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ORIGINAL ARTICLE

HPV testing versus p16 immunohistochemistry in oropharyngeal squamous cell carcinoma: results from the DAHANCA 19 study

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ABSTRACT

Introduction: The prognosis after primary (chemo-)radiotherapy for oropharyngeal squamous cell carcinoma (OPSCC) is affected by Human Papillomavirus (HPV) status, with a better prognosis in HPV-positive OPSCC. HPV-status is routinely assessed by p16 immunohistochemistry (IHC), but additional HPV DNA testing is debated. Also, there are numerous HPV genotypes, which prognostic role may need clarification. The purpose of this study was: (1) to test a custom-made targeted HPV next generation sequencing (NGS) panel in OPSCC, (2) to determine correlation with p16 IHC, and (3) to assess the impact of HPV DNA testing on outcome in the prospectively randomized clinical trial DAHANCA 19.
Materials and methods: We included 271 patients with OPSCC treated with primary (chemo-)radiotherapy in the DAHANCA 19 trial. Of these, 199 (73%) were p16-positive. HPV-status was determined by targeted HPV next generation sequencing (NGS), using a custom-made HPV genotyping panel.
Results: HPV was detected in 194 tumor samples. p16 IHC and NGS HPV status were concordant in 265 (98%) of 271 patients, whereas we did not detect HPV DNA in 5 p16-positive tumors. HPV16 accounted for 169 of 194 HPV-positive cases (87%). HPV genotypes 18, 31, 33, 35, and 59 were also detected.
Results: Loco-regional failure and overall survival were similar whether patients were separated by p16 IHC, or HPV DNA status ($p < 0.0001$ for all) and did not depend on HPV genotype ($p = 0.9$ and $p = 0.7$).
Conclusion: In the present study, HPV DNA testing or typing in a Danish OPSCC cohort did not add additional information to p16 IHC, the most widely used and accepted prognostic indicator.

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KEYWORDS
Head and neck cancer; radiotherapy; HPV; NGS; p16

Background

Treatment response and prognosis after primary (chemo-)radiotherapy for oropharyngeal squamous cell carcinoma (OPSCC) is affected by Human Papillomavirus (HPV) status, with a markedly better prognosis in the more radiosensitive HPV-positive OPSCC [1–4]. The most common method for determining HPV-status in OPSCC is by p16 immunohistochemistry (IHC), which is widely used and affordable, and recommended in UICC TNM 8th edition as well as in the Danish Head and Neck Cancer Group (DAHANCA) guidelines [5]. HPV may also be detected by other methods, such as *in situ* hybridization, PCR or DNA sequencing. Today, the increasing availability of targeted DNA sequencing makes this potentially feasible in a clinical setting, but it is not clear if this provides additional clinical benefit that outweigh expenses.

In addition, there are numerous carcinogenic HPV genotypes recognized by IARC, and it is still debated if the prognosis varies between subtypes [6].

The purpose of this study was: (1) to test a custom-made targeted HPV next generation sequencing (NGS) panel in OPSCC; (2) to determine correlation with p16 IHC; and (3) to assess the impact of HPV DNA testing on outcome in the prospectively randomized clinical trial DAHANCA 19 [7].

Material and methods

Patients and treatment

We included Danish patients with OPSCC treated in the DAHANCA 19 trial (ClinicalTrials.gov identifier: NCT00496652; regional ethics committee approval no. 20070091) [7]. This randomized multicenter trial included in the period 2008–2012 a total of 608 patients with squamous cell carcinoma of the pharynx, larynx, and oral cavity eligible for curatively intended (chemo-) radiotherapy, who were randomized to receive the monoclonal EGFR-antibody zalutumumab or placebo.

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Table 1. Patient, tumor and treatment characteristics.

	All ($n = 271$)	p16-positive ($n = 199$)	p16-negative ($n = 72$)
Age	58 (53–63)	57 (52–63)	60 (55–64)
Years, median (IQR)			
Gender			
Male	222 (82%)	167 (84%)	55 (76%)
Female	49 (18%)	32 (16%)	17 (24%)
Tobacco smoking			
Never	49 (18%)	48 (24%)	1 (1%)
Former	139 (51%)	112 (56%)	27 (38%)
Current	83 (31%)	39 (20%)	44 (6 1%)
Pack-years Median (IQR)	26 (7–43)	19 (1–39)	41 (31–52)
≥30 pack-years	128 (47%)	71 (36%)	57 (79%)
Tumor subsite			
Tonsil	168 (62%)	128 (64%)	40 (56%)
Base of tongue	91 (34%)	70 (35%)	21 (29%)
Other site, oropharynx	12 (4%)	1 (<1%)	11 (15%)
T classification			
T1-2	187 (69%)	152 (76%)	35 (49%)
T3-4	79 (29%)	44 (22%)	35 (49%)
Missing	5 (2%)	3 (2%)	2 (3%)
N classification			
N0	40 (15%)	19 (10%)	21 (29%)
N+	231 (85%)	180 (90%)	51 (71%)
Stage*			
I–II	25 (9%)	13 (7%)	12 (17%)
III–IV	246 (91%)	186 (93%)	60 (83%)
Treatment			
≥ 66 Gy	270 (99%)	198 (99%)	72 (100%)
Cisplatin weekly	216 (80%)	169 (85%)	47 (65%)
Nimorazole	264 (97%)	197 (99%)	67 (93%)
HPV DNA			
Positive	194 (72%)	194 (97%)	0
Negative	77 (28%)	5 (3%)	72 (100%)

*Staging according to TNM6.

In the DAHANCA 19 trial, patients received curatively intended radiotherapy according to the DAHANCA guidelines [8]. The prescribed dose was 66–68 Gy in 33–34 fractions, 6 fractions per week, with the hypoxic sensitizer nimorazole. Concomitant weekly low-dose cisplatin was indicated in patients with stage 3–4 disease. A hyperfractionated schedule of 76 Gy in 56 fractions, 10 fractions per week without cisplatin, was allowed in T2-4N0 patients. Staging was according to TNM6, as per the original protocol. Patients were followed to death or for a minimum of five years following completion of treatment.

At 5 years, there was no effect of zalutumumab on loco-regional control or survival [7]. For the present study, we analyzed patients with OPSCC only; with available tissue for DNA extraction; tumor cell fraction >5%; and subsequent successful HPV DNA sequencing.

HPV DNA sequencing and p16 immunohistochemistry

HPV DNA sequencing was performed on the Ion Torrent platform, using a custom-designed HPV-specific primer panel covering all 25 carcinogenic, probably carcinogenic or possibly carcinogenic HPV-genotypes [6, 9]. Following DNA extraction from formalin-fixed, paraffin-embedded tissue blocks, HPV DNA sequencing and analysis were performed as described elsewhere in detail [9].

Immunohistochemistry for p16 was performed at the including centers according to DAHANCA and international guidelines, defining p16 positivity as strong nuclear and cytoplasmic staining in 70% or more of tumor cells, with a basaloid morphology [5, 10]. Eight cases with questionable p16 status were reviewed centrally.

Statistical analysis

Loco-regional failure and overall survival were primary outcomes. Loco-regional failure was defined as persistence or recurrence at T- or N-site, and cumulative–incidence curves were estimated by Aalen-Johansen method and corrected for competing risks (distant failure and death). Overall survival was defined as time to death of any cause, estimated by Kaplan-Meier method, and compared using log-rank test. Prognostic effect was estimated using Cox regression. Statistical analysis was performed with STATA 14.2.

Results

Patient, tumor, and treatment characteristics

A total of 301 patients with OPSCC with FFPE tissue were available for analysis, of which 16 were excluded due to low tumor fraction in the specimen, while NGS analysis failed in 14 patients. Thus, a total of 271 patients with p16 status and HPV DNA sequencing data were included. Most patients were male with a history of tobacco smoking. Almost all had cancer of the tonsils or base of tongue, and 85% had node-positive disease (Table 1).

Patients with p16-negative disease were more often smokers, and 79% had a history of 30 or more pack years vs.

Table 2. Clinical characteristics in HPV DNA positive patients according to HPV genotype ($n = 194$).

	HPV-16 ($n = 169$)	Other HPV genotypes (HPV-18/31/33/35/59) ($n = 25$)
Age		
Years, median (IQR)	57 (52–61)	63 (56–70)
Gender		
Male	141 (83%)	22 (88%)
Tobacco smoking		
Never	45 (27%)	3 (12%)
Former	95 (56%)	16 (64%)
Current	29 (17%)	6 (24%)
Pack-years median (IQR)	19 (0–36)	20 (12–41)
≥30 pack-years	58 (34%)	10 (40%)
Tumor subsite		
Tonsil	111 (66%)	13 (52%)
Base of tongue	57 (34%)	12 (48%)
Other site, oropharynx	1 (1%)	0
T classification		
T1-2	128 (76%)	20 (80%)
T3-4	38 (22%)	5 (20%)
Missing	3 (2%)	0
N classification		
N0	15 (9%)	4 (16%)
N+	154 (91%)	21 (84%)
Stage*		
I–II	10 (6%)	3 (12%)
III–IV	159 (94%)	22 (88%)
HPV genotype		169 (87%)
16		13 (7%)
33		7 (4%)
35		2 (1%)
18		1 (1%)
31		1 (1%)
33 + 35		1 (1%)
59		

*Staging according to TNM6.

36% of patients with p16-positive tumors. Also, patients with p16-negative disease presented with higher T-category and were more often N0.

HPV DNA and p16

A total of 199 patients had p16-positive tumor. Of these, 194 patients had detectable HPV DNA in tumor. No HPV DNA was detected in p16-negative tumors. Overall concordance was 98% (266 of 271). There were five discordant cases which were p16-positive and HPV-negative: 3 of 5 had 30 or more pack-years of smoking, and all had cancer of the tonsils or base of tongue.

HPV-16 was the most frequent genotype, detected in 169 of the 194 HPV DNA positive samples. HPV-18, 31, 33, 35, and 59 were also detected; all are classified as carcinogenic according to IARC [6]. Clinical characteristics in patients with HPV-16 positive genotype vs. other genotypes are shown in Table 2.

Loco-regional failure and survival

Median follow-up time was 5.5 years, during which 101 deaths were recorded.

Loco-regional failure occurred in 65 patients during follow-up, corresponding to a cumulative incidence of 22% at 5 years (95% confidence interval [CI]: 17–27%). p16 and HPV DNA were equally prognostic with HRs of 0.25 and 0.22, respectively ($p < 0.001$ for both).

Overall survival was 74% at 5 years (CI: 68–79%). Both p16 and HPV DNA were also equally prognostic, with a hazard ratio (HR) of 0.25 and 0.22, respectively ($p < 0.001$ for both).

In the 194 patients with HPV DNA positive disease, we saw no evidence of a different prognostic effect of HPV genotype. Patients with HPV genotype 16 had a similar prognosis compared to patients with other HPV genotypes, both regarding overall survival (HR: 1.1 [CI:0.5–2.5]) and loco-regional failure (HR: 0.96 [CI: 0.33–2.8]) (Figure 1).

Discussion

In this cohort of OPSCC patients treated in a large clinical trial with uniform treatment and follow-up, p16 immunohistochemistry and HPV DNA were equally prognostic. We found no difference in prognosis between the investigated HPV genotypes, although the absolute number of patients and events was low. The HPV NGS panel performed well, and may be a valuable tool for research, as it is easily integrated into other NGS panels.

The strengths of this study include the uniform treatment and follow-up of patients. Limitations include the relatively low number of events for patients with p16-positive disease. Although patients were recruited more than ten years ago, the treatment strategy is still current.

Mehanna et al. recently published a large individual patient-data meta-analysis, in which discordance between p16 and HPV DNA/RNA was found in approximately 10% of patients, and had a prognostic significance [11]. This study included retrospective cohorts treated and followed in various ways, and results were more prominent in geographical

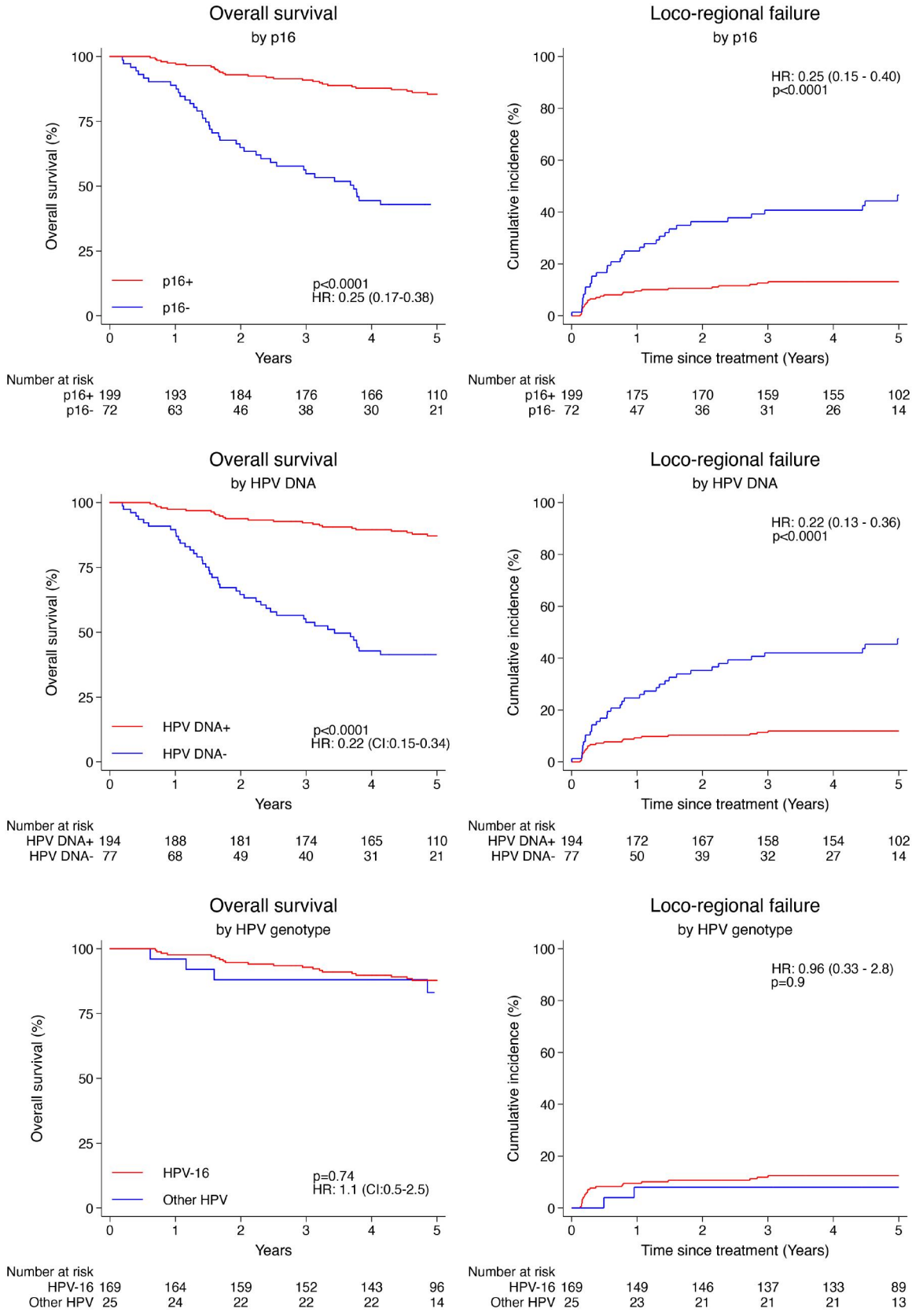

Figure 1. Overall survival and loco-regional failure according to p16 (top row), HPV DNA (Middle row) and HPV genotype (bottom row, HPV DNA positive patients only, $n = 194$). HR: Hazard ratio. CI: 95% confidence interval.

areas with low HPV-attributable fractions. In our cohort, which is from an area with a high HPV-attributable fraction, the concordance between the two methods was high. A previous study from Denmark which was included in the meta-analysis, found somewhat higher discordance with prognostic significance [12].

It should be noted, that both p16 IHC and HPV DNA testing or sequencing should be performed and interpreted with care.

For p16 IHC, it is essential that the pathologist assesses not only presence or absence of p16 staining, but also cellular morphology and the intensity and pattern of staining [5, 10]. The vast majority of the including laboratories used the clone E6H4 for staining of p16. Other clones used were JC2 and MX007. All three monoclonal antibodies are recommended as markers for p16ink4a. According to the Nordic immunohistochemical Quality Control (NordiQC), the majority of suboptimal staining results for p16 are related to weak or false negative staining reaction, and not to overstaining or false positive results. HPV NGS equally has pitfalls and limitations, as seen in the number of failed analyses, as well as high cost and time consumption.

Curatively intended radiotherapy with concomitant cisplatin is established as standard treatment for the majority of patients with OPSCC [13, 14]. This strategy is supported by two randomized, clinical trials of de-escalation, which both showed inferior survival in the de-escalated groups [15, 16]. Since treatment decisions in OPSCC are currently not affected by p16/HPV-status outside clinical trials, it is performed for prognostic staging only. For these reasons, the added cost of routine HPV DNA/RNA testing does not seem warranted, especially not in geographical areas with high HPV-attributable fraction.

Thus, we conclude, that in comparison with p16 immunohistochemistry, no significant additional advantage of routine HPV DNA testing was found. Further investigations may elucidate the need for routine HPV DNA testing in future treatment guidelines.

Disclosure statement

The authors report no conflicts of interest.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, JKL-F, upon reasonable request.

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