

Distribution of Human Papillomavirus Genotypes in Sardinian Patients with Oral Squamous Cell Carcinoma

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Abstract: Human papillomaviruses (HPVs) seem to play an important role in the pathogenesis of gynecological carcinomas and in head and neck carcinomas. The aim of this study was to detect and genotype HPVs in fresh oral squamous cell carcinoma (OSCC) from a Sardinian population, and to determine whether HPV presence was significantly associated with the development of OSCC.

The oral mucosa tissues were obtained from 120 samples (68 OSCC and 52 control samples) taken from a Sardinian population seen at the Dental Clinic of the Department of Surgery and Odontostomatological Sciences, University of Cagliari (Italy) and the “Ospedale SS Trinità”, Cagliari (A.S.L. 8) between 2007 and 2008. PCR was used for the detection of HPV DNA and the genotype was determined by DNA sequencing. The frequency of HPV infection was evaluated in relation to age, sex, smoking and alcohol use. Statistical analysis was performed using the SPSS 11.5 software.

The results showed the presence of HPV-DNA in 60.3% of OSCC with HPV-16 (51.2%) being the most frequent genotype. In these Sardinian OSCC patients, HPV-DNA was detected more in males (65.8%) than in females (34.1%) while controls show a 0% of HPV presence. HPV positive was highly associated with OSCC among subjects with a history of heavy tobacco and alcohol use and among those with no such history.

A greater frequency of high risk HPV presence was observed in patients with OSCC compared to health control patients. In addition these results suggested that oral HPV presence could be associated in OSCC subjects. Our results need more analyses to detect the HPV-DNA integration into tumoral cells.

Keywords: HPV, oral squamous cell carcinoma, seminested PCR, Sardinian patients.

INTRODUCTION

Squamous cell carcinoma (SCC) is the most common malignant tumor of the oral cavity and one of the ten most common causes of death. In addition the incidence of oral squamous cell carcinoma (OSCC) of the oral cavity and oropharynx is increasing worldwide [1]. The annual incidence and mortality rates vary considerably between different races, genders, and age groups; however, as reported in previous publications, the risk of intra-oral cancer rises with increasing age especially for males [1-4]. Many risk factors have been reported by different authors such as: tobacco use and alcohol consumption which are the main risk factors for this group of cancer [5]. However, these cancers also occur among lifelong tobacco and alcohol abstainers and, thus, other risk factors such as conditions related to oral health and hygiene (i.e. poor condition of the mouth, dentition, bleeding gums, and mouthwash use) have been suspected as contributing to its etiology.

In this context, Human papillomavirus (HPV) as a prognostic risk for OSSC has not yet been extensively studied, a reason for this “defect” could be due to: (i) unusual or less sensitive technologies for HPV detection in SSC, (ii) in excess of 100 HPV genotypes are recognized based on specific DNA sequence variations and 30-40 are associated with sexual lesions. However, the oncogenic capacity of individual HPV types differs considerably [6-8]. This virus is epitheliotropic and in humans is associated with papillomatosis, hyperplastic, and verrucous lesions on the skin and mucous membranes of various sites [9]. These genotypes, which are frequently identified in cellular neoplasm, have been segregated into those with a low risk and those with a high risk of malignant transformation [10]. Indeed, some authors have shown that high-risk viral genotypes such as HPV 16, 18, 31 and 33, are frequently associated with leukoplakia and squamous carcinoma [11, 12]. On the contrary low-risk types, such as HPV 6, 11, 13, 32 are preferentially associated with benign proliferative epithelial lesions e.g. squamous papilloma, condyloma acuminatum, verruca vulgaris and focal epithelial hyperplasia [13]. Functionally high-risk HPV infection contributes to carcinogenesis and tumor progression through two viral oncogenes E6 and E7 [9]. These oncogenes inhibit the activities of the p53 and pRb, and, have been considered

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as an important feature in disrupting cell-cycle regulatory pathways leading to a genetic progression to oral squamous cell carcinoma (OSCC) [14].

The frequency of HPV in oral lesions varies with geographic occurrence [15], the type of lesion [16] and the diagnostic methodology [17]. The diagnosis of HPV infection is based on the use of advanced molecular tools [18]. Polymerase chain reaction (PCR) and sequencing are considered the most sensitive and rapid methods for the detection and genotyping of HPV-DNA, respectively [19-21].

The present study was conducted to investigate the distribution of HPV in Sardinian patients with malignant oral mucosal lesions compared with healthy patients. An association between HPV-16, HPV -31 exposure and OSCC was also evaluated among subjects with different use of tobacco and alcohol.

MATERIALS AND METHODOLOGY

Clinical Samples

The oral mucosa fresh tissues were obtained from 120 samples (mean age 61.4, age range: 22-86, 69 men and 51 women). Sixty-eight were from the patients with OSCC seen at the "Ospedale SS Trinità", Cagliari (A.S.L. 8) between 2007 and 2008. Fifty-two control samples were from volunteers randomly chosen from patients at the Department of Dental Disease Prevention (University of Cagliari). These controls reported no history of cancer or showed no sign of mucosal lesions or relevant systemic disease. At the time of examination, all participants were informed about the research and its purpose and gave their informed consent.

An incisional biopsy of the oral squamous cell carcinoma (OSCC) lesion was performed on each patient. All biopsies were divided into two parts: one part was used for histopathological analysis and the other was frozen at -80°C until a molecular assay could be performed. Each fresh specimen sample was analyzed and histopathologically classified as regards to OSCC. Information on patient gender, age, smoking and alcohol history was collected from direct patient interviews and medical records. Tumor grade and staging system (TNM) were obtained from clinical notes, histopathology and radiology reports. Staging was carried out according to the Fifth Edition of the International Cancer Union guidelines [22].

Extraction, Amplification and Sequencing

About 25 mg of tissue was finely crushed and the DNA was extracted with a QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer's instructions, quantified by spectrophotometry and frozen at -80°C until use. The molecular method for the detection of HPV-DNA was Seminested PCR. In the first reaction the degenerate primers MY09 (5'-CGT CC(AC) AA(AG) GGA (AT)AC TGA TC-3') and MY11 (5'-GC(AC) CAG GG(AT) CAT AA(CT) AAT GG-3') were used to amplify a 450 bp fragment in the L1 gene [23]. The first reaction was performed in a final volume of 25 µl, consisting of 4 µl extracted DNA as a template (100 ng), 2.5 µl of a 10X PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 1.5 mM magnesium chloride, a deoxyribonucleotide triphosphate mixture (0.2

mM of each), 0.5 µM of each primers, 2.5 µl U Platinum Taq DNA Polymerase and 4 µl of the Rnase Dnase-free template. All reagents were purchased from Invitrogen (Milan, Italy) with the exception of the primers. The mixture was incubated for 2 minutes at 95°C and subjected to 20 cycles of amplification (95°C for 30 seconds, 52°C (MY09/MY11) for 1 minute; and 72°C for 30 seconds) with a final extension at 72°C for 5 minutes in a thermal cycler (Eppendorf, Milan, Italy). Two micro-liters of the PCR product of the first reaction were taken for the PCR second round, with the primer MY09 in conjunction with a new second primer, GP5+N (5'-TTTGTTACTGTGGTAGATAC-3') to reamplify the material generated in the first round, thus generating a 407 base pair fragment. The second reaction was performed in a final volume of 25 µl and contained 2.5 µl of a 10X PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 1.0 mM magnesium chloride, a deoxyribonucleotide triphosphate mixture (0.2 mM of each), 0.5 µM of each primers and 2.5 µl U Platinum Taq DNA Polymerase. Initial denaturation for 2 minutes at 95°C was followed by 40 cycles. Each of the cycles consisted of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 47°C and chain elongation for 1 minute at 72°C. One cycle of chain elongation was took for 7 minutes at 72°C at the end. The PCR products were electrophoresed on 2% agarose gels, stained with sybr green 1.5% and visualized under an ultraviolet transilluminator. The beta-globin gene was detected (data not shown) in all specimens to confirm the presence of amplifiable DNA in the extracted specimens.

PCR products were purified with a QIAquick Purification PCR kit (Qiagen, Milan, Italy) and were sequenced by a conventional capillary ABI Prism 310 automatic sequencer (Applied Biosystems Foster City, Calif.). The results were edited and analyzed with a Basic Local Alignment Search Tool [24].

Statistical analysis was performed using the SPSS 11.5 software. Data are expressed as the number of percentages detected. All data were compared using the Chi-Square and Fisher exact with significance considered to be $P < 0.05$.

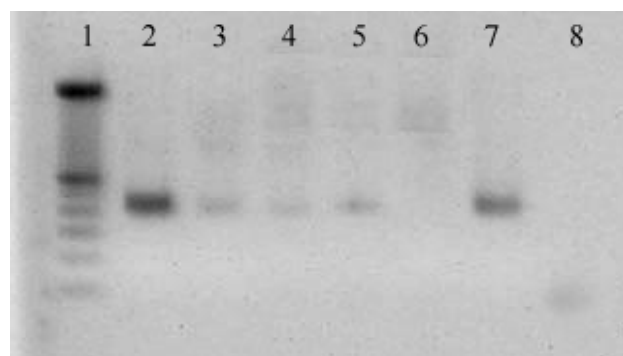
RESULTS

HPV-DNA was detected in 120 tissue samples: 68 oral squamous cell carcinoma and 52 control samples. The age of the patients with OSCC ranged from 27-86 (mean age: 65.6), with 47 males aged between 35-86 (mean age: 61.4) and 21 females aged between 27-84 (mean age: 64.9). The age range of the control patients was 22-85 (mean age: 56.8); 25 males were aged between 40-85 (mean age 57.5) and 27 females between 22-84 (mean age 56.3). Table 1 summarizes the information on HPV-positive patient gender, age. Using the Blast program, the HPV sequences obtained from positive samples showing a representative nucleotide motif were submitted to the DNA data Bank GenBank [24] with the following accession numbers: DQ286926, DQ312262, DQ312263.

Of the 68 OSCC samples 41 (41/68, 60.3%) were positive for HPV-DNA and indicated by a 407-bp fragment on 2% agarose gel electrophoresis of the PCR products (Fig. 1). Presence of faint bands was read as partial positives that need a confirmation by the sequencing.

Table 1. OSCC Positive for HPV DNA

Positive Samples	Age Years	Gender	HPV Type	Oral Lesions
1	50	M	31	OSCC
2	53	M	31	OSCC
3	58	M	31	OSCC
4	50	M	31	OSCC
5	63	M	31	OSCC
6	58	M	31	OSCC
7	50	F	6	OSCC
8	66	F	16	OSCC
9	79	F	16	OSCC
10	56	M	16	OSCC
11	72	F	6	OSCC
12	60	F	16	OSCC
13	51	F	16	OSCC
14	76	M	6	OSCC
15	68	F	16	OSCC
16	60	M	55	OSCC
17	80	F	16	OSCC
18	35	M	16	OSCC
19	78	M	16	OSCC
20	61	F	16	OSCC
21	54	F	16	OSCC
22	51	F	16	OSCC
23	39	M	16	OSCC
24	52	F	31	OSCC
25	67	M	16	OSCC
26	58	M	16	OSCC
27	74	M	16	OSCC
28	71	M	16	OSCC
29	73	M	16	OSCC
30	49	M	16	OSCC
31	63	M	16	OSCC
32	74	M	6	OSCC
33	61	M	6	OSCC
34	65	M	6	OSCC
35	78	M	6	OSCC
36	70	M	6	OSCC
37	84	F	6	OSCC
38	59	M	16	OSCC
39	78	M	6	OSCC
40	72	F	6	OSCC
41	59	M	6	OSCC



DNA from oral tissue was amplified by seminested polymerase chain reaction.

Fig. (1). This figure shows the amplicon of the second round reaction generating a 407 bp fragment on 2% agarose gel electrophoresis. Lane 1 is Marker 100 bp, lanes 2, 3, 4 and 5 are positive samples confirmed by nucleotide DNA sequencing, lane 6 is a negative sample. Lane 7 is the positive HPV control and lane 8 is the negative control. Some sequences of HPV types 6 and 31 were submitted to the DNA data Bank (GenBank <http://www.ncbi.nlm.nih.gov/BLAST/>) with the following accession numbers: DQ286926, DQ312262, DQ312263. The beta-globin gene was detected (data not shown) in all specimens to confirm the presence of amplifiable DNA in the extracted specimens.

Whereas twenty-eight carcinoma samples (28/41, 68.3%) were positive for a high-risk subtype (21/41, 51.2% HPV-16 and 7/41, 17% HPV-31) only 13 samples out of 41 (34.14%) were positive for low-risk subtype (12/41, 29.3% HPV-6 and 1/41, 2.4% HPV-55) (Fig. 2). We were unable to find HPV presence in the 52 samples forming the control group. Moreover, post hoc analysis showed a significant effect for the differences between the OSCC patients and the control group ($P < 0.05$) for the prevalence of HPV-DNA.

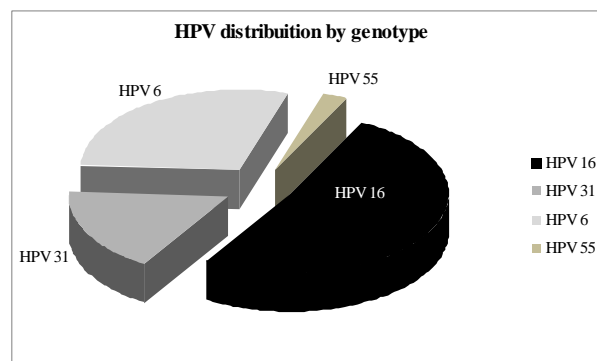


Fig. (2). Frequency in percentage of different HPV genotypes in OSCC samples. Samples positive on seminested PCR were subjected to nucleotide DNA sequencing. Alignments were obtained from the GenBank online BLAST server and HPV sequences were downloaded from the HPV database.

As regards virus distribution between genders (Fig. 3), we observed significant differences in HPV frequency between males and females with OSCC from a Sardinian population, 65.8% vs 34.1%, respectively. Furthermore, in subjects over 50 years, a significant increase in HPV DNA ($P < 0.05$) was detected with advancing age (8% <49 years and 92% > 49 years) (Fig. 4).

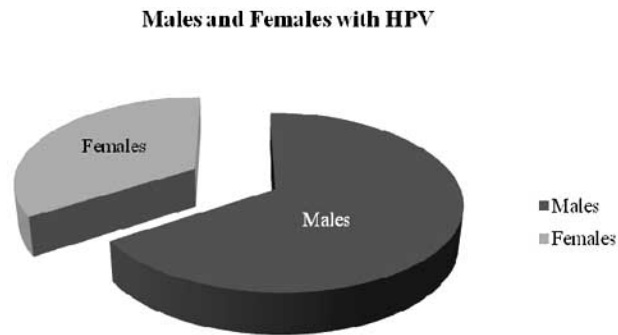


Fig. (3). Presence in the OSCC human papillomavirus (HPV) in relationship to sex, expressed as a frequency percentage. We observed significant differences in HPV frequency between males and females with OSCC from a Sardinian population, 65.8% vs 34.1%, respectively. Was not observed the HPV DNA in our control group.

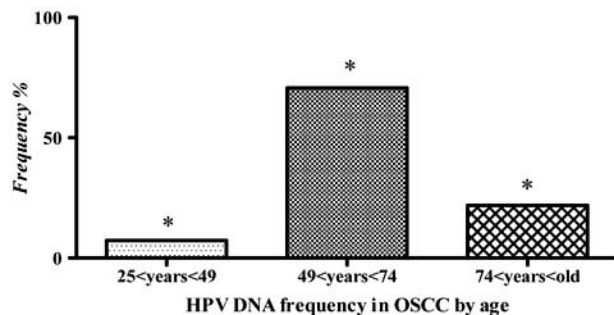


Fig. (4). Distribution of HPV in relationship to age, expressed as a frequency percentage. Over 50 years of age a significant increase HPV DNA of the OSCC samples was found.

Table 2 shows the lack of statistical associations between the presence of the virus and other known risk factors such as smoking and alcohol. We did not observe a significant difference between HPV high risk presence and tobacco and alcohol use in patients with OSCC.

Table 2. Samples Positive for HPV High Risk and Tobacco and Alcohol Use

	HPV +, High Risk, n = 28	HPV - n = 27	P Value
Tobacco and alcohol use	35 (64.3%)	10 (37.0%)	P = 0.351
Abstain completely from Alcohol and tobacco	10 (35.7.6%)	17 (63%)	P = 0.509

DISCUSSION

This study was set up to establish the prevalence and HPV genotypes in Sardinian patients with OSCC compared with a group of healthy patients. According to scientific literature, several types of HPV, particularly type 16, have been found to be associated with oropharyngeal squamous-cell carcinoma, a form of head and neck cancer.

The role of HPV in the development of uterine cervix lesions has been demonstrated by many studies [25]; it still remains a controversial issue as regards oral lesions [26-28].

Contradictory results reported on HPV infection in oral mucosa are mainly due to differences in detection methods and the epidemiological characteristics of the observed populations [29, 30]. Several reports have indeed utilized different molecular techniques (*in situ* hybridization [31] and Southern Blot analysis [32, 33] from several sample typologies (mouthwashes, in scrapings and in paraffin-embedded tissue) [12, 34].

Over the past 20 years, there has been an increasing interest in HPVs as regards their potential role in the pathogenesis of OSCC [35-41]. Nevertheless, a recent paper has confirmed HPV as an independent risk factor for OSCC compared to other known factors such as alcohol and smoking [42]. Several HPV genotypes (1, 2, 3, 4, 6, 7, 10, 11, 13, 16, 18, 31, 32, 33, 35, 55, 57) have been isolated from oral lesions [12, 43-45]. However, in OSCC, the most commonly HPV genotype detected is 16, which has been demonstrated in 90-95% of all HPV positive neoplasms, followed by HPV 18 and HPV 31 [45-47].

This study was performed on a Sardinian population, which presents genetic homogeneity compared to other known populations in Europe, including the Italian population [48]. Furthermore, a highly sensitive/specific molecular methodology such as nested PCR followed by capillary sequencing was used [49]. These results confirm the prevalence of HPV-DNA in OSCC and also that HPV 16 is the most frequent genotype. The findings of the present study are in agreement with those reported by Miller *et al.* [50] and Anaya-Saavedra *et al.* [43] in two recent studies, suggesting that HPV 16 represents a risk factor in the etiopathogenesis of a subset of OSCC.

Despite the presence of low-risk, HPV (29.3% HPV-6 and 2.4% HPV-55) is according to the results of Herrero *et al.* [26] and other authors [44, 51, 52] who have demonstrated a malignant degeneration of papillomas induced by low-risk HPV. For example Chang *et al.* [44] reported that HPV 6 was also found in squamous cell carcinoma. In addition the same bibliographic data showed that an association between multiple infection (HPV 6, HPV 11 and HPV 16) is required for the development of malignant lesions [39]. Our results give us the basis to further determine how many virus' presence could be fundamental in the carcinogenesis process through the study of the HPV DNA integration.

In our study we observed a different frequency between genders; in fact a major presence of HPV-DNA results in males compared to females, as has been described in previous studies [53]. Moreover, this finding indicates that higher positivity may be the result of accumulated lifetime exposure to HPVs. The oral cavity is the anatomic site with the most frequent exposure to viruses and we observed an increase in HPV positivity with age (Fig. 4) in accordance to many papers [47, 54, 55]. This increment may be explained by the fact that different factors can be involved in the malignant process such as: immune defences [56], nutritional deficiencies [57], traumatism inflicted by fractured teeth, incongruous restorations or prostheses [58, 59], and poor oral hygiene [60] which worsens with an increase in age. Moreover, according to our findings, HPV is a specific risk factor for the development of OSCC in association with other important risks i.e. smoking and alcohol [61-65].

CONCLUSION

Our data support the presence of high frequency of HPV 16 in oral cancer, but also suggest the possibility of an involvement of low-risk HPVs as a cofactor in this malignant process; in this context the age of the patients plays a central role in the cancer onset. Moreover, the results of this study, probably because of small sample size, did not show the association significantly among HPV and tobacco and alcohol use. Next step, to verify the real HPV role in carcinogenesis, will be the study of the possible HPV infection determined by the integration in the human genome confirmed by the study of E6 and E7 mRNA expression [14].

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