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Detection and genotyping of human papillomavirus DNA in samples from healthy Sardinian patients: a preliminary study

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The human papillomavirus (HPV) is involved in the development of different benign and malignant lesions that include in particular squamous tumours of the cervix, skin and the respiratory tracts. In particular, the 'high risk' HPV type 16 (HPV 16) causes genito-rectal epithelial cancers and is suspected of causing epithelial cancers of the head and neck. To determine the presence and genotypes of HPV was determined in saliva samples from 164 subjects recruited from the Department of Surgery and Odontostomatological Sciences (University of Cagliari). For this study a sensitive seminested polymerase chain reaction (PCR) method was used to detect HPV-DNA; moreover in all positive samples, HPV genotyping was based on sequencing of the HPV genome L1 region. The results obtained with these patients (who were ethnically homogeneous), showed an interesting percentage of positive samples for HPV-DNA (30 samples out of 164–18.3%). Only two HPV genotypes have been identified in these patients, HPV 16 and HPV 31 with 76.7% and 23.3% of the positive specimens, respectively, both correlating with high carcinogenic risk. This preliminary result leads us to reflect on the presence of HPV in saliva, in particular in young asymptomatic subjects (15.38%), and its prognostic value for the possible incidence in Sardinia of oral carcinoma.

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Introduction

Different epidemiological and molecular studies have been performed to determine the possible involvement of human papillomavirus (HPV) in the aetiology of both benign respiratory papillomas and squamous cell carcinomas (1–5).

Phylogenetic relationships have demonstrated that high-risk types of HPV are not only aetiologically related to the development of uterine cervical carcinoma (6–11), but are also associated with certain types of carcinomas in the head and neck (12, 13). A variety of molecular mechanisms in combinations with different co-factors could be associated in HPV carcinogenesis; in particular the viral proteins or polymorphic alterations in host tumour suppressor genes, such as p53, pRb (14–16). In fact, for example, oncogenic mucosal HPVs encode to early proteins, E6 and E7, which jointly transform and immortalize human keratinocytes in culture and are retained and expressed in HPV-positive carcinomas (17).

More than 100 different HPV genotypes have been identified and described in scientific literature. 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 (18, 19).

To contribute to an understanding of the pathogenesis of HPV-associated lesions, it is important to investigate the presence of HPV in the saliva of healthy patients. Because HPV genotyping information is clinically useful in prognosis and therapy based on risk type, it is important for the HPV genotype to be identified by as sensitive and as specific a method as possible. The possibility of monitoring health status, disease onset, progression and outcome of treatment through non-invasive means is a highly desirable goal in healthcare promotion and delivery. Oral fluid (saliva) is a perfect medium for exploration in health and disease surveillance (20). The presence and correlated genotype of HPV in the oral cavity isolated in healthy subjects has not been systematically examined in such a large number of subjects and in particular in saliva and with a genetically homogeneous population (21).

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The use of saliva as non-invasive pathology samples could be interesting for retrospective studies of the risk and prognosis in oral carcinoma (22–24).

The purpose of this study was to describe, by a sensitive and highly specific molecular procedure, the 'HPV *saliva status*' of 164 healthy Sardinian patients before the onset of infection, when the virus penetrates into the new host through micro-injuries.

Materials and methods

Patients typology and specimens

Saliva specimens were collected from 164 subjects: 69 men and 95 women (mean age 37 years; range: 4–77) recruited from the Surgery Department of Odontostomatological Sciences, Odontostomatology Section (University of Cagliari). The patient's conditions were non-diseased for oral pathology. However, different sites from the oral cavity were analysed, they included: tongue dorsum, lateral sides of tongue, buccal epithelium, hard palate, soft palate, maxillary anterior vestibule and tonsils. These patients not showed any signs or symptoms included lesions reporting cancer o precancerous signals. Approximately 1 ml of saliva was collected in the morning from each patient and placed into a sterile tube it was stored at –20°C and 400 μl of this were used for DNA extraction.

Information on age, smoking and alcohol use was obtained by personal interviews. At the time of their visit all participants were informed concerning the research and its purpose and gave their informed consent.

DNA extraction

The 400 μl of the sample was inactivated for 30 min at −80°C and stored at −20°C until use. DNA was extracted from the saliva by standard methods routinely used in our laboratory. Briefly afterwards the saliva sample was lysed with sodium dodecyl sulphate 10% (Carlo Erba Reagenti, Milano, Italy) and subjected to Proteinase K (Sigma-Aldrich, Milano, Italy) treatment (10 mg/ml) for 10′ at 65°C. Cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich) was used to remove complex polysaccharides that may inhibit polymerase chain reaction (PCR) amplification. Samples were treated with 750 μl of SEVAG (Sigma-Aldrich; chloroform isoamilico alcohol 24/1) and after centrifugation resuspended in 450 μl of isopropyl alcohol (Sigma-Aldrich), followed by overnight incubation at −20°C. The precipitated DNA was suspended in Rnasi Dnasi-free water and stored at −20°C until use. Specific precautions were taken to prevent and monitor cross-contamination during the DNA extraction process.

Seminested PCR

Detection of HPV-DNA was performed by means of seminested PCR designed by using the most commonly used PCR primer sets (25).

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. The first 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), 1.5 mM magnesium chloride, deoxyribonucleotide triphosphate mixture (0.2 mM each), primers (0.5 μM each), 2.5 U Platinum *Taq* DNA Polymerase, and 4 μl of the Rnasi Dnasi-free template. All reagents were purchased from Invitrogen (Milano, Italy), with the exception of the primers. The mixture was incubated for 2 min at 95°C and subjected to 20 cycles of amplification [95°C for 30 s, 52°C (MY09/MY11) for 1 min and 72°C for 30 s] with a final extension at 72°C for 5 min in a thermal cycler (Eppendorf, Milano, Italy). In the seminested second-round reaction the primer MY09 was used in conjunction with a new second primer, GP5 + N (5′-TTTGTTACTGTGGTAGATAC-3′) to re-amplify the material generated in the first round, thus generating a 407 bp fragment.

Two microlitres of the PCR product of the first reaction were taken for the PCR with seminested GP5 + N/MY09 primers. 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, deoxyribonucleotide triphosphate mixture (0.2 mM each), primers (0.5 μM each), 2.5 μl U Platinum *Taq* DNA Polymerase. Initial denaturation for 2 min at 95°C was followed by 40 cycles. Each of the cycles consisted of denaturation for 30 s at 94°C, annealing for 30 s at 47°C and chain elongation for 1 min at 72°C. One cycle of chain elongation for 7 min at 72°C was performed at the end. The PCR products were electrophoresed on 2% agarose gels, stained with syber green 1.5% and visualized under an ultraviolet transilluminator. Strict procedures were followed to avoid false-positive reactions due to contamination. DNA extraction, reagent preparation and addition of sample DNA were carried out by using filtered pipette tips in separate cabinets and every test included a negative control amplification mixture without DNA sample.

HPV genotyping by capillary sequencing method

Polymerase chain reaction products were purified with a QIAquick Purification PCR kit (Qiagen, Milano, Italy) and were sequenced by a conventional capillary sequencer (26). The sequence reaction was performed in 10 μl volumes using a Big Dye chemistry Kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA) according to the manufacturer's instructions. The mixture contained: 5 μM of each primer (MY09 or GP5 + N), 2 mM $MgCl_2$ and 1.5 μl of purified PCR products. The cycling parameters were 25 cycles consisting of denaturation at 96°C for 10 s; 50°C for 5 s, 60°C for 4 s. The sequences were determined with an ABI Prism 310 automatic sequencer (Applied Biosystems). The results were edited and analysed by Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Positive controls

In order to evaluate the sensitivity and specificity of the procedure described in this work, we have utilized the subsequent positive controls.

(i) 450 bp HPV-DNA amplicon , obtained by PCR (with the oligos, described above, MY09/MY11) and purified by using the Qiagen purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Serial 10-fold dilutions of this target, ranging from 10^6 molecules/2 μl served as a standard for the sensitivity test.

(ii) A series of 11 tissues fragments recovered from oral papillomas/carcinomas , previously detected and typized by a InnoLiPa technology (HPV-HS Bio, AB analitica, Padova, Italy) was used for sensitivity test.

(iii) A series of five tissue fragments recovered from colon rectal papillomas.

Statistical analysis

Statistical analysis was performed using NCSS software. Data are expressed as the number of percentages detected (mean ± SEM). All data were compared using paired Student's *t*-test with the significance considered as being $P \leq 0.05$. Graphic displays of data were created using GRAFIT software.

Results

In order to evaluate the incidence of oral HPV infection in clinically healthy subjects recovered in the same geographical area (Island of Sardinia), a collection of 164 saliva clinical isolates was examined by a seminested PCR and subsequent by conventional DNA capillary sequencing procedure. Patients' ages ranged from 4 to 77 years, with a median of 37 years. The distribution of HPV-DNA in saliva was as follows: 95 of the patients were women (58%) and 69 were men (42%). Thirty (18.3%) of the 164 patients presented high-risk HPV in saliva, as indicated by a 407-bp fragment, on 2% agarose gel electrophoresis of the PCR products (Fig. 1).

Of the 30 samples positive for HPV-DNA, seven were HPV 31 and 23 were HPV 16. The results of the detection of the high-risk HPV in saliva samples are shown in Table 1. The class of age between 0 and 15 years showed a frequency for HPV 16 of 66.6% and for HPV 31 of 33.3% in saliva. Beyond 65 years the incidence of the HPV was 15.38% (HPV 16 only, Fig. 2a). As for gender, we observed a different frequency of HPV in saliva: 14 women and nine men with HPV 16 and six women and one man with HPV 31 (Fig. 2b).

By sequencing analysis, only two different genotypes were recognized, they were to correlate both at high-risk carcinogenic HPVs: HPV 16, $n = 23$ (14%) and HPV 31, $n = 7$ (4.3%) (Fig. 2c). This sequences of HPV types 16 and 31 were submitted to DNA data bank (GenBank http://www.ncbi.nlm.nih.gov/BLAST/) with the following accession numbers: DQ440542, DQ440541, DQ440540, DQ440539, DQ440538, DQ440537, DQ440536, DQ435771, DQ435770, DQ435769, DQ426541, DQ422967.

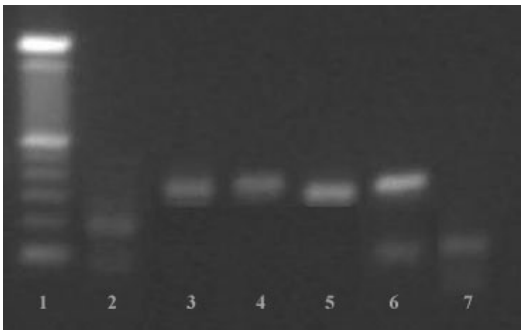

Figure 1 DNA from saliva samples was analysed by eminested polymerase chain reaction. This figure shows the amplification of the second-round reaction generating a 407 bp fragment. Lane 1, marker (100 bp); lane 2, negative sample; lanes 3–5, positive samples confirmed by nucleotide DNA sequencing; lane 6, positive HPV 16 control; lane 7, negative control.

Table 1 Detection of high-risk HPV in saliva samples

Positive patients	*Age (years)*	*Gender*		*Oral HPV type*	*Oral lesions*
		Male	*Female*		
1	56		+	16	–
2	49		+	16	–
3	43		+	31	–
4	49	+		16	–
5	46	+		16	–
6	48		+	31	–
7	51	+		31	–
8	13		+	31	–
9	39		+	31	–
10	30		+	31	–
11	57	+		16	–
12	77		+	16	–
13	11	+		16	–
14	25	+		16	–
15	27		+	16	–
16	61		+	16	–
17	36	+		16	–
18	77		+	16	–
19	44		+	16	–
20	24	+		16	–
21	57		+	16	–
22	9		+	16	–
23	25		+	16	–
24	55	+		16	–
25	61	+		16	–
26	40		+	16	–
27	23		+	16	–
28	44		+	31	–
29	41		+	16	–
30	49		+	16	–

Of the 30 samples positive for HPV-DNA, seven were HPV 31 and 23 were HPV 16.

Discussion

Many studies, thanks to the rapid development of molecular biological techniques, have shown that

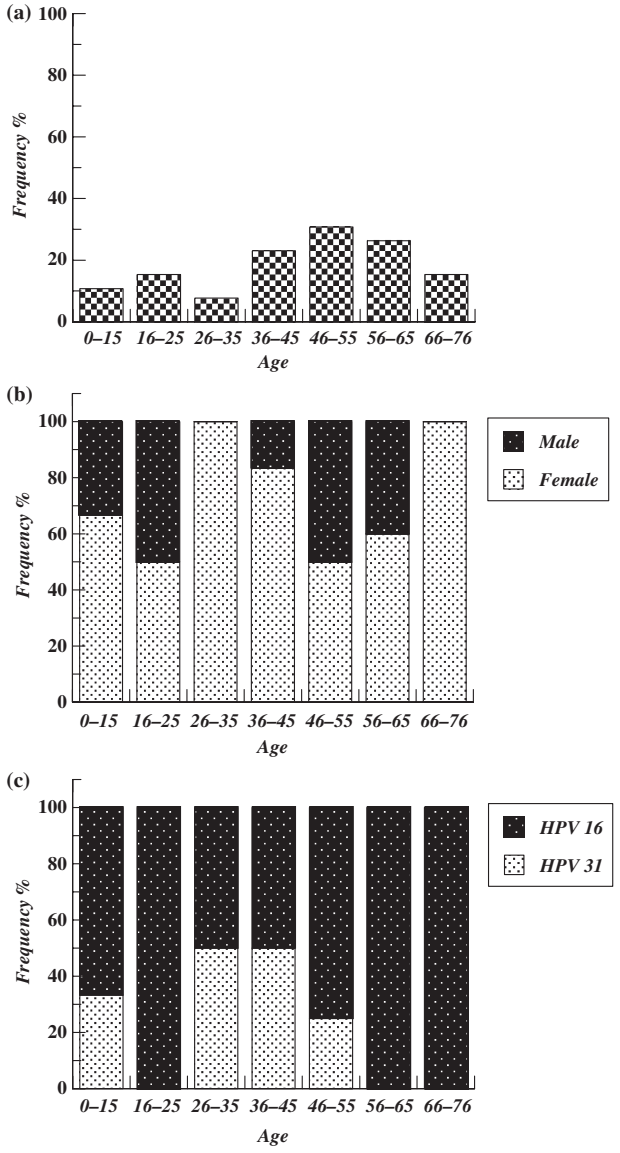

Figure 2 (a) Presence in the saliva of high-risk human papillomavirus (HPV) in relationship to age, expressed as a frequency percentage. Samples positive on seminested polymerase chain reaction (PCR) were subjected to nucleotide DNA sequencing. Alignments were obtained from the GenBank online BLAST server and HPV sequences were downloaded from the HPV database. (b) Distribution of high-risk HPV in saliva samples of males and females in relationship to age, expressed as a frequency percentage. 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. (c) Frequency in percentage of different high-risk HPV genotypes in saliva samples in relationship to age. 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.

different subtypes of HPV are involved in oral carcinogenesis (2, 5, 27). So far in scientific literature, over 100 types of HPV have been described (28) and of these, 25 types have been associated with oral lesions (HPV-1, 2, 3, 4, 6, 7, 10, 11, 13, 16, 18, 31, 32, 33, 35, 40, 45, 52, 55, 57, 58, 59, 69, 72 and 73; 2). Moreover, HPV infections are a necessary, but not sufficient, condition for the onset of the carcinogenesis process; the main contributors to oral carcinogenesis and the cell proliferation process are in fact (29, 30) physical and chemical agents (UV, tobacco, alcohol; 31, 32), nutritional deficiencies (33), chronic infections from other pathogens (34, 35), genetic predisposition (33), traumatisms inflicted by fractured teeth or maladapted restorations or prosthesis (36), poor oral hygiene (37).

Normally, the involvement of HPV in carcinogenesis depends on three factors: cell permeability, virus type and the host's immune status (38). Furthermore, the HPV infection may persist for long silent periods, without producing appreciable damage, while a latent infection may be activated by a condition, it too temporary, in immunosuppressed patients (HIV-positive; 29, 39, 40).

When 164 saliva samples from volunteers without signs or lesions in oral cavity were examined, HPV in saliva was found in 30 subjects. Our results, using the capillary sequencing method, only showed two HPV genotypes, 16 and 31 with the 14% and 4%, respectively. These results, in especially when considering the high prevalence of HPV 16 are in accordance with many authors who have described normal oral mucosa samples (41) or results obtained on oral carcinomas (42–44). If we consider specimens from other oral lesions, we can observe the same differences, for example, the most recovered HPV types are 13 and 32 in oral epithelial hyperplasia (45) and HPVs 2-4-11-57 in oral papillomas, condillomas (46).

Despite the fact that HPV 31 is correlated in few cases in normal specimens (47) or in papilloma tissues (48) in our samples this genotype was detected only for the same age group (Fig. 2a) and was absent over the six decade. We speculated that the presence and frequency of high-risk HPV 16 and HPV 31 in saliva increased with age in both sexes (Fig. 2b). Figure 2c shows the relationship between age vs. sex and genotypes. In Fig. 2a, we can observe that the maximum percentage of positive samples is shown in the age range from 46 to 55 years, in accordance with many authors who have describe the age prevalence of HPV infection (49). Prevalence of HPV in saliva showed a disparity in the male:female ratio and it was highest among females aged 26–45 years (Fig. 2b).

The presence of oral HPV in very young patients (aged 0–15 years) is a not an understandable result considering that in classical and most studies, the transmission method for oral HPV infection is by oral-genital contact (50); in particular, in 30 positive samples we found three HPV-positive subjects aged 9, 11 and 13, respectively (51). Our results show that HPV is present in saliva, indicating a possible and continuous virus replacement in saliva from different oral districts and it is possible that these results could explain the role of saliva as a means of virus transmission in patients with a high infectious dose of HPV .

Our preliminary results demonstrate that subclinical or latent HPV infections of normal oral mucosa are common and that HPV 16 is the predominant genotype

in the Sardinian population. The assumption that HPV is present in saliva before integration in host cells is possible. It is well known that saliva acts as a reservoir for different pathogens and that saliva is important in the maintenance of oral health. Actually, saliva as a diagnostic fluid has been evaluated in dentistry as a marker for periodontitis, pre-malignant and malignant oral lesions (20).

Consequently, as in the case of the uterine cervix it is important to monitor the presence of HPV (52) in the oral cavity as well.

In conclusion, our results show that HPV is present in saliva, it indicating a possible and continuous virus replacement in saliva from different oral districts and is possible that these results could explain saliva's role as a means of virus transmission in patients with a high infectious dose of HPV. Moreover, the saliva analysis to reveal the presence of HPV is potentially reliable, and may be useful as an effective and valuable non-invasive way of diagnosing and monitoring the presence of HPV infections and thus preventing any damage that may occur if they are not diagnosed in time, especially in individuals exposed to other oral cancer risk factors, such as smoking, drinking and other habits that may lead to the formation of malignant oral tumours.

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