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Human papillomavirus (HPV) load is higher in HPVDNA/p16 positive than in HPVDNA positive/p16 negative oropharyngeal squamous cell carcinoma but does not differ significantly between various subsites or correlate to survival

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ABSTRACT

Objective: Patients with human papillomavirus DNA positive (HPVDNA$^+$) and p16^{ink4a} overexpressing (p16$^+$) oropharyngeal squamous cell carcinoma (OPSCC), especially those with cancer in the tonsillar and base of tongue subsites as compared to other OPSCC subsites have a better outcome than those with only HPVDNA$^+$ or only p16$^+$ cancer. Likewise having a high viral load has been suggested to be a positive prognostic factor. We therefore hypothesized, that HPV viral load could vary depending on OPSCC subsite, as well as with regard to whether the cancer was HPVDNA$^+$ and p16$^+$, or only HPVDNA$^+$, or only p16$^+$ and that this affected outcome.
Material and methods: To address these issues HPV viral load was determined by HPV digital droplet (dd) PCR in tumor biopsies with previously known HPVDNA/p16 status from 270 OPSCC patients diagnosed 2000–2016 in Stockholm, Sweden. More specifically, of these patients 235 had HPVDNA$^+$/p16$^+$, 10 had HPVDNA$^+$/p16$^-$, 13 had HPVDNA$^-$/p16$^+$ and 12 had HPVDNA$^-$/p16$^-$ cancer.
Results: We found that HPVDNA$^+$/p16$^+$ OPSCC had a significantly higher viral load than HPVDNA$^+$/p16$^-$ OPSCC. Moreover, there was a tendency for a higher viral load in the tonsillar and base of tongue OPSCC subsites compared to the other subsites and for a low viral load to correlate to a better clinical outcome but none of these tendencies reached statistical significance.
Conclusion: To conclude, the mean viral load in HPVDNA$^+$/p16$^+$ OPSCC was higher than in HPVDNA$^+$/p16$^-$ OPSCC, but there was no statistically significant difference in viral load depending on OPSCC subsite or on clinical outcome.

Introduction

In 2007, human papillomavirus (HPV) was acknowledged as a risk factor, besides smoking and alcohol, for oropharyngeal- and tonsillar squamous cell carcinoma (OPSCC and TSCC) by the International Agency for Research against Cancer (IARC) [1–4]. Since then, a considerable rise in the incidence of HPV-positive (HPV$^+$) OPSCC, mainly TSCC and base of tongue cancer (BOTSCC) has been noted while this is not the case for other OPSCC subsites (otherOPSCC) e.g. the soft palate the uvula and the pharyngeal walls [5–18]. Moreover, HPV$^+$

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OPSCC and especially HPV$^+$ TSCC/BOTSCC have a better prognosis than corresponding HPV-negative (HPV$^-$) cancer with roughly 80 % vs. 50 % 5 - year disease free survival, [1–4,8,16,18,19]. The reason for this discrepancy has not been resolved entirely but there are many molecular differences between HPV$^+$ and HPV$^-$ OPSCC/TSCC/BOTSCC [1–4,16,19]. Furthermore, in the present era of personalized cancer therapy, many studies have been done to identify clinical or molecular factors that differ between HPV$^+$ and HPV$^-$ cancer as well as between HPV$^+$ OPSCC/TSCC/BOTSCC with better or worse prognosis in order to better individualize therapy [19–33].

Some studies have examined whether the physical status of HPV, as episomal, integrated, or both, or whether viral load in OPSCC/TSCC/BOTSCC affect clinical outcome but the obtained data has varied and has not always been in concordance [34–40]. Other reports focused on whether survival in HPV$^+$ OPSCC differed depending on subsite, since the presence of HPV and its prognostic impact was mainly observed in TSCC and BOTSCC and not in otherOPSCC [41–45]. Upon doing so, it was shown to be important to clarify how an HPV$^+$ status was defined [43–45]. According to the current 8th Edition staging system by American Joint Committee on Cancer (AJCC), having p16^{INK4a} overexpression (p16$^+$) is accepted as a surrogate marker for HPV$^+$ status in OPSCC, but when conducting clinical trials, the latter is not always optimal [46]. Notably, the golden standard for HPV$^+$ status and an active oncogenic HPV infection is the expression of the mRNA of the oncogenes HPV E6 and E7 [43–45]. However, since determination of HPV E6 and E7 mRNA is more challenging to assay for, overexpression of p16^{INK4a} detected in $>$ 70 % of the tumor cells by immunohistochemistry (IHC) has been suggested as a surrogate marker for HPV$^+$ status in OPSCC [43–45] and has also been accepted as such in the AJCC 8th Edition staging system. Of note, however, HPV E6 and E7 mRNA expression and p16$^+$ are not always concordant, since roughly 10–15 % of p16$^+$ OPSCC/TSCC/BOTSCC are HPV DNA negative (HPVDNA$^-$), and at otherOPSCC subsites and other HNSCC this discordance is even greater [43–46].

Nonetheless, in smaller studies it has been shown especially in TSCC and BOTSCC, as well as in otherOPSCC that the parallel presence of both HPVDNA$^+$ and p16$^+$ is almost equivalent to the presence of the golden standard of HPV E6 and/or E7 mRNA expression [43–45]. Furthermore, in a large multinational, multicenter study it was shown that patients with both HPVDNA$^+$ and p16$^+$ OPSCC have a better clinical outcome than those with only HPVDNA$^+$ or only p16$^+$ OPSCC [46]. In fact, there are reports that suggest that while p16$^+$ is a positive prognostic marker for TSCC and BOTSCC, this is not the case for other OPSCC sites [41,42]. This could partly be due to that fact that there is a higher concordance in HPVDNA$^+$ and p16$^+$ in TSCC/BOTSCC as compared to otherOPSCC [41,42]. None of the latter is completely surprising, since the anatomy of the tonsil and the base of tongue as part of the Waldeyer's ring, with lymphoepithelial tissue, differs from that in other OPSCC subsites [41]. Due to these anatomical differences, we therefore hypothesized that HPV viral load could vary depending on OPSCC subsite, as well as with regard to whether the cancer was defined as being both HPVDNA$^+$ and p16$^+$, or only HPVDNA$^+$, or only p16$^+$ and that this could have a clinical impact.

To address the above issues, we attempted to investigate whether HPV viral load: i) differed between tumors that were both HPVDNA$^+$/p16$^+$, only HPVDNA$^+$, or only p16$^+$; ii) differed between OPSCC subsites; and finally iii) was correlated to clinical outcome.

Materials and methods

Patients' characteristics

Patients diagnosed with OPSCC, (TSCC: ICD-10 C09.0–9 and C02.4; BOTSCC C01.9; otherOPSCC: C10.0–9, C05.1–9) 2000–2016, in the County of Stockholm/Gotland, Sweden were identified through the Swedish Cancer Registry. Only patients with tumors assessed for HPV16 DNA and p16 status [13–15] and with extracted DNA available for assaying viral load were eligible for inclusion and for HPVDNA$^+$ cases only HPV type 16 (HPV16) positive tumors were included. Consequently, the study contained 270 OPSCC patients. Their OPSCC subsite as well as the initial HPV16 DNA$^+$/HPVDNA$^-$ and p16$^+$/p16$^-$ status of all 270 tumors that were included in the analysis for viral load are depicted in Table 1.

In the survival analysis, the seven patients treated with palliative intent were excluded. Subsequentially, in Table 2, the 263 patients treated with curative intent with e.g. surgery, radiotherapy or chemoradiotherapy are included and the table presents data on e.g. age, gender, TNM7 and TNM8 stages, obtained from the patients' charts.

The study was conducted according to ethical permissions 1999–237, 2005/431–31/4 and 2005/1330–32, 2009/1278–31/4, 2018/870–32 from the Karolinska Institutet and the Stockholm Regional Ethical Review Board.

HPV-DNA and overexpression of p16

Data on HPV DNA and p16 status were attained from earlier studies done by us, or from patients' charts, where the analysis was performed at the Department of Clinical Pathology at the Karolinska University Hospital [13–15]. More specifically, DNA was extracted from pre-treatment formalin fixed paraffin embedded (FFPE) biopsies and examined for HPV DNA by PCR using broad-spectrum general primers bsGP5+/6 + and subsequent HPV-typing was done using a bead based multiplex assay evaluated on a MagPix instrument (Luminex Corp.). For details see [13–15]. Presence of p16^{Ink4a} overexpression (p16$^+$) or not (p16$^-$) was assessed by IHC and obtained from earlier studies or from patients' charts and p16$^+$ was defined as a strong nuclear and cytoplasmatic staining in $>$ 70 % of tumor cells, as suggested by the College of American Pathologists [13–15].

Hpv-digital droplet (dd) PCR and viral load analysis

The ddPCR assay developed for the E7 gene of HPV 16, 18 and 45 and has been described in detail in a recent report [47]. Here only HPV16 was examined and the assays were individually multiplexed with an assay for cell-free albumin DNA (cfALB) as an internal control for amplifiable cell-free DNA (cfDNA) in each sample and as a surrogate marker for normal cfDNA load. HPV copies were assessed in correlation

Table 1
Patient tumors, including subsite location and human papillomavirus type 16 DNA and p16^{ink4a} overexpression status.

OPSCC[1] subsite	HPV16 DNA$^+$[2] /p16$^+$[5]	HPV16 DNA$^+$ /p16$^-$[6]	HPV16 DNA$^-$/ p16$^+$[7]	HPV16 DNA$^-$ /p16$^-$[8]	Total
TSCC[2]	149 (83.2 %)	8 (4.5 %)	13 (7.3 %)	9 (5.0 %)	179 (100 %)
BOTSCC[3]	81 (95.3 %)	2 (2.4 %)	0 (0.0 %)	2 (2.4 %)	85 (100 %)
otherOPSCC[4]	5 (83.3 %)	0 (0.0 %)	0 (0.0 %)	1 (16.7 %)	6 (100 %)
Total OPSCC[1]	235 (87.0 %)	10 (3.7 %)	13 (4.8 %)	12 (4.4 %)	270 (100 %)

[1] OPSCC: oropharyngeal squamous cell carcinoma.
[2] TSCC: tonsillar squamous cell carcinoma.
[3] BOTSCC: base of tongue squamous cell carcinoma.
[4] otherOPSCC: nonTSCC/BOTSCC OPSCC.
[5] Human papillomavirus 16 DNA positive and p^{16ink4a} overexpressing.
[6] Human papillomavirus 16 DNA positive and p16^{ink4a} non-overexpressing.
[7] Human papillomavirus DNA negative and p^{16ink4a} overexpressing.
[8] Human papillomavirus DNA negative and p^{16ink4a} non-overexpressing.

Table 2
Patient and tumor characteristics of patients treated with curative intent.

OPSCC[1] subsite	TSCC[2] N = 175	BOTSCC[3] N = 82	Other OPSCC[4] N = 6	Total N = 263
Age range (mean)	38–92 (59.1)	43–82 (61.7)	51–75 (67.7)	38–92 (60.1)
Gender	46	16	1	63
Female	(26.3 %)	(19.5 %)66	(16.7 %)5	(24 %)200
Male	129 (73.7 %)	(80.5 %)	(83.3 %)	(76 %)
Tumor size[5]	46	31	2	79
1	(26.3 %)75	(37.8 %)27	(33.3 %)3	(30.0 %)
2	(42.9 %)32	(32.9 %)10	(50 %)0	105
3	(18.3 %)16	(12.2 %)13	(0 %)0	(40.0 %)42
4a	(9.1 %)6	(15.9 %)1	(0 %)1	(16.0 %)29
4b	(3.4 %)	(1.2 %)	(16.7 %)	(11.0 %)8
				(3.0 %)
Nodal disease[5]	25	10	4	39
0	(14.3 %)19	(12.2 %)8	(66.7 %)1	(14.8 %)28
1	(10.9 %)13	(9.8 %)11	(16.7 %)1	(10.6 %)25
2a	(7.4 %)104	(13.4 %)38	(16.7 %)0	(9.5 %)142
2b	(59.4 %)13	(46.3 %)15	(0.0 %)0	(54.0 %)28
2c	(7.4 %)1	(18.3 %)0	(0.0 %)0	(10.6 %)1
3	(0.6 %)	(0.0 %)	(0.0 %)	(0.4 %)
Distant metastasis[5]	170	82	6	258
0	(97.1 %)5	(100 %)0	(100 %)0	(98 %)5
1	(0.03 %)	(0.0 %)	(0.0 %)	(1.9 %)
Tumor Stage[5]				
AJCC7	8	0	1	9
1	(4.6 %)6	(0.0 %)6	(16.7 %)2	(3.4 %)14
2	(3.4 %)23	(7.3 %)7	(33.3 %)1	(5.3 %)31
3	(13.1 %)	(8.5 %)69	(16.7 %)2	(11.8 %)
4	138 (78.9 %)	(84.1 %)	(33.3 %)	209 (79.5 %)
AJCC8[6]		49	4	
1	104	(59.8 %)16	(66.7 %)0	157
2	(59.4 %)31	(19.5 %)13	(0.0 %)2	(59.7 %)47
3	(17.7 %)21	(15.9 %)4	(33.3 %)0	(17.9 %)36
4	(12.0 %)19	(4.9 %)	(0.0 %)	(13.7 %)23
	(10.9 %)			(8.7 %)

[1] OPSCC: Oropharyngeal squamous cell carcinoma.
[2] TSCC: Tonsillar squamous cell carcinoma.
[3] BOTSCC: Base of tongue squamous cell carcinoma.
[4] otherOPSCC: nonTSCC/BOTSCC OPSCC.
[5] According to American Joint Committee on Cancer (AJCC) 7th Edition Cancer Staging Manual.
[6] According to American Joint Committee on Cancer (AJCC) 8th Edition Cancer Staging Manual.

to copies of the albumin gene [47].

Statistical and survival analysis

Differences in continuous variables were assessed by an independent two-tailed *t*-test. Overall survival (OS) was classified as time from diagnosis until death irrespective of cause. Disease-free survival (DFS) was classified as time from diagnosis until disease recurrence, under the condition that the patient had completed treatment with curative intent and had been assessed as first having had a complete response at check-up and then having a recurrence confirmed by radiology and/or histopathology. Time-to recurrence was calculated as time from diagnosis until confirmed recurrence was noted in the patient chart. Patients who died from other causes were censored at the time of death. Survival was assessed with a Cox regression model or a log-rank test. Results were presented in Kaplan-Meier curves. All analyses were made in SPSS (version 25 for Mac). P-values of < 0.05 were considered significant.

Results

Assessement of HPV16 viral load in HPV16 DNA$^+$/p16$^+$, HPV16 DNA$^+$/p16$^-$, HPV DNA$^-$/p16$^+$ and HPV DNA$^-$/p16$^-$ status by HPV ddPCR

HPV viral load was assessed by ddPCR in all 270 samples with known HPVDNA/p16 status from previous studies [13–15]. The samples were divided into four groups according to previously obtained data where 235 were HPV16 DNA$^+$/p16$^+$; 10 were HPV16 DNA$^+$/p16$^-$; 13 were HPVDNA$^-$/p16$^+$; and 12 were HPVDNA$^-$/p16$^-$ (Table 1). None of the two latter initially HPV DNA$^-$ control groups had any HPV DNA$^+$ samples when assessed by HPV ddPCR and these two goups were therefore excluded from further analysis.

In the remaining groups 232/235 HPV16 DNA$^+$/p16$^+$ and 10/10 HPV16 DNA$^+$/p16$^-$ respectively were defined as having an HPV16 DNA positive viral load and when including all samples their viral load (HPV copies/cell) ranged from 0.00 to 424 and 0.06–17.6 respectively. The mean and viral load (HPV copies/cell) of the former was 17.4 and of the latter 2.5 respectively. Moreover, when comparing the mean viral load of the HPV16 DNA$^+$/p16$^+$ to that of the HPVDNA$^+$/p16$^-$ cases respectively the former group had a significantly higher mean viral load than the latter group i.e. 17.4 vs. 2.5 respectively, $p < 0.001$ (*t*-test).

To conclude, none of the previously defined HPVDNA negative samples were found HPV positive by HPV ddPCR and the HPVDNA$^+$/p16$^+$ group had a significantly higher mean viral load than the HPVDNA$^+$/p16$^-$ group.

Assessement of HPV16 viral load by HPV16 ddPCR according to OPSCC subsite in HPV16 DNA$^+$/p16$^+$ samples

Taking into account all 235 HPV16 DNA$^+$/p16$^+$ samples, the mean viral load (copies/cell) and virus copy ranges (copies/cell) are presented for the different subsites in Table 3. There were no major differences in mean viral load between TSCC and BOTSCC, 19.7 vs. 13.3, $p = 0.2$ (*t*-test). In addition, although the mean viral load of otherOPSCC tended to be lower as compared to that of TSCC and BOTSCC together (12.9 vs. 17.5), this difference was not statistically significant either $p = 0.8$ (*t*-test).

To conclude, although there was a trend for TSCC/BOTSCC to have a higher mean viral load than otherOPSCC this difference did not reach statistical significance.

Assessment of HPV viral load and clincial outcome in OPSCC according to HPV16 DNA and p16 status

Prior to assessing a possible correlation of viral load to clinical outcome we examined whether the average viral load correlated to tumor size and nodal spread as well as according to TNM stage according to both the 7th and 8th Edition of AJCC staging system. Assessing viral load this way showed a great variation throughout the various

Table 3
Viral load in oropharyngeal squamous cell carcinoma (OPSCC) cases that were human papillomavirus type 16 DNA positive and overexpressed p16^{Ink4a}.

OPSCC[1] subsite	Nr of cases	Mean viral load[5]	Range[5]
All	235	*17.4*	0.00 – 424
TSCC[2]/BOTSCC[3]	230	*17.5*	0.00 – 424
TSCC[2]	149	*19.7*	0.00 – 424
BOTSCC[3]	81	*13.3*	0.094 – 97.6
OtherOPSCC[4]	5	*12.9*	0.017 – 31.6

[1] OPSCC: oropharyngeal squamous cell carcinoma.
[2] TSCC: tonsillar squamous cell carcinoma.
[3] BOTSCC: base of tongue squamous cell carcinoma.
[4] otherOPSCC: nonTSCC/BOTSCC, OPSCC.
[5] HPV viral copies/cell.

categories with no specific correlations observed (data not shown).

We then focused on patients with HPV16 DNA$^+$ tumors by ddPCR only by excluding those treated with palliative care (n = 7), 235 patients remained for the survival analysis and the characteristics of the patients and their tumors are summarized in Table 2. Having a high viral load as compared to a having a lower viral load tended to correlate to a worse DFS (Cox regression: HR 1.004, 95 % CI: 1.000–1.009, p = 0.07) and to a worse OS (Cox regression: HR 1.003, 95 % CI: 0.998–1.008, p = 0.18) but these trends did not reach statistical significance.

Moreover, when dichotomizing the patients into two groups divided by the mean viral load value (17.1) as a cut-off, patients with tumors with a low viral load (>17.1) tended to have a better survival (Fig. 1A and B), but this difference was not statically significant either (log rank test: DFS: p = 0.19, and OS: p = 0.4).

Furthermore, when separating the survival analysis based on p16 status, patients with HPV16 DNA$^+$/p16$^+$ tumors (Fig. 2A and B) and a low viral load tended to have a better survival outcome, but this difference was again not statistically significant (log rank test: DFS: p = 0.27, OS: p = 0.6).

However, when rating patients with HPV16 DNA$^+$/p16$^-$ tumors, those with tumors with a low viral load had a significantly better outcome than those with a high viral load (log rank test: DFS: p = 0.003, OS: p = 0.003), but the data should be taken with caution since only 10 cases were included in the latter analysis (Supplementary Fig. 1).

Discussion

In this study, HPV16 DNA viral load was assessed by HPV16 DNA ddPCR in OPSCC according to whether the tumor samples had previously been defined to be HPV16 DNA$^+$/p16$^+$, HPV16 DNA$^+$/p16$^-$,

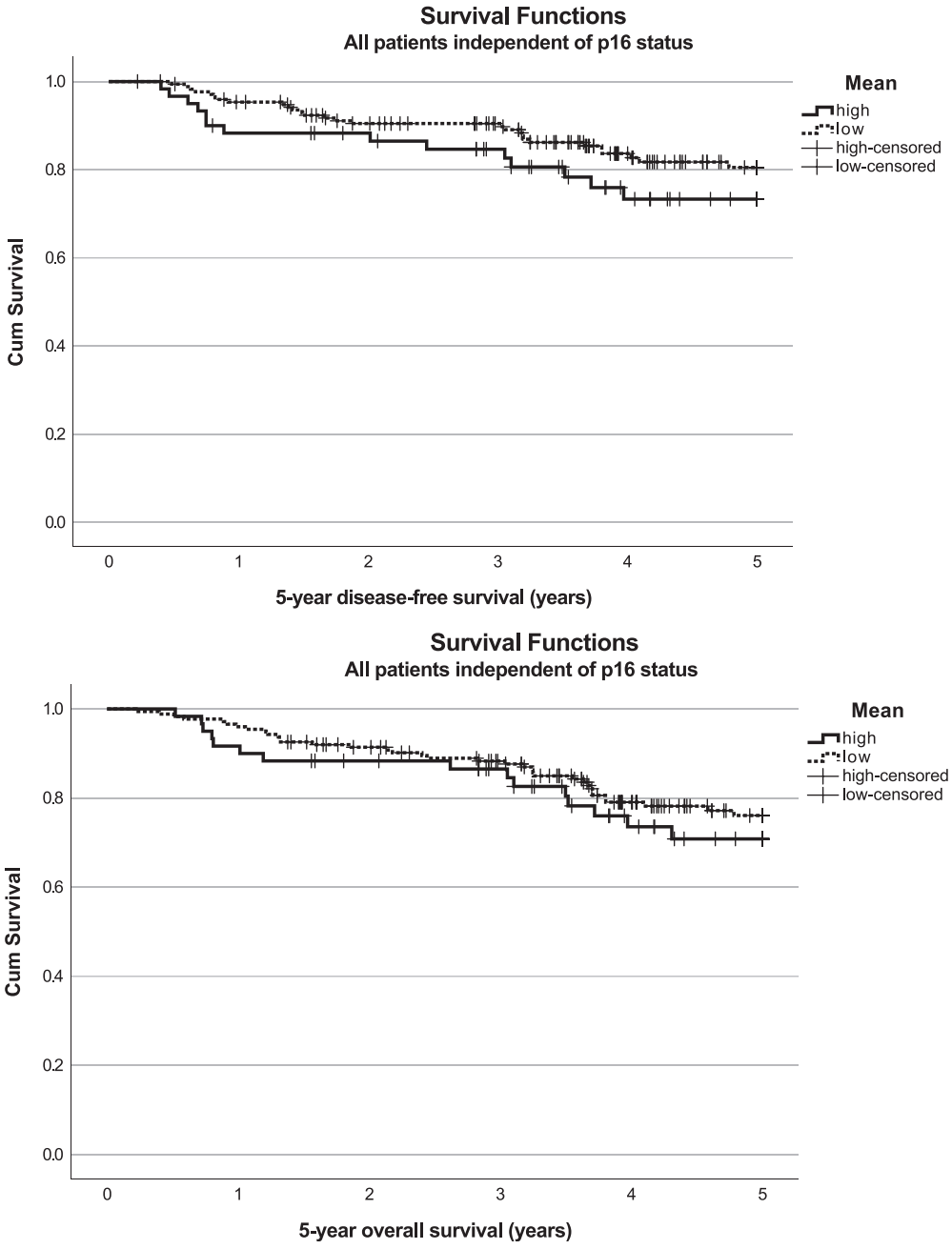

Fig. 1. Kaplan-Meier figures with (A) 5 - year disease free survival and (B) 5-year overall survival in patients with HPV16 DNA$^+$ cancer irrespective of p16 status dichotomizing the patients into two groups with the mean viral load value (17.1) as a cut-off value.

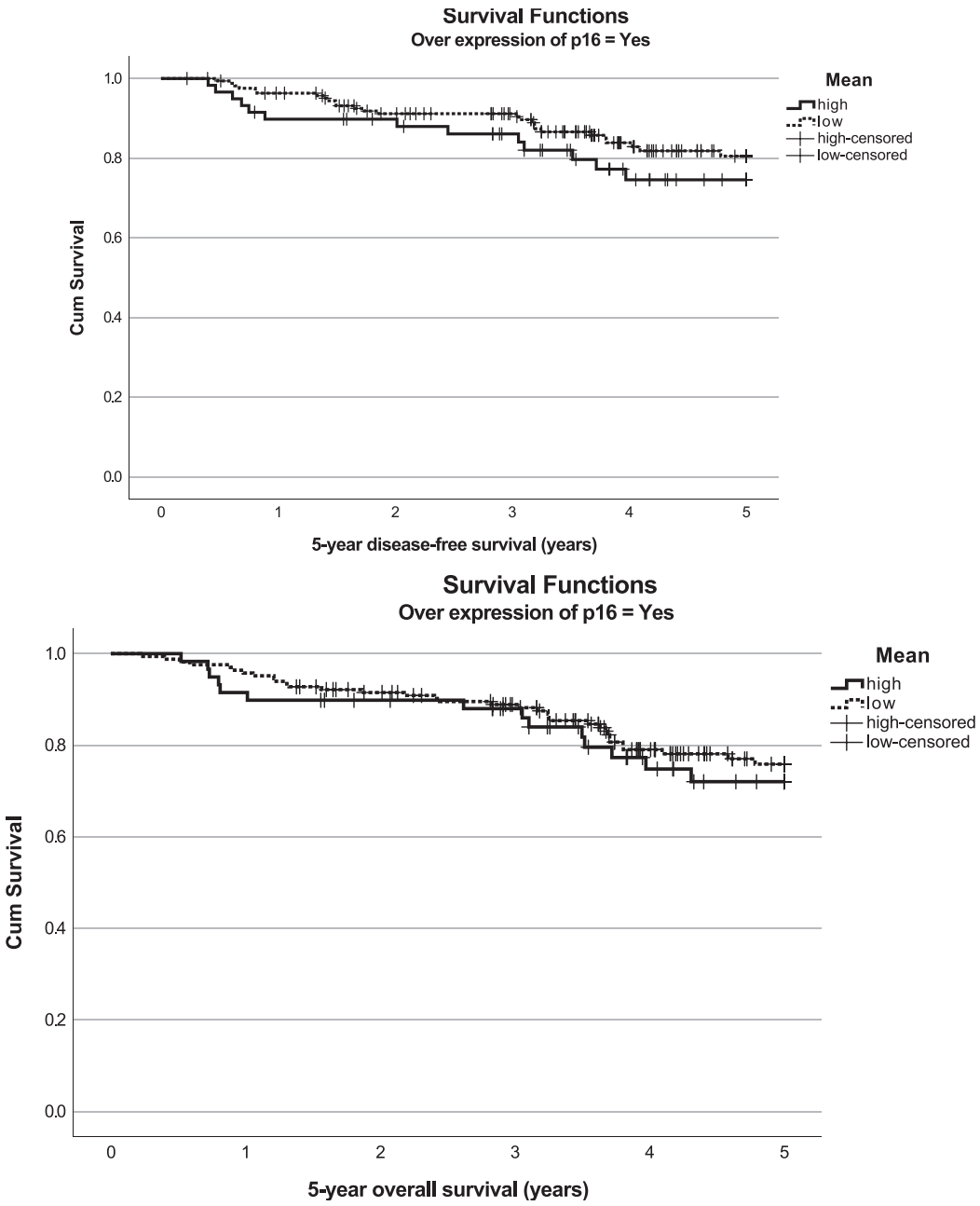

Fig. 2. Kaplan-Meier figures with (A) 5 - year disease free survival and (B) 5-year overall survival in patients with HPV16 DNA^+ /$p16^+$ status dichotomizing the patients into two groups with the mean viral load value (17.1) as a cut-off value.

HPV16 $DNA^-/p16^+$ or HPV16 $DNA^-/p16^-$ as well as in correlation to subsite and in correlation to clinical outcome.

HPV16 DNA mean viral load was found to be higher in HPV16 $DNA^+/p16^+$ samples than in HPV16 $DNA^+/p16^-$ samples, while all HPV16 $DNA^-/p16^+$ or HPV16 $DNA^-/p16^-$ samples were HPV16 DNA^- by HPV16 ddPCR. However, a statistically significant difference in viral load was not observed between different OPSCC subsites. Moreover, although patients with a higher HPV16 viral load in their tumors tended to have a worse clinical outcome (DFS and OS) as compared to those with a lower viral load the difference was not statistically significant. Of note however, when assessing only patients with HPV16 $DNA^+/p16^-$ cancer, clinical outcome (DFS and OS) was better for those with tumors exhibiting a low viral load, but since the number of patients was limited (10 patients) the data should be taken with caution.

Notably, there was a fine concordance between samples assessed by HPV ddPCR and those tested previously by the bead based multiplex assay, since only 3/245 initially HPV16 DNA^+ samples were HPV16 DNA^- with the present methodology. This could be due to that the quality of the extracted DNA had deteriorated somewhat over the years, or possibly but less likely due to that the HPV ddPCR assesses HPV E7, while the bead based multiplex assay utilized primers analyzing the presence of HPV16 L1 and E6 [13–15,47].

The fact that HPV16 DNA load was higher in samples that were HPV16 $DNA^+/p16^+$ than in HPV16 $DNA^+/p16^-$ samples was perhaps not entirely unexpected since first of all overexpression of $p16^{ink4a}$ is highly dependent on HPV E7 activity and abrogation of Rb function [43]. Overexpression of $p16^{ink4a}$ could therefore be expected in samples that have a considerable HPV16 DNA load based on assessment of HPV E7, although of course $p16^+$ depends more on the actual production of E7 rather than only the presence of the E7 gene [43–45,47].

Of note, there were no major differences in viral load depending on OPSCC subsites. First of all, there was no difference in viral load between

the TSCC and BOTSCC subsites. Secondly, when we compared the viral load of the latter together, we did find a tendency for a higher mean viral load in TSCC and BOTSCC as compared to otherOPSCC, but this did not reach statistical significance. However, the data should be treated with caution. To begin with the number of otherOPSCC cases was limited, but it is also not impossible either that there are areas interspersed within otherOPSCC that could resemble the lymphoepithelial tissue found in TSCC and BOTSCC [41]. In fact, it may be of potential interest that when HPV16 DNA$^+$/p16$^+$ is found in otherOPSCC that the viral load is not that dissimilar to that found in TSCC and BOTSCC.

Finally, we also investigated whether HPV16 viral load was associated to clinical outcome in OPSCC. Both when comparing HPV viral load per se and when dichotomizing the patients into two groups and using a defined mean viral load value, having a low viral load tended to be correlated to a better DFS and OS, although the data did not reach statistical significance.

Nevertheless, this finding was not what we had anticipated, since we in a previous study with a limited number of patients (12 cases) showed that a higher viral load was associated to a better clinical outcome and this was also the case in two later larger studies (35 and 199 cases respectively) [37,48,49]. Moreover, findings analogous to those in our first study were presented in two recent reports, more one from Japan and another from Scotland with 91 and 113 cases respectively [39,50]. The former used a TaqMan-based real-time quantitative PCR (qPCR) while the latter used an HPV ddPCR method, but both quantifying HPV16 E6 (and the latter study also quantifying HPV16 L1), a higher viral load was correlated to better clinical outcome [39,50]. In a different type of study, in a series of 78 HPV16 positive head and neck squamous cell carcinoma (HNSCC) samples, it was instead shown that samples with a higher viral load more frequently presented metastasis to regional lymph nodes as compared to those having a lower viral load, but in that study viral load was not studied in relationship to clinical outcome [38].

Notably, in a recent study with 362 OPSCC patients it was shown that those with HPV DNA$^-$ cancer had a worse prognosis than those with HPV DNA$^+$ cancer [40]. Moreover, in the patients assessed for viral load and physical HPV status, both a high viral load and episomal/ mixed physical status were associated with a better progression free survival (PFS) [40]. However, upon further analysis of the impact of viral load and physical status on the outcome of 88 patients with HPV16 DNA and RNA-positive tumors, neither viral load nor physical status predicted patient outcome, while having high levels of HPV16 L1 antibody did [40].

Clearly the data in the field are not in complete concordance and the numbers of cases in the various studies are limited so even though some studies suggest a high viral load is correlated to a better outcome the data should be interpreted with caution and viral load should likely not be used as a strong prognostic marker in clinical practice [37–40,48–50].

There were some limitations in this study, even though the total number of cases was in consistence with the larger studies noted above, the numbers of cases within subgroups varied and the numbers of HPV16 DNA$^+$/p16$^-$ and otherOPSCC cases were limited. Thus, also our data especially with regard to some of the small subgroups should also be taken with special caution. Nevertheless, the numbers of HPV16 DNA$^+$/p16$^+$ cases were considerable and here the viral load did not play a major role with regard to clinical outcome.

Another limitation in this study was that we only assessed viral load in HPV type 16 positive samples and this has to be emphasized, but on the other hand the presence of other HPV types in OPSCC is still very limited in the Stockholm region [15]. In a relatively recent report we found that HPV16 dominated (86 %) followed by HPV33 (8 %), HPV35 (3 %), HPV18 (2 %) and HPV56 and HPV59 both (0.5 %) [15]. Moreover, it would not be fair to compare HPV viral loads between these different HPV types since they may differ considerably in nature.

To summarize, we found a statistically significantly higher mean viral load in HPV16 DNA$^+$/p16$^+$ OPSCC as compared to that in HPV16 DNA$^+$/p16$^-$ OPSCC. However, although there was a tendency for the viral load to be lower in otherOPSCC as compared to TSCC/BOTSCC and for patients with HPV16 DNA$^+$ tumors with a lower viral load to have a better outcome than those with a higher viral load, these trends did not reach statistical significance. We therefore conclude that viral load should not be used as a major clinical prognostic factor.

CRediT authorship contribution statement

Mark Zupancic: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing – review & editing, Project administration, Writing – original draft. **Ourania N. Kostopoulou:** Data curation, Investigation, Methodology, Supervision, Writing – review & editing. **Stefan Holzhauser:** Data curation, Investigation, Methodology, Writing – review & editing. **Monika Lukosevicciute:** Data curation, Investigation, Methodology, Writing – review & editing. **Cecilia Jylhä:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Linda Marklund:** Data curation, Project administration, Resources, Writing – review & editing. **Anders Näsman:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Lars Sivars:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Tina Dalianis:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2024.106749.

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