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ORIGINAL ARTICLE

H3 and H3.3 histone mRNA amounts and ratio in oral squamous cell carcinoma and leukoplakia

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Histone variants (e.g. H3) play an important role in chromatin structure and gene expression regulation of normal cells. Aims of this study were to: (1) estimate H3 and H3.3 histone mRNA expressions and their ratio in oral squamous cell carcinoma (OSCC) and oral leukoplakia (OL); (2) investigate whether H3 and H3.3 variants could play a role in the pathogenesis of OSCC and OL, also conditionally to HPV infection, age, gender, and main habits (tobacco smoking and alcohol drinking) in human beings studied. Twenty-three cases of OSCC and 20 cases of OL were examined in lesion site (LS) and juxtaposed clinically undamaged site (JUS) by RT-PCR for H3 and H3.3 histone mRNA; 13 healthy oral mucosa samples (HS) were investigated in a single site as controls. HPV DNA presence was investigated in the respective exfoliated oral mucosa cells by *nested* PCR (*n*PCR: MY09-MY11/GP5-GP6). The data showed that both H3 and H3.3 histone mRNA crude concentrations are higher in OSCC (LS = 2901 ± 459 ng of H3; JUS = 2699 ± 658 ng of H3; LS = 3190 ± 411 ng of H3.3; JUS = 2596 ± 755 ng of H3.3) than those in OL (LS = 2095 ± 349 ng of H3; JUS = 2192 ± 897 ng of H3; LS = 2076 ± 911 ng of H3.3; JUS = 1880 ± 654 ng of H3.3) and in HS (2579 ± 959 ng of H3; 2300 ± 758 ng of H3.3), although not reaching any statistical significance. Interestingly, ratio of H3/H3.3 mRNA amounts decrease both in OSCC (0.99) and OL (1.009) *vs* HS (1.121). No association was found for H3 and H3.3 histone mRNA expressions in OSCC and OL with respect to HPV infection and the social-demographical variables considered ($P > 0.2$). The overall higher expression of H3.3 in damaged tissues up to the ratio inversion in OSCC especially in HPV+ alcohol drinkers (60.0%) represents the most interesting finding, in consideration of the proven ability of alcohol to act as permeability enhancer of human oral mucosa, to alter the mucosal structure and by this dynamics could favour the penetration through the epithelial layers of HPV.
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Keywords: H3 histone mRNA; H3.3 histone mRNA; HPV; oral squamous cell carcinoma; oral leukoplakia

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the 10 most common cancers of the body. It is a rapidly increasing malignancy which represents the most frequent malignant tumour of this region (Gupta *et al*, 1996). Its prevalence increases with age and is more common in men. Similarly, of all potentially malignant lesions and conditions of the oral mucosa, oral leukoplakia (OL) (Axell *et al*, 1996; Pindborg *et al*, 1997; van der Waal *et al*, 1997; Reibel, 2003) is the most common and extensively studied. For both type of oral lesions, biological markers that can help to identify the lesions with an aggressive phenotype and worse prognosis need to be identified. As carcinogenesis has been believed to be based on the imbalance of the regulatory cell-cycle control processes, the study in OSCC and potentially malignant lesions on the expression of proteins starts elucidating processes of carcinogenesis (Lo Muzio *et al*, 1997, 2003; Pich *et al*, 2004).

In particular, abnormalities in gene regulating cell proliferation and cell death are frequently seen in OSCC and less in OL. At this regard, histones are key structural proteins important in the organization of eukaryotic DNA into chromatin, and histone gene expression is closely associated with the timing of DNA replication (Stein *et al*, 1984; Zanders, 2002). Many data are already available in literature on the H3 histone mRNA accumulation in tumours: by Northern blot and *in situ* hybridization analyses Torelli *et al* (1986) showed a considerable expression of the H3

histone gene in two of 11 cases of acute myeloid leukaemia; Wong *et al* (1990) showed that H3 histone mRNA in hamster oral epithelia is a good indicator of the proliferation status; Smith *et al* (1995) used non-isotopic *in situ* hybridization (NISH) for detecting H3 histone mRNA, showing that the number of positive cells was generally elevated in biopsies from hyperproliferative dermatoses and dysplastic or malignant lesions; finally, Sakamoto *et al* (2004) showed that BrdU and histone H3 mRNA labelling indices (LIs) were the lowest for normal epithelium, higher for hyperplastic and dysplastic epithelia, and the highest for squamous cell carcinoma.

The most modern RT-PCR technique has been used only recently and gives also real quantitative results: Berta *et al* (1997) determined the expression of histone H3 in normal mucosa, benign lesions and in OSCC, reporting only a higher expression of H3 in OSCC.

Histones are generally synthesized in correlation with DNA duplication, therefore their genes are named cell-cycle dependent genes. In addition there are histone variants that are coded by genes with cell-cycle independent expression. Such variants, named replacement variants (Wolffe and Pruss, 1996), are present at low concentration in growing cells but they become relatively abundant in non-dividing cells (Krimer *et al*, 1993). The H3.3 histone is the replacement variant that has been studied for a long time and is very well conserved in the evolution (Fucci *et al*, 1994; Albig *et al*, 1995). Few data are available in literature on the amount of H3.3 histone mRNA in normal and tumour cells. Using Northern blotting analysis Graber *et al* (1996) have shown that histone H3.3 is overexpressed in four of six carcinomas of the oesophagus but not in normal oesophageal mucosa. In addition 11 of 12 human cancer cell lines overexpressed it.

Our work hypothesis is based on the possibility that the two genes for H3 and H3.3 histone variant are expressed at different levels in growing cells and in cells at different periods of oral carcinogenesis. On these bases, we measured H3 and H3.3 histone mRNA amounts and ratios in normal, potentially malignant and cancerous oral tissues, in order to evaluate their usefulness as marker of the proliferation status in association or not with the most known risk factors for OSCC, such as tobacco smoking, alcohol misuse, up to viral infections (e.g. high-risk HPV) (Warnakulasuriya, 2000).

Materials and methods

Study design

In a multi-centric hospital-based research the study group was composed of 43 cases, including 23 OSCC and 20 OL, consecutively diagnosed at two Italian Units of Oral Medicine (University of Naples and Palermo). Two samples were taken for each patient of the study group: lesion site (LS) and juxtaposed clinically undamaged site (JUS). Only OSCC and OL cases histologically confirmed were included in the present research. Informed consent was obtained from all participants. As controls, 13 otherwise healthy individuals, underwent to common oral surgery interventions at the same Units consented to have an oral mucosal biopsy in one single site (exclusion criterion: suspected diagnosis for any type of OSCC or potentially malignant lesion).

Anamnestic and clinical evaluation

The historical and clinical data of each subject were recorded on a clinical report form and thus collected by means of data entry program for PC. Information regarding age, smoking and alcohol use was obtained by personal interviews; patients were included in two smoking categories (smokers and non-smokers), in two drinking categories (drinkers and non-drinkers) and in HPV+ and HPV−.

Determination of H3 and H3.3 amplified cDNAs

H3 and H3.3 mRNA amounts in oral specimens were determined by RT-PCR. Total RNAs were extracted from oral specimens by total RNA isolation System (Promega, Southampton, UK) and RNA was checked by agarose gel electrophoresis in denatured conditions. Total RNA was reverse transcribed into cDNA (RT) and the cDNA was amplified by PCR with primers specific for human H3 and H3.3 histone. HH3 ERA (5′-GGCATGATGGTGACGCGGTTTAGC-3′) and Anti UB1 (5′-GGCATGATGGTGACTCTCTTAGC-3′) were used as reverse primers in the RT reaction for H3 and H3.3, respectively. RT reaction mixtures contained 1 μg RNA sample, 1 μM primer, 10 U of AMV reverse transcriptase (Promega), 1 U μl^{-1} Rnasin recombinant Rnase inhibitor, 1 mM each dATP, dTTP, dCTP and dGTP in a final volume of 25 μl RT buffer (250 mM Tris–HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT). All chemicals were from Promega. Reverse transcription was performed at 42°C for 1 h. Samples were then heated to 70°C for 10 min to denature the reverse transcriptase and stored at −20°C. Control samples in which RNA was replaced by DEPC-treated water were prepared. Preliminary PCR experiments have been performed in order to ensure that the amount of the amplified products were in a linear relationship with the initial cDNA amount. Such correlation was found using 5 μl of the RT mixture and 30 and 35 amplification cycles, respectively, for H3 and H3.3 histone cDNA. PCR was performed in a Gene Amp PCR system 2400 (Applied Biosystems, Monza, Italy) and consisted of an initial denaturation step of 3 min at 94°C followed by 30 or 35 cycles, as previously reported for H3 and H3.3, respectively, of 30 s at 94°C, 30 s at 60°C and then 60 s at 72°C. At the end of the amplification samples were incubated for a further 10 min at 72°C. PCR was performed using 5 μl of cDNA, 1.5 mM $MgCl_2$, 200 μM each of dATP, dTTP, dCTP and dGTP dNTPs, 1 U of Taq Polymerase (Sigma, Milan, Italy), 0.5 μM forward and reverse primers, in a final volume of 50 μl PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3). All reagents were from Sigma. For the amplification of human H3 histone cDNA was used as forward oligo HH3MAR: (5′-AAGCTCCGCGCAAGCAGCTTGC-3′) and as

reverse the above-mentioned HH3ERA. For the amplification of the H3.3 histone cDNA was used as forward oligo UB1 (5′-AAGCCCCCCGCAAACAGCTGGC-3′) and as reverse the Anti-UB1. After amplification, 9 μl of each PCR reaction was analysed by electrophoresis on 1% agarose gel stained with ethidium bromide. Amounts of amplified cDNA were measured by using Kodak Digital Science software in comparison with the Low DNA Mass ladder marker (Invitrogen, Milan, Italy) and then the H3/H3.3 cDNA was determined. Some PCR products have been sequenced in the automated sequencer model 373A (Perkin Elmer, Beaconsfield, UK); the sequence was analysed via the European Bioinformatics Institute Fasta3 software (http://www2.ebi.ac.uk/fasta3). Various PCR conditions (i.e. volume of RT mixture and number of cycles) have been tested in order to verify that the amplified products were in linear relation with the initial cDNA amount. In addition controls have been performed because histone genes are present in high number in the genome and H3 and H3.3 genes are similar. Then RT-PCR control experiments have been performed on total RNA digested with RNAse-free DNAse and some amplified products have been sequenced. All the results led us to the conclusion that the products were amplified from RNA of the H3 and H3.3 histone genes (Figure 1).

Virological evaluation

Oral cytological specimens were obtained from LS, by means of a cytobrush (RAM; Mirandola, MO, Italy) (Ammatuna *et al*, 2001).

DNA extraction was performed as previously described (Giovannelli *et al*, 2002). All clinical samples were checked for DNA by amplification of the human β-globin gene and tested in duplicate. Three types of controls were included in each reaction series: blank control, HPV DNA-negative cell W138 as negative control and HPV-18 DNA-positive HeLa cells, in dilutions from 20 000 to 50 000 down to 2–5 HPV-DNA copies, as positive control. All controls were prepared and analysed in parallel with the clinical specimens, to ensure that proper reaction conditions were maintained. HPV DNA presence was checked by nested (MY09-MY11/GP5/-GP5) PCR assay (*n*PCR) as previously described (Ammatuna *et al*, 2000). All PCR assays were performed in a DNA thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany), and amplification products analysed by electrophoresis in 2% agarose gel (Figure 2).

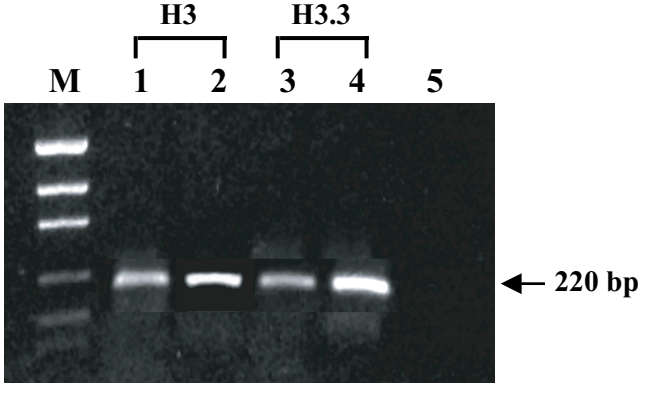

Figure 1 Agarose gel electrophoresis of RT-PCR fragments amplified from human total RNA using primers specific for H3 and H3.3 histone gene in UT (lanes 1-3) and OSCS (lanes 2-4). Lane 5: negative control; M: Low DNA Mass Ladder (Invitrogen)

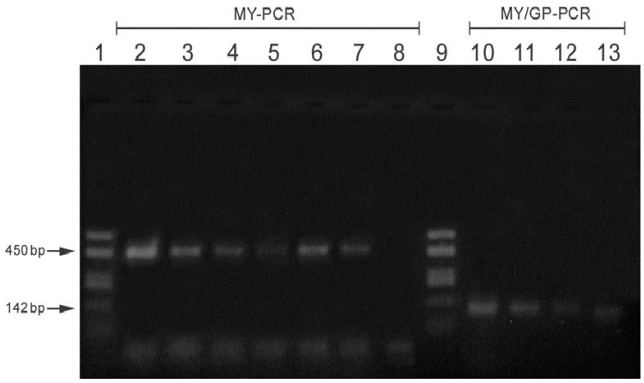

Figure 2 Agarose gel electrophorasis showing representative first step amplification with MY09-MY11 primers (MY-PCR) (450 bp) and second step amplification with nested GP05-GP06 primers (MY/GP-PCR) (about 140 bp) products of HPV-DNA from oral mucosa samples. Lanes 1 and 9: /X 174 RF DNA cleaved with Hae III molecular size standard; lanes 2-5: HeLa cells (positive control) dilutions corresponding to 2 3 10_5, 2 3 10_4, 2 3 10_3, and 2 3 10_2 HPV DNA copies; lanes 6 and 7: positive samples; lane 8: Wi cells (negative control); lanes 10 and 11: HeLa cells (positive control) dilutions corresponding to 20 and 2 HPV DNA copies; lanes 12 and 13: positive samples.

Statistical analysis

Statistical analysis of data was calculated using S-Plus 4.0 (Cambridge, UK) and SPSS 9.0 (Chicago, IL, USA).

Comparison of baseline characteristics was made by Student's *t*-test for continuous variables, and by Pearson's chi-square test for categorical variables. Before the *t* statistical analysis, Kolmogorov–Smirnov's test was used in order to test the normal distribution for age in the groups analysed.

In bivariate analysis, the chi-square test was used to determine differences in proportions of categorized variables. Fisher's exact test was calculated when the number observed was quite small.

The Kruskal–Wallis test was used to demonstrate significant differences between the two groups for ordinal variables (e.g. amounts of H3 and H3.3) in the different type of samples. Furthermore, Spearman's test for ranks was used to evaluate correlations between ordinal variables (e.g. amounts of H3 and H3.3) into each group (OSCC, OL, HS). *P*-values ≤ 0.05 were considered statistically significant. Odds ratios (OR) were calculated along with 95% confidence interval (CI) and used to describe further associations or differences.

Results

Level of H3 and H3.3 histone gene expression

The data showed that both H3 and H3.3 histone cDNAs, amplified from 1 μg of RNA, expressed higher

amounts in OSCC (LS = 2901 ± 459 ng of H3; JUS = 2699 ± 658 ng of H3; LS = 3190 ± 411 ng of H3.3; JUS = 2596 ± 755 ng of H3.3) than those in OL (LS = 2095 ± 349 ng of H3; JUS = 2192 ± 897 ng of H3; LS = 2076 ± 911 ng of H3.3; JUS = 1880 ± 654 ng of H3.3) and in HS (2579 ± 959 ng of H3; 2300 ± 758 ng of H3.3), although not reaching any statistical significance ($P > 0.2$) (see Figure 3).

HPV DNA was found in 10/23 (43.4%) of overall OSCC; in all cases HPV genotypes were high risk HPV-18 in (7/10), and HPV-16 in the remaining three cases. In OL HPV DNA was found in 5/20 (25.0%): three HPV-18, one HPV-16 and one HPV-33.

In Figure 4 the ratios of the mean values of H3 and H3.3 histone mRNA amounts measured on oral specimens of OSCC, OL, JUS and HS are reported. These data, although not statistically significant, indicated that H3/H3.3 mRNA amounts decreased in leukoplakia and in carcinoma. The ratio changed because the total amounts of H3 and H3.3 cDNAs changed differently in the specimens. In the case of OSCC the ratio was inverted for the increased amount of H3.3. No association was found for H3 and H3.3 histone mRNA expressions in OSCC and OL with respect to HPV infection and the social-demographical variables considered ($P > 0.2$). In Table 1a,b, samples of OSCC and OL are grouped by the ratio of H3/H3.3, assuming as normal a ratio ≥1. Worthy of note, it was found that the overall higher expression of H3.3 is present in damaged tissues up to the ratio inversion in OSCC especially in alcohol drinkers (60.0%).

Table 1a Characteristics and risk factors for OSCC grouped by H3/H3.3 ratio ($n = 23$) in LS

	Ratio H3/H3.3 normal (≥1) (n = *13*)	*Ratio H3/H3.3 altered (<1)* (n = *10*)
Age (mean ± s.d.)	65.2	72.8
Gender		
Male	8 (61.5)	6 (60.0)
Female	5 (38.5)	4 (40.0)
Smoking		
Current	7 (53.8)	4 (40.0)
Former	2 (15.4)	1 (10.0)
No	4 (30.8)	5 (50.0)
Alcohol		
Current	4 (30.8)	6 (60.0)
Former	0	0
No	9 (69.2)	4 (40.0)
Ratio in JUS		
Normal	7 (53.8)	3 (30.0)
Altered	4 (30.8)	5 (50.0)
ND	2 (15.4)	2 (20.0)
HPV DNA		
+ (43.4%)	4 (30.7)	6 (60.0)
–	8 (61.5)	4 (40.0)
ND	1 (0.7)	0

Values are given as *n* (%). ND, not determined; LS, lesion site; JUS, juxtaposed site.

Table 1b Characteristics and risk factors for OL grouped by H3/H3.3 ratio ($n = 20$) in LS

	Ratio H3/H3.3 normal (≥1) (n = *12*)	*Ratio H3/H3.3 altered (<1)* (n = *8*)
Age (mean ± s.d.)	51.3	54.3
Gender		
Male	8 (66.7)	4 (50.0)
Female	4 (33.3)	4 (50.0)
Smoking		
Current	8 (66.7)	7 (87.5)
Former	2 (16.7)	0
No	2 (16.7)	1 (12.5)
Alcohol		
Current	2 (16.7)	2 (25.0)
Former	0	0
No	10 (83.3)	6 (75.0)
Ratio in JUS		
Normal	10 (83.3)	2 (25.0)
Altered	0	5 (62.5)
ND	2 (16.7)	1 (12.5)
HPV		
+ (25%)	4 (33.3)	1 (12.5)
–	7 (58.3)	4 (50.0)
ND	1 (8.3)	3 (37.5)

Values are given as *n* (%). ND, not determined; LS, lesion site; JUS, juxtaposed site.

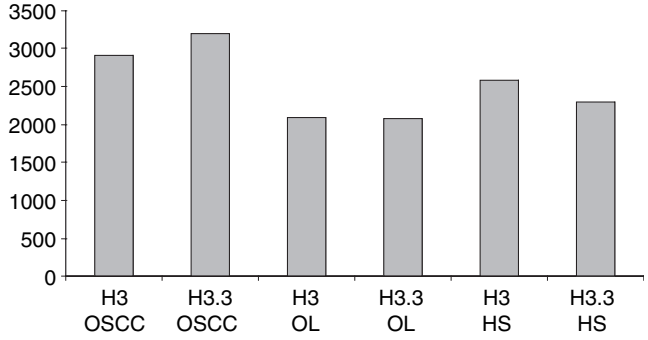

Figure 3 Comparison between total amount of H3 and H3.3 histone cDNA amplified from 1 μg of RNA extracted from 23 OSCC (lesion site = LS), 20 OL (LS) and from 13 healthy mucosal site (HS). In OSCC (LS = 2901 ± 459 ng of H3; LS = 3190 ± 411 ng of H3.3); in OL (LS = 2095 ± 349 ng of H3; LS = 2076 ± 911 ng of H3.3) and in HS (2579 ± 959 ng of H3; 2300 ± 758 ng of H3.3)

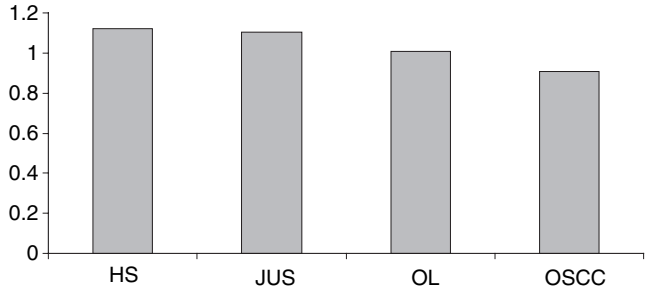

Figure 4 Average of ratios between H3 and H3.3 mRNA amounts in 13 healthy samples (HS; ratio = 1.121), in 43 juxtaposed to lesion site (JUS; ratio = 1.103), in 20 OL (ratio = 1.009), and in 23 OSCC (ratio = 0.909)

Discussion

Human H3 and H3.3 histone genes belong to the classes of cell-cycle dependent and independent genes, respectively. The function of H3.3 histone variant, as that of other 'cell-cycle independent' variants, is not yet completely known. It has been reported that the H3.3 histone increases in abundance in non-dividing cells in vertebrates (Urban and Zweidler, 1983) presumably because it is deposited both during and outside S phase (Ahmad and Henikoff, 2002) whereas the H3 histone is incorporated exclusively during replication. Moreover using GFP-tagged versions of H3.3 and H3 histones in a cytological assay, it has been shown that chromatin associated with transcriptionally active loci accumulates the H3.3 histone variant. In addition the H3.3 histone relatively enriched in modifications associated with transcriptional activity and deficient in dimethyl lysine-9, which is abundant in heterochromatin (McKittrick *et al*, 2004).

In this work we evaluated by RT-PCR the ratio between H3 and H3.3 histone mRNA amounts in normal oral tissues of healthy individuals and in OSCC and OL in the damaged site as well as in the juxtaposed clinically normal tissue. The highest increase was for H3.3 variant expression in OSCC, for the so-called 'cell-cycle independent' variant. With respect to the ratio value, the results reported in Figure 4 showed that the H3/H3.3 mRNA ratio decreased progressively in correlation with the malignancy although without statistical significance, because of the small sample size and the high variability of values. We suggest that other actually uncontrolled factors might affect the level of the histone mRNAs and led to data variability. Moreover our recent observation (Mancini *et al*, 2004) on the existence of H3L-like histone gene with peculiar characteristics such as cell-cycle dependent and independent histone genes might interfere with the absolute values.

Changes in the ratio values are due to a decreased histone H3 mRNA and increased histone H3.3 mRNA in leukoplakia and to an increase of H3.3 mRNA higher than H3 mRNA in carcinoma. Consequently it is possible to conclude that, carcinoma is characterized by higher expression of 'cell-cycle independent' H3.3 histone genes than that of 'cell-cycle dependent' H3 histone genes. Our data are in agreement with Krimer *et al* (1993) showing that the three polyadenylated mRNAs transcribed on the H3.3 gene are co-ordinately induced during the first few hours of murine erythroleukemia (MEL) cell differentiation. Moreover, Knowles and Phillips (2001) have analysed by RT-PCR the H3.3 histone gene expression *in vitro* tumour microenvironment finding an hyper-expression of H3.3 histone gene. Using Northern blotting analysis Graber *et al* (1996) have shown that histone H3.3 is overexpressed in four of six carcinoma of the oesophagus but not in normal oesophageal mucosa. In addition, 11 of 12 human cancer cell lines overexpressed it. Our data on H3 histone mRNA are in agreement also with those obtained by Berta *et al* (1997) that have found by RT-PCR an enhanced expression of H3 in OSCC (four of five cases) but not in the benign lesions where we revealed a slight decrease.

Certainly aetiological factors, such as alcohol and HPV infection could contributed to the decrease of the ratio value in OSCC (Table 1a). Alcohol has always been considered a factor in oral carcinogenesis and excessive alcohol consumption is the second most important risk factor after smoking (Moreno-Lopez *et al*, 2000). Our data show that alcoholism influenced the H3/H3.3 cDNA. The results obtained on drinkers–smokers appeared particularly interesting because they could be explained by a synergically action of alcohol and tobacco although a higher number of individuals should be studied. This result is in agreement with Hindle *et al* (2000) that suggested that rising alcohol consumption since the 1950s is more closely related to increasing oral cancer incidence and mortality than smoking. In addition while smokers who do not use alcohol have a two- to fourfold risk of oral cancer compared with non-smokers and non-drinkers, the risk of smokers who are heavy drinkers is increased six to 15 times compared with non-smokers and non-drinkers (van der Waal, 1999). Studies on oral mucous membrane permeability have shown that chronic alcohol ingestion alters the mucosal structure and it may increase permeability for tobacco-associated nitrosamines and polycyclic hydrocarbons (Squier *et al*, 1986). With respect to the contextual presence of HPV infection in OSCC with the ratio H3/H3.3 altered likely, alcohol could act as permeability enhancer of human oral mucosa, alters the mucosal structure, and by this dynamics could favour the penetration through the epithelial layers of HPV. Furthermore, to date only in tonsillar SCC, morphology of the HPV + ve tumours suggests that HPV may have a predilection for a population of non-keratinized squamous cells, easier to be penetrated especially in alcohol drinkers, or that the virally transformed cells inhibit keratinization of the tumour cells (Klussmann *et al*, 2001). Furthermore, Smith *et al* (2004) observed a significant interaction effect between younger age and heavy alcohol use associated with HPV-HR cancer status. First of all, data evaluation pointed out that, in the present series of OSCC and OL, prevalence of HPV DNA was 43.4 and 25.0%, respectively, as being in the range of prevalence recently reported (Campisi *et al*, 2004a,b; Ha and Califano, 2004). Indeed, in this study, HPV DNA detection rate was not considered a main finding, but as a variable of the analysis system; the issue of HPV infection in OSCC and in OL has been formerly debated elsewhere by the same research group (Giovannelli *et al*, 2002; Campisi *et al*, 2004a,b).

With respect to viral involvement, it is still highly controversial whether high-risk HPV (HR-HPV) can be considered as an etiological or a malignancy risk factor in oral carcinogenesis (Nielsen *et al*, 1996): some research groups (Ostwald *et al*, 1994; Nielsen *et al*, 1996; Bouda *et al*, 2000) identified HR-HPV antigens and viral DNA in potentially malignant and malignant oral lesions, and somebody else defined HR-HPV as to

play an important role in OSCC, especially in absence of common oral habits (Chang *et al*, 2003). In a very recent review, on epidemiological and molecular bases, Ha and Califano (2004) attribute for HR-HPV a role in oral carcinogenesis, but only in a small subset of cases with differences reported in clinical outcome, behaviour to radiotherapy, and prognosis (Gillison *et al*, 1999, 2000; Gillison and Shah, 2001; Lindel *et al*, 2001; Mork *et al*, 2001; Ringstrom *et al*, 2002; Li *et al*, 2003). The present study, although with the limit to be a cross-sectional on a small sample size, confirms the chance of OSCCs to be HPV + ve, suggests a different behaviour of HPV + OSCC with respect to HPV− ones, consistently with the hypothesis of a complex relationship between HR-HPV infection and cell-cycle regulation in malignant oral lesions.

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