agreement between both techniques. Similarly, when comparing the results of the 71 samples
394 with a $p16^{INK4A}$ immunostaining test done, a low 40.3% sensitivity and a 93.3% specificity was
395 found. Authors claim that the low sensitivity was probably due to the type of sample used or
396 to the large dilution of samples before freeze storage [29]. During the same year, another
397 manuscript evaluated the performance of AmpFire assays for the detection and genotyping of
398 HPV on 214 DNA samples extracted from FFPE from several localisations (including 17 samples
399 of oropharynx). The test was evaluated in comparison with the Cobas4800 system and Linear
400 Array assay. The invalid rate was 0.5% and the detection agreement was 100% for both,
401 detection and identification [18].
402 As mentioned above, the double positivity for HPV-DNA and $p16^{INK4A}$ can provide better
403 diagnostic accuracy and prognostic value than one test alone and can also allow a more
404 accurate patient classification [11,30]. In our study, 145 OPSCC samples with a valid HPV result
405 had previously been analysed for $p16^{INK4A}$ status by IHC. Nine samples tested by the Ampfire
406 Multiplex HR-HPV assay delivered discordant results with respect to $p16^{INK4A}$ expression (93.8%
407 concordance). Similarly, 8 samples were discordant when the HPV test used was Linear Array
408 (94.5% concordance). Within the oropharynx, HPV-DNA/$p16^{INK4A}$ discordance rates have been
409 reported to range from 4% to 20% [11,31–34]. In the present study, a 6.2% HPV-DNA/$p16^{INK4A}$
410 discordance was observed, which falls in the above mentioned range.
411 Earlier studies that used HR-HPV detection methods to test OPSCC specimens showed a similar
412 or slightly lower performance compared to the Ampfire Multiplex HR-HPV test. A very recent
413 study using a large set of OPSCC samples compared Anyplex II HPV28 with $p16^{INK4A}$-IHC
414 expression [35]. The results released by Anyplex II HPV28 showed a Cohen's Kappa index of 0.76
415 versus the 0.85 assessed in our study. Another study that used Cepheid Xpert HPV assay in the
416 detection of HPV in 159 FFPE samples of OPSCC origin and compared it to $p16^{INK4A}$ IHC,
417 exposed a 0.87 Cohen's Kappa coefficient similar to the Ampfire Multiplex HR-HPV test
418 (Cohen's Kappa=0.85, 95% CI 0.751-0.945). Guerendiain and colleagues also tested Cepheid

Xpert HPV assay on 50 OPSCC samples finding a 90% agreement with $p16^{INK4A}$ immunostaining [24], a concordance slightly lower than the 93.8% displayed by the Ampfire test in the present study.

The Roche Linear Array HPV Genotyping test is a robust method for determining HPV genotype in the routine analysis of OPSCC in FFPE [36,37]. Moreover, this technique is effective in detecting 37 HPV genotypes (including all high-risk types) with high precision [36] in both, preserved cytology and FFPE samples. Unfortunately, the assay is costly and low throughput, and requires a long time of technical handling. Additionally, the amplicons detected by the Ampfire assay are smaller (100 bp E1 and/or L1) than the ones detected by Linear Array (450 bp) which would normally represent an increase of sensitivity due to a better amplification efficiency [38].

From a biological point of view the detection of HPV E6/E7 mRNA constitutes the current gold standard to identify transcriptionally-active HR-HPV and thus an etiological role of HPV in OPSCC. It has shown superior patient survival stratification, slightly better than $p16^{INK4A}$ immunohistochemistry alone and better than DNA in situ hybridization and DNA PCR [39]. Nevertheless, expression of $p16^{INK4A}$ as detected by IHC is a widely available surrogate biomarker that has very good agreement with HPV status as determined by HPV E6/E7 mRNA expression (National Comprehensive Cancer Network Guidelines for Head and Neck Cancers, Version 3.2021, https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1437, last accessed date July 10, 2021). Both high sensitivity and specificity have been demonstrated when $p16^{INK4A}$ IHC is combined with HPV DNA detection by PCR [30]. Moreover, HPV E6/E7 mRNA detection from FFPE samples is still difficult to implement in certain settings.

Ampfire assays, instead, rely on real-time isothermal PCR, which is an easier and faster procedure as compared with the Linear Array technique which appears more tedious and involves specific material for hybridisation of the strip following PCR amplification. Using the Ampfire assay as the initial screen could significantly decrease the turnaround time, labor and costs associated with our current testing strategy.

The data shown in this manuscript demonstrate that the commercially available Ampfire Multiplex HR-HPV and Genotyping HR-HPV tests show a similar performance to Linear Array on the detection and genotyping of HPV on FFPE OPSCC samples. In conclusion, the Ampfire tests appear to be a trustable HPV detection method that could be used in clinical laboratories with good performance in routine OPSCC FFPE samples, thus meeting the latest recommendations for direct HPV testing in OPSCC.

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AUTHOR CONTRIBUTIONS

SP and MAP contributed to the investigation and writing of the manuscript. MAP, FXB and LA contributed to the conceptualization of the work. BQ contributed to the statistical analysis.
MT was involved in patient recruitment and clinical data collection. MM contributed to epidemiological analysis and MG in histopathological examination and $p16^{INK4A}$ IHC.
MAP and SP are the guarantors of this work and, as such, have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Atila did not play any role in the study design, conduction, data analysis; in the writing of the report; and in the decision of submitting the article for publication.

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Table 1. HPV-DNA positivity percentage agreement between Linear Array and Ampfire Multiplex HR-HPV tests in total HNSCC, OPSCC and non-OPSCC

HNSCC		**Ampfire Multiplex HR-HPV Test**			
		Positive	**Negative**	**Total**	98.9 % concordance
Linear Array	**Positive**	47 (26.1%)	2 (1.1%)	49 (27.2%)	Cohen's Kappa 0.972
	Negative	0 (0.0%)	131 (72.8%)	131 (72.8%)	(95% CI Kappa 0.932-1.000)
	Total	47 (26.1%)	133 (73.9%)	180 (100%)	p-value <0.001
OPSCC		**Ampfire Multiplex HR-HPV Test**			
		Positive	**Negative**	**Total**	99.4% concordance
Linear Array	**Positive**	44 (28.0%)	1 (0.6%)	45 (28.7%)	Cohen's Kappa 0.984
	Negative	0 (0.0%)	112 (71.3%)	112 (71.3%)	(95% CI Kappa 0.953-1.000)
	Total	44 (28.0%)	113 (72.0%)	157 (100%)	p-value <0.001
Non-OPSCC		**Ampfire Multiplex HR-HPV Test**			
		Positive	**Negative**	**Total**	95.7 % concordance
Linear Array	**Positive**	3 (13.0%)	1 (4.3%)	4 (17.4%)	Cohen's Kappa 0.832
	Negative	0 (0.0%)	19 (82.6%)	19 (82.6%)	(95% CI Kappa 0.489-1.000)
	Total	3 (13.0%)	20 (87.0%)	23 (100%)	p-value <0.001

667 **Table 2. HPV genotyping and detection agreement, in single and multiple infections,**
668 **between Linear Array and Ampfire Genotyping HR-HPV Real Time Fluorescent Detection**
669 **tests**

	HPV-Positive samples, n	
HPV-type	**Linear Array (HR-HPV)**	**Ampfire Multiplex HR-HPV Assay (HPV16, HPV18 & Other HR-HPV)**
HPV16	39	39
HPV18	1	1
Other HR-HPV non-HPV16/18	10	7

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Table 3. HPV-types agreement and discrepancies, between Linear Array and Ampfire Genotyping HR-HPV Real Time Fluorescent Detection Assay

	HPV Genotypes in HPV-Positive Cases, n	
HPV-type	**Linear Array (HR-HPV)**	**Ampfire Genotyping HR-HPV Assay (HR-HPV non-HPV16/18)**
HPV16	1	1
HPV45	2	1
HPV33	6	5
HPV35	1	1
HPV52	6	0

The number of HPV genotypes exceeds that of HPV positive cases because of multiple infections (16 and 52, 33 and 52 and 35 and 52)

Table 4. Concordance between Linear Array versus p16^{INK4A}-IHC and Ampfire Multiplex HR-HPV DNA versus p16^{INK4A}-IHC in OPSCC

		P16-IHC			
		Positive	**Negative**	**Total**	94.5%concordance
Linear Array	**Positive**	38 (26.2%)	4 (2.8%)	42 (29.0%)	Cohen's Kappa 0.866*
	Negative	4 (2.8%)	99 (68.3%)	103 (71.0%)	(95% CI Kappa 0.775-0.957)
	Total	42 (29.0%)	103 (71.0%)	145 (100%)	p-value <0.001
		Positive	**Negative**	**Total**	93.8 % concordance
Ampfire HR-HPV	**Positive**	37 (25.5%)	4 (2.8%)	41 (28.3%)	Cohen's Kappa 0.848*
Multiplex Test	**Negative**	5 (3.4%)	99 (68.3%)	104 (71.7%)	(95% CI Kappa 0.751-0.945)
	Total	42 (29.0%)	103 (71.0%)	145 (100%)	p-value <0.001

** The p-value between Cohen's Kappa coefficient of the dual testing Linear Array/ p16^{INK4A}-IHC versus Ampfire HR-HPV Multiplex/ p16^{INK4A}-IHC is 0.791 (not significant)*