Background. Squamous cell carcinoma of the oral cavity is generally caused by the long-term impact of known risk factors, e.g. tobacco and alcohol, along with chronic traumatisation. A number of studies now implicate HPV infection in head and neck tumour carcinogenesis but the exact role of HPV infection in the oral cavity remains unclear.

Methods. In this study, we evaluated 78 patients with oral squamous cell carcinoma (OSCC) for the expression of protein p16 in the context of HPV positivity and its influence on the overall survival rate, disease location, staging and grading.

Results. Regarding the tumour location, no significant difference was found between HPV-positive and HPV-negative patients, nor between p16-positive and p16-negative patients. There was also no trend in terms of HPV status and stage, and differentiation of carcinoma. There was no effect on HPV-positive patients relative to the time to progression ($P=0.84$) and overall survival rate ($P=0.78$). P16 positivity was not found to have an effect on the overall survival rate of patients ($P=0.41$) and there was no correlation between p16 positivity relative to the time to progression ($P=0.66$).

Conclusions. In summary, the data suggest that there is no effect of HPV status on the prognosis of OSCC patients compared to other HNSCC locations.

Key words: oral squamous cell carcinoma, oral cancer, HPV, human papilloma virus, p16

INTRODUCTION

In 2012, oral cavity tumours were diagnosed in over 300,000 patients worldwide, of which 60,000 were diagnosed in Europe. The most frequent malignant carcinoma of the oral cavity is (OSCC) (ref.1). In oral squamous cell carcinoma the Czech Republic, the incidence of OSCC affects 4.82/100,000 individuals with a 2.12/100,000 mortality rate and occurs more often in men2. Unfortunately, most patients come to the doctor with the advanced form – clinical stage IV – which is observed in 43% of these cases. Lately, there has been a changing tendency observed in head and neck squamous cell carcinomas (HNSCC), especially in oropharyngeal carcinomas (OPSCC): HPV-positive oropharyngeal tumours clinically and molecularly differ from HPV-negative tumours, and can be associated with varied prognostic results3,4. HPV-positive HNSCCs display different risk factors than HPV-negative cancers, and are more likely to be differentiated from those caused by tobacco and alcohol5,6.

HPVs are small non-enveloped double-stranded DNA viruses that belong to the Papillomaviridae family, a large group of viruses with new types that are continuously being identified7. HPVs cause a wide range of diseases, from benign lesions to invasive tumours8,9.

Currently, the highest designated HPV-type number is HPV 225 (www.hpvcenter.se, accessed on 2018-04-15). The most common, HR HPV type 16, is found in more than 95% of HPV positive HNSCC tumours10,11. High-risk types of HPV encode two viral oncoproteins, E6 and E7, that promote tumour progression by inactivating the TP53 and retinoblastoma tumour suppressor gene products12.

Protein p16 is a cyclin-dependent kinase inhibitor that inhibits pRB phosphorylation and blocks cell cycle progression at the G1 to S check point13. The loss of p16 expression results in a worse prognosis for HNSCC (ref.14). The overexpression of protein p16 (p16-positive) has been correlated with an improved outcome in OPSCC and possibly in OSCC (ref.15). The expression of p16 occurs as a result of the functional inactivation of the retinoblastoma protein (pRb) by the HPV E7 protein16. This leads to the up-regulation of protein p16. Thus, HPV positive tumours are characterized by the high expression of protein p16 (ref.17). This is typical in head and neck tumours for OPSCC (ref.17), but for tumours of the oral cavity it is necessary to further explore this question. On the other hand, the down-regulation of p16 appears to be associated with HPV-negative (tobacco, alcohol) HNSCC (ref.18). The goal of this study was to evaluate the expression of protein p16 in the context of HPV positivity and to evaluate the overall survival rate in HPV and p16-positive patients with oral cavity tumours, which has not been frequently studied, particularly in regards to HPV status.
Table 1. Baseline characteristics of the study patients and their tumors.

| Factor                        | median (IQR) | HPV +   | HPV -   | HPV n/s | p16+   | p16-   | p16 n/s |
|-------------------------------|--------------|---------|---------|---------|--------|--------|---------|
| **Age**                       | 59.64 (52.29 to 66.87) |         |         |         |        |        |         |
| **Sex**                       |              |         |         |         |        |        |         |
| M                             | 59 (75%)     | 5 (6%)  | 50 (63%)| 4 (5%)  | 5 (6%) | 50 (63%)| 4 (5%)  |
| F                             | 19 (24%)     | 1 (1%)  | 18 (23%)| 0 (0%)  | 2 (3%) | 16 (20%)| 1 (1%)  |
| **Primary site**              |              |         |         |         |        |        |         |
| tongue – the front two-thirds| 12 (15%)     | 2 (3%)  | 10 (13%)| 0 (0%)  | 1 (1%) | 9 (11%)| 2 (3%)  |
| base of the tongue            | 7 (9%)       | 0 (0%)  | 7 (9%)  | 1 (1%)  | 0 (0%) | 7 (9%) | 0 (0%)  |
| floor of the mouth – frontal area| 21 (27%) | 1 (1%)  | 20 (25%)| 2 (3%)  | 3 (4%) | 18 (23%)| 0 (0%)  |
| floor of the mouth – distal area| 14 (18%) | 1 (1%)  | 13 (16%)| 0 (0%)  | 1 (1%) | 12 (15%)| 1 (1%)  |
| mandibular alveolar ridge     | 12 (15%)     | 1 (1%)  | 11 (14%)| 1 (1%)  | 0 (0%) | 10 (13%)| 2 (3%)  |
| retromolar trigone            | 3 (4%)       | 0 (0%)  | 3 (4%)  | 0 (0%)  | 0 (0%) | 3 (4%) | 0 (0%)  |
| maxillary alveolar ridge      | 4 (5%)       | 1 (1%)  | 3 (4%)  | 0 (0%)  | 1 (1%) | 3 (4%) | 0 (0%)  |
| cheek                         | 5 (6%)       | 0 (0%)  | 5 (6%)  | 0 (0%)  | 1 (1%) | 4 (5%) | 0 (0%)  |
| **Clinical stage**            |              |         |         |         |        |        |         |
| I                             | 6 (8%)       | 1 (1%)  | 5 (6%)  | 0 (0%)  | 0 (0%) | 5 (6%) | 1 (1%)  |
| II                            | 15 (19%)     | 0 (0%)  | 15 (19%)| 1 (1%)  | 1 (1%) | 12 (15%)| 2 (3%)  |
| III                           | 16 (20%)     | 3 (4%)  | 13 (16%)| 1 (1%)  | 2 (3%) | 13 (16%)| 1 (1%)  |
| IVa                           | 34 (43%)     | 2 (3%)  | 30 (38%)| 2 (3%)  | 4 (5%) | 29 (37%)| 1 (1%)  |
| IVb                           | 7 (9%)       | 0 (0%)  | 7 (9%)  | 0 (0%)  | 0 (0%) | 7 (9%) | 0 (0%)  |
| **Tumour stage “T”**          |              |         |         |         |        |        |         |
| T1                            | 6 (8%)       | 1 (1%)  | 5 (6%)  | 0 (0%)  | 0 (0%) | 5 (6%) | 1 (1%)  |
| T2                            | 27 (34%)     | 2 (3%)  | 24 (30%)| 1 (1%)  | 1 (1%) | 20 (25%)| 2 (3%)  |
| T3                            | 19 (24%)     | 2 (3%)  | 17 (22%)| 1 (1%)  | 2 (3%) | 17 (22%)| 1 (1%)  |
| T4                            | 26 (33%)     | 2 (3%)  | 22 (28%)| 2 (3%)  | 4 (5%) | 24 (30%)| 1 (1%)  |
| **Nodal stage “N”**           |              |         |         |         |        |        |         |
| N0                            | 34 (43%)     | 2 (3%)  | 30 (38%)| 2 (3%)  | 3 (4%) | 27 (34%)| 4 (5%)  |
| N1                            | 15 (19%)     | 3 (4%)  | 11 (14%)| 1 (1%)  | 1 (1%) | 13 (16%)| 1 (1%)  |
| N2a                           | 1 (1%)       | 0 (0%)  | 1 (1%)  | 0 (0%)  | 1 (1%) | 0 (0%) | 0 (0%)  |
| N2b                           | 17 (22%)     | 1 (1%)  | 15 (19%)| 1 (1%)  | 1 (1%) | 16 (20%)| 0 (0%)  |
| N2c                           | 11 (14%)     | 0 (0%)  | 11 (14%)| 0 (0%)  | 1 (1%) | 10 (13%)| 0 (0%)  |
| N3                            | 0 (0%)       | 0 (0%)  | 0 (0%)  | 0 (0%)  | 0 (0%) | 0 (0%) | 0 (0%)  |
| **Distant metastases “M”**    |              |         |         |         |        |        |         |
| M0                            | 75 (95%)     | 6 (8%)  | 65 (82%)| 4 (5%)  | 7 (9%) | 63 (80%)| 5 (6%)  |
| M1                            | 3 (4%)       | 0 (0%)  | 3 (4%)  | 0 (0%)  | 0 (0%) | 3 (4%) | 0 (0%)  |
| **Grading**                   |              |         |         |         |        |        |         |
| G1                             | 24 (30%)     | 1 (1%)  | 21 (27%)| 2 (3%)  | 0 (0%) | 0 (0%) | 0 (0%)  |
| G2                             | 31 (39%)     | 2 (3%)  | 29 (37%)| 0 (0%)  | 0 (0%) | 0 (0%) | 0 (0%)  |
| G3                             | 13 (16%)     | 1 (1%)  | 10 (13%)| 2 (3%)  | 0 (0%) | 0 (0%) | 0 (0%)  |
| G4                             | 1 (1%)       | 0 (0%)  | 1 (1%)  | 0 (0%)  | 0 (0%) | 0 (0%) | 0 (0%)  |
| N/S                            | 9 (11%)      | 2 (3%)  | 7 (9%)  | 0 (0%)  | 0 (0%) | 0 (0%) | 0 (0%)  |
| **HPV status of primary tumour** |          |         |         |         |        |        |         |
| negative                       | 68 (86%)     | -       | -       | -       | 5 (6%) | 58 (73%)| 5 (6%)  |
| Positive                       | 6 (8%)       | -       | -       | -       | 2 (3%) | 4 (5%) | 0 (0%)  |
| N/S                            | 4 (5%)       | -       | -       | -       | 0 (0%) | 4 (5%) | 0 (0%)  |
| **p16 Expression in primary tumour** |          |         |         |         |        |        |         |
| negative                       | 66 (84%)     | 4 (5%)  | 58 (73%)| 4 (5%)  | -      | -      | -       |
| Positive                       | 7 (9%)       | 2 (3%)  | 5 (6%)  | 0 (0%)  | -      | -      | -       |
| N/S                            | 5 (6%)       | 0 (0%)  | 5 (6%)  | 0 (0%)  | -      | -      | -       |
MATERIALS AND METHODS

Set of Patients
The patients included in the study (n=78) were treated at the Department of Oral and Maxillofacial Surgery, University Hospital Brno, from January 2011 until December 2014. Inclusion criterion was the histopathological diagnosis of oral squamous cell carcinoma (OSCC) and tumour localization exclusively in oral cavities. The exclusion criteria were OSCC relapses and any additional locations other than the oral cavity and lips. Baseline characteristics of the study patients and their tumours is provided in Table 1.

HPV analysis
DNA extracted from native tumour tissue was used for HPV typing analysis. HPV typing was performed at the Department of Microbiology, University Hospital Brno. INNO-LiPA research genotyping assay (Innogenetics, Gent, Belgium) was used for HPV typing.

The INNO-LiPA HPV Genotyping Extra is a line probe assay for in-vitro diagnostic use, designed for the identification of 28 different genotypes (6LR, 11LR, 16HR, 18HR, 26pHR, 31HR, 33HR, 35HR, 39HR, 40LR, 43LR, 44LR, 45HR, 51HR, 52HR, 53pHR, 54LR, 56HR, 58HR, 59HR, 66pHR, 68HR, 69/71, 70LR, 73HR, 74, 82HR) of the human papillomavirus (HPV) by the detection of specific sequences in the L1 region of the HPV genome.

The results were interpreted either visually or by using LiRAS® for LiPA HPV software.

p16 analysis
In our study, archived, formalin-fixed, paraffin-embedded tissue samples with OSCC were analysed for protein p16 expression. Immunohistochemical analysis was performed at the Institute of Pathology, University Hospital Brno, with CINtec® p16 Histology, Ventana. CINtec Histology is a qualitative test and the CINtec Histology status is interpreted as either positive or negative based on the p16 staining pattern in the squamous epithelium.

Tumour p16 expression was evaluated by means of immunohistochemical analysis with mouse monoclonal antibody Anti- p16INK4a (E6H4) against protein p16INK4a, in formalin-fixed, paraffin-embedded (FFPE) tissue samples.

Positive p16 expression was defined as strong, with diffuse nuclear and cytoplasmic staining in 70% or more of the tumour cells18–22. Figure 1 shows representative images of a p16INK4a-immunolabelled.

Statistical analysis
The categorical and continuous variables were analysed using the χ2 test or the Mann-Whitney U test, respectively. Survival-rate analysis was performed by the Kaplan-Meier approach followed by a log-rank test. The relationship between clinicopathological characteristics and a patient’s outcome was determined in multivariate models using the Cox proportional hazard regression. The P values of less than 0.05 were considered significant, unless noted otherwise. Analyses were performed at Statistica 12.2 (Dell, OK, USA).

RESULTS

Patient characterization, p16 and HPV status
Of the 78 patients with tumours, 35 tumours were located in the floor of the mouth (27%), 21 in the frontal area, and 14 in the distal area. The second most common site was the tongue (12 at the apex, seven at the base) followed by the mandibular alveolar ridge (12 tumours), five in the cheek, four in the maxillary alveolar ridge and the final location was the retromolar trigone with three tumours.

Regarding the tumour locations, there was no significant difference detected between HPV-positive and HPV-negative patients (P=0.79, Pearson Chi-square)

Six out of the 78 patients (8%) tested positive for HPV (Table 1). High-risk HPV viruses 16/31 were present in three out of the six patients with HPV positivity. In one patient, we found pHR26, and in two patients, low-risk HPV 11 was found. Only one patient from the HPV-positive group was a woman (Table 2). No distant metastases were found; tumours were found in the tongue (2/6), the floor of mouth (2/6), the mandibular (1/6) and the maxillary alveolus (1/6). All of the patients were treated by primary surgery, while five of those subsequently received adjuvant therapy. The group of patients included all clinical stages, the majority having stage III (3/6). At the end of a three-year evaluation, half of the patients were still living, while the other half had died due to complications from the tumour. In four patients, it was not possible to evaluate the HPV status.

As Table 1 shows, most patients were p16 negative (n=66, 84%), while seven patients (9%) were p16 positive.

Table 2. Baseline characteristics of patients with HPV positive tumors.

| gender | age | localization          | stage | T NM | grade | HPV genotype | p16 positivity | p16 positivity |
|--------|-----|----------------------|-------|------|-------|---------------|----------------|----------------|
| M      | 59  | tongue               | III   | T2   | N1    | M0            | 2              | LR 11          |
| M      | 62  | floor of mouth       | III   | T3   | N1    | M0            | 2              | HR 31          |
| M      | 56  | mandibular alveolus  | IV    | T4   | N2b   | M0            | 1              | LR 11          |
| F      | 49  | maxillary alveolus   | IV    | T4   | N0    | M0            | 1              | HR 16          |
| M      | 51  | floor of mouth       | III   | T2   | N1    | M0            | 2              | pHR 26         |
| M      | 49  | tongue               | I     | T1   | N0    | M0            | 3              | HR 16          |
In five patients, it was impossible to evaluate p16 due to technical reasons.

Table 1 also summarizes the association between HPV-positive/negative patients and p16-positive/negative patients; the correlation was at the threshold of statistical significance ($P=0.07401$).

One interesting fact is that the HPV-positive patients were not predominantly p16-positive; to the contrary, only two HPV patients were p16-positive (LR 11 and HR16) (Table 2).

TNM classifications and HPV

The TNM system is the most widely used cancer staging system (in this case 7th edition). Tumour size in 34% (n=27) of the patients was at T2 stage. The next most frequent stage was T4, observed in 26 patients (33%), followed by T3 in 19 patients (24%), and six tumours were in T1 stage (8%). There was no significant difference between the HPV-positive and HPV-negative patients for the T stages ($P=0.73478$). Regarding the lymph node metastases (N), 34 tumours were in N0 stage (43%), 15 tumours were in N1 stage (19%) and 29 tumours were in N2 stage (37%); no patients were classified as N3 stage. There was no significant difference between the HPV-positive and HPV-negative patients in the N stage ($P=0.56$). Regarding distant metastases (M), 75 patients with tumours were at M0 stage (95%) and three patients were at M1 stage (5%). There was no significant difference between the HPV-positive and HPV-negative patients for the M stage ($P=0.60$).

Staging and HPV

While comparing the advanced clinical stages III + IV to low stages I + II in relation to the presence of HPV, we observed a non-significant relationship as well ($P=0.53$).

Grading and HPV

Finally, we compared the rate of cell differentiation in relation to HPV positivity. Most tumours were very well differentiated (24–30%) or moderately differentiated (31–39%); only 13 patients had tumours with poor differentiation (16%), and in one case, there was no differentiation at all. There was no significant difference between the HPV-positive and HPV-negative patients in grading ($P=0.95367$).

Univariate survival analysis

Next, the prognostic significance of p16 and HPV was analysed using a log-rank test. In this analysis, the effect of HPV on the overall survival rate of patients was not demonstrated ($P=0.78$). Accordingly, there was no effect in HPV status on the time to progression ($P=0.83749$). P16 positivity was not found to have any effect on the overall survival rate of patients ($P=0.40798$). There was no effect on p16-positive patients relative to the time to progression ($P=0.66329$), see Fig 2.

Multivariate survival analysis

In the next step, HPV and p16 status was analysed using a stepwise Cox proportional hazards model with HPV status, p16, clinical stage, grade, cT and pN as independent predictors. First, the overall survival rate was analysed. It was found that the only factor predicting the overall survival rate was the tumour grade, hazard ratio, HR = 3.37, 95% confidence interval, CI 1.33 to 8.38, $P=0.01$. Next, the aim was to analyse the progression-free survival rate using multivariate analysis with a similar approach. Unfortunately, due to the limited number of cases, it was not possible to perform this analysis.

DISCUSSION

Many recent studies have pointed out the increasing prevalence of the human papilloma virus in the area of head and neck squamous cell carcinomas. Chaturvedi et al. studied changes in the incidence of oropharyngeal,
oral cavity and lung cancers in both women and men, but only in oropharyngeal tumours was there an evident increase of incidences. This fact confirms the effect of HPV on the growing frequency of oropharyngeal tumours in the younger population. Our study confirmed HPV positivity in 8% of patients with OSCC. One of many systematic works reports that 26% of HNSCCs, when using the PCR method, yields HPV positivity. However, the high HPV positivity applies more to OPSCC than to OSCC (ref. 3). Furthermore, many studies, if they consider OSCCs, include the base of tongue and pharynx cancers and occasionally the larynx and oral cavity. Very rarely can we find a study regarding OSCC solely within the oral cavity, as it should correctly be, i.e. the specifically defined area from the line of lips (which are not included due to entirely different behaviour of the tumour) to the glosopalatine arches. Our study respected this delimitation, therefore we included only patients with OSCC, and not those with OPSCC. Moreover, the prevalence of HPV infection in the oral cavity is quite varied, depending on the applied laboratory method, the size of the study group, race and geographical location. The prevalence of these cancers is lesser in Asia, where it can be influenced by either culturally acceptable or discouraged forms of sexual behaviour. Some studies analyzing HPV subtypes with a high prevalence also include verrucous carcinomas in OSCCs (ref. 29).

Studies that generally focus on head and neck cancers without pinpointing their exact location, report a high range due to the HPV infection, from 32% to 84% (ref. 17,30,31). When the location is limited to our area of interest, specifically the oral cavity, Syrjänen reports a 12% prevalence, Koppikar a 6% prevalence, Simonato 17% (ref. 34), Glombitza 17% (ref. 35), Van Monsjou 10% (ref. 34) and Koslabova 16.9% (ref. 37). The average value most generally accepted is a 13% prevalence specifically in the oral cavity, with the prevalence being higher in men than in women. Furthermore, it was found that high-risk sexual behaviour, including orogenital sex, is one of the most probable means of transmission between the genitals and the oral cavity.

Squamous cell carcinoma of the oral cavity is generally caused by the long-term impact of known risk factors, e.g. the use of tobacco and alcohol along with chronic traumatisation. Many studies imply that there is a possible impact of HPV infection on the head and neck tumours’
cancerogenesis but the exact role of HPV infection in the oral cavity remains unclear. One reason is that there seems to be less interest in this clearly defined group.

Even if we respect the impact of HPV on the onset of OSCC, only a few studies designate it to be a definite prognostic factor in HPV-positive patients, although with a generally better therapeutic response\textsuperscript{43,45}. On the other hand, several studies reported worse results in HPV+ patients\textsuperscript{56-58}. Our work provides similar results, where HPV positivity did not have an impact on the overall survival rate of patients and time of progression, which is in accordance with other works\textsuperscript{59}.

Another fundamental factor of carcinogenesis in relation to HPV is its subtype, where only HR types should be responsible for the onset of cancer, with the most commonly cited types being 16, 18 and 33 in the head and neck area\textsuperscript{57,59,60}. In OSCC, type 16 is considered to be less common than in OPSCC (ref.\textsuperscript{51,52}). High-risk types 31, 33 and others are rarely observed\textsuperscript{53,54}, which, unlike the LR types (e.g. LR 11), are most often detected in recurrent respiratory papillomatosis\textsuperscript{55} and only marginally correspond with larynx carcinogenesis. Incidentally, the presence of LR 11 was confirmed in one of our patients, and this sample was repeatedly tested immunohistochemically, proving a high expression of protein p16 of over 85%. This indicates that there was only the presence of the HPV infection and not the carcinogenesis cause. This agrees with the summarizing work of Syrjänen, in which the viral DNA was PCR-detected both in the cancer-altered and the normal oral cavity epithelium\textsuperscript{56}.

There is still insufficient agreement on what method for routine use is the most suitable for identifying HPV within the tumour tissue\textsuperscript{50,57}. The combination of protein p16 immunohistochemistry and the PCR DNA-based method seems to be the most convenient because it yields both high specificity and sensitivity\textsuperscript{58}. In our study, we employed both recommended methods and discovered that remarkably, many p16-marked tumours that did not yield positivity had none of the 28 tested HPV genotypes. Furthermore, HPV positivity was determined from samples in which, consequently, there was a clearly confirmed presence of squamous cell carcinoma. Five patients with a high p16 expression rate were HPV-negative for one of the 28 standardized types. Similar results were reported by Reusenbach et al.\textsuperscript{59}, where most OSCC samples were p16-negative. Furthermore, in 6 out of 17 of the strongly positive p16 tumours, HPV negativity was observed. Upile et al. focused on the occurrence of HPV in non-keratinising carcinomas, where out of 102 patients with OSCC, 8 patients were p16-positive but only 4 patients were HPV-positive\textsuperscript{60}. Kouketsu et al. in their recent study, narrowed the area of interest in the oral cavity to just the tongue and evaluated CDKN2A (p16) aberrations. However, unlike our work, they did not observe any significant correlation with clinical characteristics. Similarly, the results obtained by Kouketsu et al. indicate a positive correlation between p16 positivity and the overall survival rate, even though this relationship was not considered to be significant\textsuperscript{61}. These results imply that every anatomical sub-part requires an independent evaluation with a larger set of patients to exactly define the prognostic role of the p16 expression. Thus, our results should be interpreted carefully because of the small sets of patients corresponding to each particular location of the oral cavity.

CONCLUSION

P16INK4 immunohistochemistry is an exact and generally-available tool in the prognostic and predictive characterization of squamous cell cancers in the head and neck. This study focuses on the relationship between HPV and p16 prognosis in OSCC. No causality between p16, OS and PFS was proved in OSCC. The low prevalence of HPV infection (8%) in our study group indicates its small role in the carcinogenesis of the oral cavity. However, to confirm these conclusions, studies with larger sets of patients will be needed.

Author contributions: JB: literature search, data analysis and manuscript writing; JZ, JG: data analysis, manuscript writing; JB, CM, ZD, OB: final approval, critical reading and manuscript revision.

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