Prognostic value of human telomerase reverse transcriptase gene expression in oral carcinogenesis

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Abstract. Human telomerase reverse transcriptase (hTERT) gene expression in resected specimens of oral squamous cell carcinoma (OSCC) and their surrounding tissue, either apparently normal or clearly histologically dysplastic, was evaluated by both real-time RT-PCR and immunohistochemical protein analyses. The expression level of hTERT in oral dysplasia and in OSCC was markedly higher than in normal tissues. The correlation between hTERT expression in OSCC and clinico-pathological parameters or survival of OSCC patients was statistically analyzed. Our study demonstrates that there is no significant relationship between hTERT expression and classical clinico-pathological parameters. Interestingly, survival analysis showed both overexpressing cases and lower survival rate in the early stage of OSCC (p=0.03 for immunohistochemistry; p=0.04 for RT real-time PCR). The histological location of hTERT in these tumors has been discussed in the context of the cancer stem cell theory.

Introduction

The human telomerase is a complex RNA-protein heterotrimeric enzyme (hTR: human telomerase RNA, that acts as template in the process of repeat addition; TP1: telomerase-associated protein 1 of unknown function; hTERT: human telomerase reverse transcriptase, the catalytic subunit of the complex), possessing an RNA-dependent DNA polymerase activity that is specialized in the synthesis and maintenance of the telomeric ends of linear chromosomes, protecting them from degradation and end-to-end fusion. hTERT expression correlates well with telomerase activity. Diverse experimental models from yeast to human have proven that telomeres play a critical role in the maintenance of chromosomal integrity. Most human somatic cells do not express telomerase (1-3) even though they lose telomeric DNA with each cell division ultimately experiencing chromosomal instability, replicative senescence, and cell death (4,5). A weak hTERT expression, however, has been demonstrated to occur in highly proliferating tissues such as intestinal, endometrial, bronchial, and epidermal epithelia, activated B and T lymphocytes, regenerating liver, and germ cells (1,6-10). In contrast, the great majority (85%) of human tumors shows strong telomerase activity (1), expresses the gene encoding hTERT (2,3,11), and maintains the length of their telomeres (12-14). Hahn *et al* have demonstrated that the ectopic expression of hTERT and two oncogenes (the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras) results in the direct tumorigenic conversion of normal human epithelial and fibroblast cells (14). The authors suggested also that the wide expression of hTERT in human tumors makes this protein a possible molecular target of anti-cancer treatment (15).

There is an overall agreement on the critical role played by telomerase in the mechanism of cell immortalization during the process of oral carcinogenesis both *in vitro* and *in vivo* (16). In particular, in a variety of oral dysplasias a marked hTERT ectopic expression has been detected as associated with other molecular changes, such as loss of INK4A and/or retinoic acid receptor-ß, even in the absence of p53 mutations (16).

Goessel *et al* have recently created *in vitro* a human cellular model of oral-esophageal carcinogenesis using the genetic alterations frequently observed in the corresponding human cancer (17). In this interesting model a critical role in the process of malignant transformation of oral keratinocytes, transfected with recombinant retroviral DNA coding for cyclin D1, dnp53, c-myc and EGFR, is played by p53 inactivation and EGFR-induced activation of telomerase and P13K/AKT pathway (17). In addition, the recent increase in understanding the structure and function of telomeres and telomerase has led to the possible use of them as diagnostic and prognostic markers in several types of neoplasms including oral cancer

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Table I. Patient characteristics.

Age (years)	
Range	42-81
Mean	65
Gender	
Male	25
Female	17
Size (cm)	
≤1.5	25
>1.5	17
Recurrence	
No	22
Yes	20
Site	
Cheek	5
Mouth floor	6
Tongue	15
Trigonous	2
Gingiva	10
Lip	1
Palate	3

	N0	N1	N2	Total
T1	18	2	4	24
T2	6	3	3	12
T3	0	0	1	1
T4	4	0	1	4
Total	28	5	9	42

(18-22). Many brilliant strategies, some of them already one step ahead of clinical trials, have been also devised in order to inhibit this complex enzyme for anti-cancer therapy (23,24).

On the basis of all these data and considerations, we planned to evaluate the role played by hTERT gene expression in human oral carcinogenesis. This study had two main objectives: i) evaluation of hTERT gene expression in the various steps of oral carcinogenesis; and ii) finding a possible correlation between hTERT gene expression, prognostic clinico-pathological parameters, and survival rate in OSCC-bearing patients with appropriate molecular methodology (RT real-time PCR) and canonical immunohistochemical analytical approach (immunohistochemistry).

Materials and methods

Patients and samples. Forty-two OSCC patients were randomly chosen among those possessing a clinical follow-up of at least 3 years (Table I). Biopsies were taken from normal oral mucosa, cancerous (cancers of different histological grade and clinical stage) or precancerous (peri-tumoral dysplasia) lesions, and metastatic lymph nodes. Formalin-fixed paraffin-embedded serial sections were obtained and used for H&E and IHC. A total of 15 specimens of oral dysplasia and 42 OSCC was obtained. Twenty-two biopsies were flash frozen in liquid nitrogen and stored at -80˚C for RT real-time PCR analysis. Metastatic lymph nodes were also collected.

Twenty-two normal mucosa samples were gathered from the same patients biopsing normal tissue far from tumor sites. The epithelial dysplasia grade and the histological diagnosis were established by standard criteria. Dysplasias were classified according to the WHO classification (25). Pathological staging of tumors was determined according to the TNM classification of the International Union against Cancer (UICC) (26).

RNA extraction. Twenty-four mg of deep-frozen bioptic tissue was used for total RNA extraction. Total RNA was isolated by using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity of all tested total RNA samples was verified by agarose gel electrophoresis.

Reverse transcription. Samples containing 5 μg of total RNA in a final volume of 100 μl were reverse-transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI), according to the manufacturer's instructions. Random hexamer primers were used and the reaction was incubated for 60 min at 42˚C. The ss-cDNA obtained was used for real-time PCR amplification.

Real-time PCR. Real-time PCR analysis of hTERT gene expression was performed by using the iCycler® apparatus (Bio-Rad) with sequence-specific primer pairs for the genes tested. The housekeeping gene glyceraldehyde-phosphate-dehydrogenase (GAPDH) was used as internal control. The primers used were the following: GAPDH forward (5'-TTG GTA TCG TGG AAG GAC TCA-3') and reverse (5'-TGT CAT CAT ATT GGG CAG GTT T-3'); hTERT forward (5'-AGT GAC CGT GGT TTC TGT GT-3') and reverse (5'-TTG TCG CCT GAG GAG TAG AG-3'). The cDNA was serially diluted and every dilution was run at least in duplicate. The Real-time PCR analysis was performed as follows: initial denaturation step, 95˚C for 3 min followed by 50 cycles of denaturation at 95˚C for 1 sec; annealing, 10 sec at 50˚C; elongation, 8 sec at 72˚C. The IQ SYBR-Green SuperMix (Bio-Rad) was used for real-time PCR monitoring of amplification. Briefly, amplification was performed in a total volume of 20 μl; the reaction mix was performed with 10 μl of 2X IQ SYBR-Green SuperMix, 0.5 μl of each primer (16 μM) and 2 μl of cDNA (or water as control, which was always included). The real-time PCR products were run on 2% agarose gel containing TAE (standard Tris-acetate-EDTA electrophoretic buffer). The amplicons of expected size were extracted, purified and controlled for sequences by Biogem DNA Sequencing Core (Biogem, Naples). Results were evaluated by ICYCLER IQ real-time Detection System Software® (Bio-Rad, Hercules, CA). Data were calculated on the basis of the threshold cycle (Ct) value. The expression of the analyzed genes was first normalized with respect to GAPDH transcript level and then the value corresponding to each pathological sample was compared with its specific relative normal mucosa counterpart. The observed difference

Table II. hTERT gene expression and tissue distribution of Est2 protein.

Case		RT Real-time PCR ($2^{\Delta Ct}$) (mean ± SEM)	IHC distribution of human Est2 (mean ± SD of 10 HPF)		
			Atypical cells	Phlogistic cells	Cell type
1	OPL	4.00±0.50	33±18	5±2	L, G
2	OPL	0.50±0.03	10±2[a]	ND	ND
3	OSCC	0.29±0.03	8±2[a]	ND	ND
4	OSCC	9.84±1.70	60±4	10±4	L
5	OSCC	0.59±0.02	9±3	25±3	L, G
6	OSCC	0.33±0.02	20±5[a]	5±3	L, G
7	OSCC	1.87±0.15	35±5	5±2	L
8	OSCC	0.57±0.03	5±2	20±4	L
9	OSCC	26.00±2.50	80±7	10±5	L, G
10	OSCC	39.00±3.40	90±5	80±9	L
11	OSCC	0.17±0.01	5±3	25±3	L
12	OSCC	1.23±0.08	34±6	30±2	L, G
13	OSCC	0.05±0.02	Negative	5±3	L, G
14	OSCC	ND	Negative	5±2	L
15	OSCC	2.5±0.2	70±5	23±2	L
16	OSCC	39.4±0.24	10±3	22±2	L
17	OSCC	1.7±0.4	40±3	10±2	L
18	OSCC	31.5±0.7	40±4	45±2	L
19	OSCC	25.9±0.25	70±3	24±2	L
20	OSCC	1.8±0.06	10±1	12±2	L, G
21	OSCC	2.1±0.2	20±2	11±2	L
22	OSCC	1.9±0.1	10±1	8±2	L
23	OSCC	6.9±0.5	90±5	4±2	L, G
24	OSCC	1.4±0.1	5±1	4±2	L
25	Met L	5.65±0.80	20±3[a]	-	-
26	Met L	0.35±0.04[b]	ND	-	-
27	Met L	4.00±0.05	15±3[a]	-	-

$2^{\Delta Ct}$, the pathological/normal-mucosa gene expression ratio, as evaluated by mRNA analysis of TERT. [a]Evaluated only in few selected focally immunostained areas; [b]histopatological analysis revealed <70% of tumor cells spreading in the lymph node studied. IHC, immunohistochemistry; L, lymphocytes; G, polymorphonucleated granulocytes; OSCC, oral squamous cell carcinoma. OPL, oral pre-malignant lesion with ascertained histological dysplasia, obtained from peritumoral mucosa; Met L, lymph-node metastases; ND, not determined.

was expressed as $2^{\Delta Ct}$ (pathological/normal mucosa gene expression ratio, as evaluated by mRNA analysis).

Immunohistochemistry. Four-μm serial sections from formalin-fixed and paraffin-embedded blocks were cut and mounted on poly-L-lysine-coated glass slides. Immunostaining was performed by linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP). After sequential deparaffinization and hydration, the slides were treated with 0.3% H_2O_2 for 15 min to quench endogenous peroxidase. Antigen retrieval was performed by microwave heating - a first time for 3 min at 650 W, a second and a third time for 3 min at 350 W - the slides immersed in 10 mM citrate-buffer pH 6.0. After heating, the sections were blocked for 60 min with 1.5% horse serum (Santa Cruz Biotechnology) diluted in PBS buffer before reaction with the primary antibody (Ab). Primary Ab had been diluted with 0.05 M Tris-HCl buffer pH 7.4 containing 1% bovine serum albumin and incubated at optimal dilution and time. The primary Ab and the conditions for their use were: 1:250-diluted rabbit polyclonal anti-hEst2 (EST22-A, IgG; Alpha Diagnostic International, San Antonio, TX, USA) for 120 min at 24˚C; the specificity of anti-Est2 and its immunohistochemical utilization technique has been previously described (27). After two washes with PBS, the slides were treated with biotinylated species-specific secondary antibodies and streptavidin-biotin enzyme reagent (Dako, Denmark), and the color developed by 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's haematoxylin and mounted using xylene-based mounting medium. Negative control slides without primary antibody were included for each staining.

The results of the immunohistochemical staining were evaluated separately by two observers. Immunostained cells were counted in at least 10 high power fields (HPF) analyzed

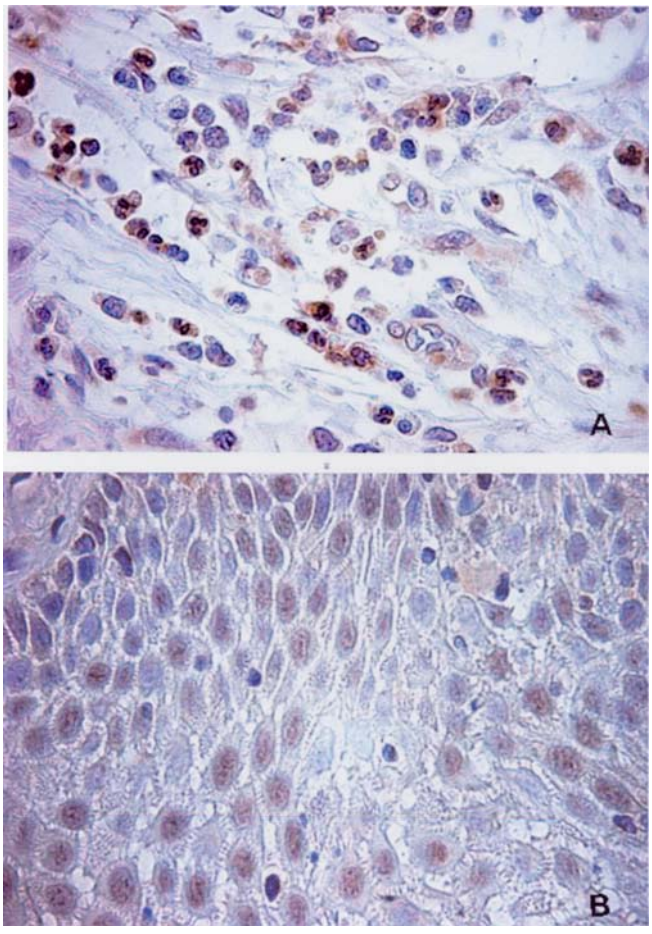

Figure 1. hTERT expression in non-neoplastic cells. (A) Immunostaining of h-TERT protein in oral tissue distant from the tumor in a patient affected by OSCC. Note the occurrence of hTERT expression in lymphocytes and granulocytes in the chorion. (B) Immunostaining of hTERT protein in oral epithelial tissue distant from the tumor in a patient affected by OSCC. The epithelium shows basal hyperplasia, spongiosis, but no cytological atypia. Interestingly, this case shows hTERT expression localized in the nuclei of almost all the cells (LSAB-HRP, x560; DNA counterstaining with haematoxylin).

by a light microscope. Staining patterns were also evaluated and recorded as nucleolar, nuclear, cytoplasmic, or diffuse nuclear and cytoplasmic.

For each case, the cumulative percentage of positive cells in all sections examined was determined. Two of the authors recorded, blindly and independently, the same slides of each case to evaluate the inter-observer variation by the K-test.

Statistical analysis. The data were analyzed by the Stanton Glantz statistical software 3 (MS-DOS) and GraphPad Prism software version 4.00 for Windows (Graph Pad software San Diego, CA, USA; www.graphpad.com). Differences between groups were determined using the one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. Only p-values <0.05 were considered significant. Differences in survival rate were also analyzed by Kaplan-Meier's method and differences in survival curves were studied by the χ^2-test. Hazard ratio and 95% confidence interval were also calculated.

The survival rate curve analysis was performed only in stage I (T1N0) patients to avoid the bias introduced in mortality calculation at the advanced stage of disease.

The cut-off value for immunohistochemistry to segregate high expressing from low expressing cases was 30% immunostained cells, as calculated by counting at least 1000 tumor cells. The cut-off value for RT real-time PCR hTERT-over-expressing cases was a number of fold increase >1.5 units.

Results

hTERT expression in phlogistic cells. By means of immunohistochemical staining we were able to show expression of hTERT in lymphocyte and granulocyte cells localised in the chorion beneath OSCC lesions or in tumor infiltrating lymphocytes (Table II, Fig. 1A).

hTERT expression in normal tissue of OSCC patients. Immunohistochemical staining showed focal expression of hTERT localized to basal and parabasal layers of normal epithelium. In the surrounding tumor epithelium immunostained nuclei were focally evident also in the intermediate spinous layer and in superficial layers (Fig. 1B).

hTERT activation occurs in OPL. The gene expression evaluation by real-time PCR of hTERT in different samples of OPL showed up-regulation of this gene in 50% of tested pre-malignant lesions surrounding full-blown OSCC. Immunohistochemical staining for hEst2, however, was detected in all cases of tested OPL, if positivity was detected in at least one selected high power field (HPF). The percentage of stained cells in OPL was significantly higher than in normal epithelium ($p<0.05$). Focally, the epithelium surrounding the tumors showed immunostained nuclei also in the intermediate spinous layer and in the superficial layers. In contrast, in oral dysplasias diagnosed on the basis of classical histological parameters, all the epithelial layers (basal, intermediate-spinous and superficial) were immunostained in the great majority of cases. Furthermore, in severe oral epithelial dysplasias full thickness epithelium was stained *in situ* with a nuclear and cytoplasmic pattern of expression (Fig. 2A, B).

These findings show that hTERT gene expression is significantly up-regulated with an altered pattern of expression throughout the epithelium. These changes in surrounding tumor epithelium occur early in oral carcinogenesis and appear to be independent from histological diagnosis of premalignant lesion.

hTERT activation occurs in OSCC. The hTERT gene expression evaluated by real-time PCR in tumor samples was found to be up-regulated in 14 out of 21 cases (66.7%) (Fig. 3, Table IV). These data were statistically significant if compared to 22 normal mucosa samples (ANOVA, $p=0.015$; Student-Newman-Keuls, $p<0.05$) (Table IV). However, in 6 cases out of 21 (28.6%) hTERT mRNA was significantly decreased, whereas in 4.7% of the cases this gene was either transcriptionally silent or expressed at a level not different from that of the normal control epithelium.

By means of immunohistochemistry hEst-2 protein was up-regulated in the great majority of OSCC (Table III, Fig. 2C, D, Fig. 4). Furthermore, the immunohistochemical data indicated an hTERT distribution rather different according to the tumor histological grade (Fig. 5), onset site (Fig. 6) and size (Fig. 7).

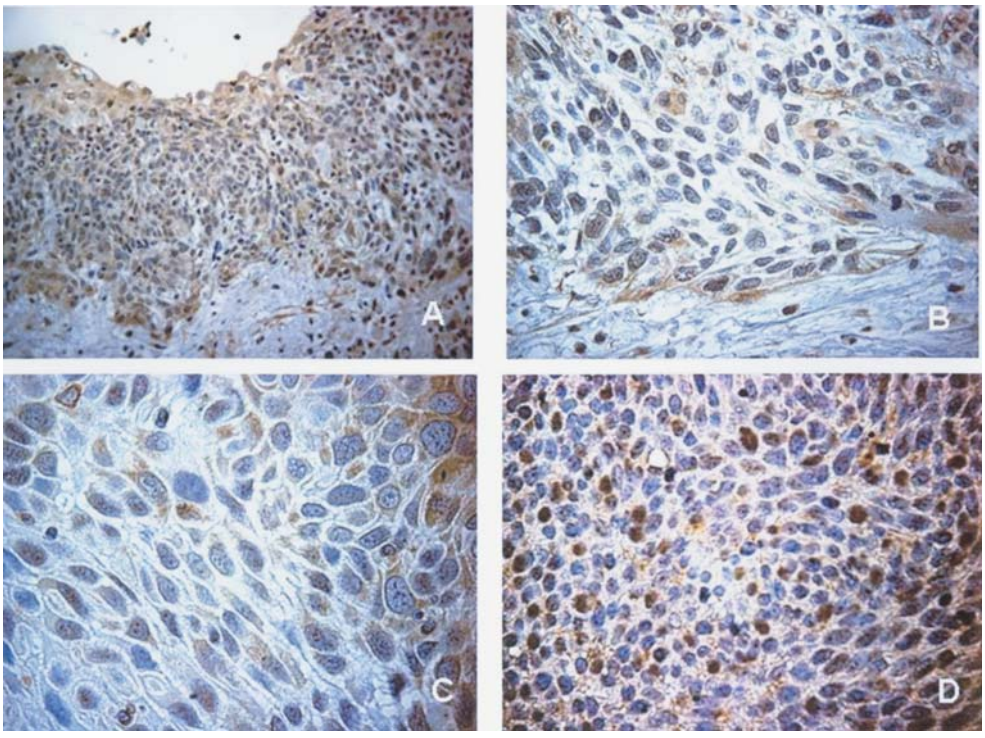

Figure 2. hTERT expression in oral carcinogenesis. (A) A case of severe dysplasia showing nuclear hTERT expression in most atypical cells. Note also the positive immunostaining of lymphocytes beneath the lesion (LSAB-HRP, x100; DNA counterstaining with haematoxylin). (B) A detail of picture A at higher magnification showing the nuclear expression of hTERT in atypical cells (LSAB-HRP, x200; DNA counterstaining with haematoxylin). (C) A case of well/moderately differentiated OSCC showing retained desmosomes and other architectural characteristics of squamous epithelium, but with high nuclear hyperchromatism, pleomorphism. Note high hTERT expression in cancer cells (LSAB-HRP, x400; DNA counterstaining with haematoxylin). (D) A case of poorly differentiated OSCC showing loss of architectural characteristics of squamous epithelium, retaining only focal desmosomes, high nuclear/cytoplasmic ratio, but with high nuclear hyperchromatism and pleomorphism. hTERT-immunostained nuclei are detectable in at least 30% of cells (LSAB-HRP, x200; DNA counterstaining with haematoxylin).

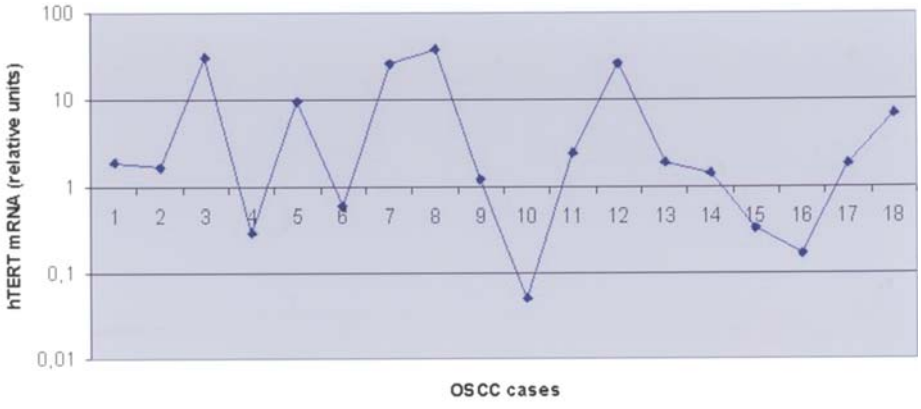

Figure 3. Quantitative deregulation of hTERT RNA in OSCC. Real-time PCR analysis of RNA isolated from pathological samples of OSCC compared to matched normal mucosa.

Table III. Statistical evaluation of hTERT protein expression in OSCC as evaluated by immunohistochemistry.

Groups	N	Mean ± SEM Ct	ANOVA	Student-Newman-Keuls
1) Normal epithelium	22	10.0±0.75	p=0.006	p<0.05
2) OSCC	42	23.7±3.48		

Group 1, hTERT positive cells of normal epithelium. Group 2, hTERT positive cells of OSCC samples.

Table IV. Statistical evaluation of hTERT gene expression in OSCC as evaluated by RT real-time PCR.

Groups	N	Mean ± SEM Ct	ANOVA	Student-Newman-Keuls
1) Normal epithelium	22	26.5±0.61	p=0.015	p<0.05
2) OSCC	22	23.87±0.86		

Group 1, h-TERT/GAPDH normalized Ct values of normal epithelium samples. Group 2, h-TERT/GAPDH normalized Ct values of OSCC samples. SEM, standard error of means.

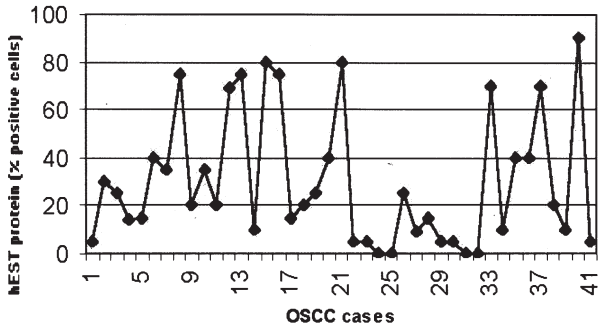

Figure 4. Quantitative deregulation of hTERT protein in OSCC as evaluated by immunohistochemistry.

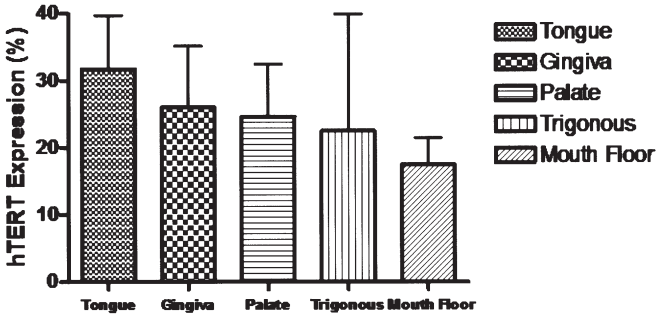

Figure 6. Immunohistochemical expression of hTERT (hEST-2) in 42 cases of OSCC grouped according to the site of localization in oral cavity.

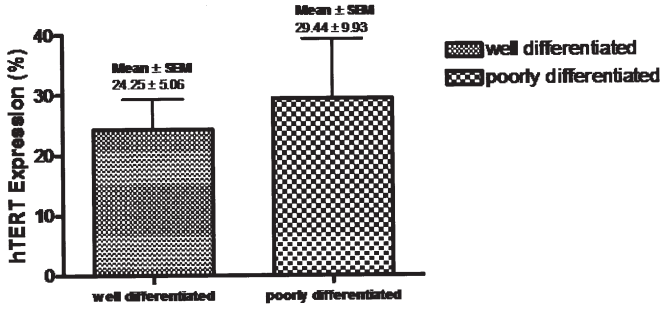

Figure 5. Immunohistochemical expression of hTERT (hEST-2) in 42 cases of OSCC grouped according to the degree of histological differentiation.

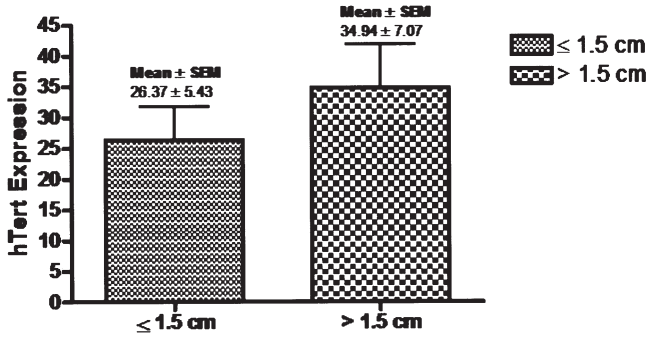

Figure 7. Immunohistochemical expression of hTERT (hEST-2) in 42 cases of OSCC grouped according to tumor size.

The highest gene expression level has been found in low differentiated tumors (mean ± SEM = 29.44±9.93) rather than in those well differentiated (mean ± SEM = 24.25±5.06) and in neoplasms with a size higher than 1.5 cm rather in those with sizes smaller or equal to this value (mean ± SEM = 34.94±7.07). The comparison between the hTERT gene expression data and the clinico-pathological findings is, however, not statistically significant.

In contrast, by separating the patients into two groups on the basis of their high or low level of hTERT expression evaluated at both mRNA and protein level, we were able to demonstrate statistically significant differences in stage I (T1N0) patients (Figs. 8 and 9).

The survival rate of high hTERT expressing patients decreased early after initial surgical treatment because of locoregional recurrences and metastases (Figs. 8 and 9).

The 3-year average survival of high hTERT-expression patients was 2 months, which vas significantly lower than that of the low hTERT-expression patients (16 months) (p=0.04 χ^2-test; OR 95% CI of ratio = 1.105-19930) (Fig. 8). This difference has been further confirmed in a higher number of cases analyzed at protein level by immunohistochemical techniques (p=0.03 χ^2-test; OR 95% CI of ratio = 0.03072-0.9019) (Fig. 9).

Discussion

In human epithelium three principal and overlapping tumor suppressor barriers [the $p16^{INK4a}$-retinoblastoma protein ($p16^{INK4a}$-Rb) pathway; the ARF-p53 pathway; telomeres] appear to be operative. The role of tumor suppressor inhibition has been demonstrated also in oral carcinogenesis, in which

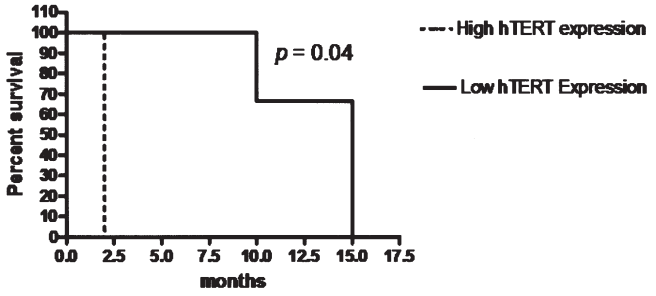

Figure 8. Overall survival rate in stage I patients according to hTERT expression as evaluated by real-time PCR. High hTERT expression patients (dotted line) showed a survival rate worse than that of low hTERT expression patients (solid line).

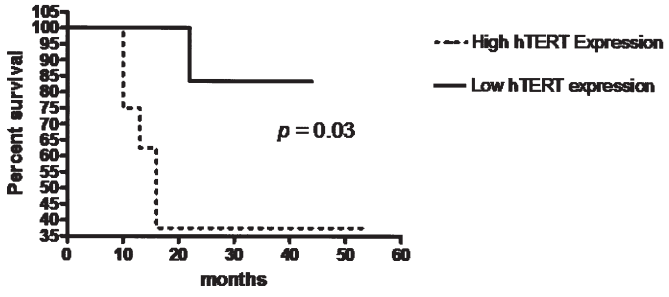

Figure 9. Overall survival rate of patients in stage I according to hTERT expression as evaluated by immunohistochemistry. High hTERT expression patients (dotted line) showed a worse survival than that of low hTERT expression patients (solid line).

both $p16^{INK4a}$-retinoblastoma protein ($p16^{INK4a}$-Rb) and ARF-p53 pathways have been shown to be actively engaged (28,29). However, only few molecular studies have been performed on the activation state of the TMM in oral carcinogenesis (16).

The repression of telomerase in mammalian cells results in shortening of telomeres with each cell division, and ultimately in chromosomal instability, aging, and cell death (30).

According to the classical paradigm of initiation-promotion-progression with its contention of involvement of mortal differentiated cells as target in the initiation process, it is postulated that, at some point during the multistep process of carcinogenesis, evolving pre-malignant cell populations exhaust their endowment of allowed doublings and accomplish their tumorigenic program by breaching the mortality barrier and acquiring unlimited replicative potential. However, this model has been recently disputed since, according to the cancer stem cell theory, the stem cells or their early progenitors have been suggested to be the real target in the initiation event. The stem cells are naturally immortal and became mortal only when they are induced to terminally differentiate and lose their telomerase activity. Consequently, the initiation process is one that stably and irreversibly inhibits the mortalization of the stem cell (31). Stem cells are defined as having the capacity to divide both symmetrically, producing two daughter cells that are stem cells, and asymmetrically, producing one daughter cell that terminally differentiates and the other that maintains 'stemness'. Therefore, within the concept of initiation of a stem cell, the first step of carcinogenesis is to block asymmetric cell division without interfering with the cell's ability to divide symmetrically.

If the original target cell in the initiation process is a mortal cell, its telomerase activity has been repressed and its telomeres have been lost during the normal tissue differentiation process. Therefore, to immortalize a terminally differentiated or a committed cell means mainly to re-establish telomerase activity and restore telomere length.

The conceptual problem of whether the telomerase activity in cancer is restored in a differentiated or committed cell or if it is preserved in the clonal espansion of a mutated stem cell, is crucial to the understanding of carcinogenesis and to envision the future of cancer therapy. This problem is also complicated by the fact that the heterogeneous tumor tissue contains functionally different subpopulations of cells: a mixture of non-neoplastic cells, non-tumorigenic cancer cells, and cancer stem cells. Furthermore, in some cases cancer stem cells might arise from the mutational transformation of normal stem cells, whereas in other cases mutations may cause restricted progenitors or differentiated cells to acquire properties of cancer stem cells such as self-renewal potential.

As we have already mentioned, telomerase expression is low or absent in most human somatic tissues, with its expression principally restricted in the adult to the activated lymphocytes, germ cells, and tissue stem cells. The restricted pattern of telomerase activity relates primarily to the strict regulation of TERT gene transcription, whereas the expression of the gene coding for the RNA component of telomerase (TERC) is broader in distribution. However, telomere maintenance is evident in virtually all types of malignant cells. It has been found that 85-90% of them maintain telomere length by up-regulating expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA, while the remainder have invented a way of activating a mechanism, termed ALT, which appears to maintain telomeres through recombination-based inter-chromosomal exchanges of sequence information. Both mechanisms seem to be strongly suppressed in most normal human cells in order to deny them unlimited replicative potential. In contrast, telomere length abnormalities appear to be one of the earliest and most prevalent genetic alterations acquired in the multistep process of malignant transformation. In fact, Meeker *et al* reported that, in epithelial carcinogenesis, the percentage of intraepithelial neoplasia lesions showing telomere length abnormalities is 95.6% (32). The implications of these findings include the potential that telomere length assessment *in situ* may be a widely useful biomarker for monitoring disease prevention strategies and for improved early diagnosis (32).

The induction of hTERT expression has been reported to be progressive and to occur throughout the entire process of oral carcinogenesis (33,34).

Our study demonstrated that the normal strict regulation of hTERT gene transcription is widely deregulated in the cancer cells of OSCC, since it appears to be highly up-regulated in 66.7% of analyzed tumors. Only in <5% of tumors is this gene unaltered or undetectable. The marked levels of hTERT gene expression have been further confirmed by immunohistochemistry analysis in an even higher number of cases. Furthermore, immunohistochemical study of hEst-2 protein

demonstrated not only overexpression in 50% of pre-cancerous lesions and in 90% of OSCC but also an altered expression pattern of hTERT protein both in OPL and in OSCC.

In our study, hTERT expression was detectable in some cases of peritumoral normal-appearing tissues and in dysplastic tissues of patients who further developed oral cancer. Furthermore, our results are in part inconsistent with a previous report (35), which demonstrated telomerase activity by TRAP assay in human oral tissues with moderate or higher grade of dysplasia but not in those with mild dysplasia, since we have found a very early expression of hTERT in the process of oral carcinogenesis. In this study, hTERT expression was detected in the basal cells of normal oral mucosa, and the cells expressing this protein were also seen in the upper layer of oral pre-malignant epithelium. These data are in agreement with other reports indicating that there are at least two steps in the increase of telomerase activity during carcinogenesis in oral squamous cells: i) a change in the distribution of cells expressing telomerase; and ii) an overexpression of hTERT in individual cells (36).

Interestingly, our study showed up-regulation and an altered pattern of expression of hTERT not only in dysplasias and carcinoma *in situ*, but also in epithelial tissues with changes not fulfilling all the histological criteria for dysplasia. These findings suggest that hTERT may be a useful additional marker to detect patches of epithelium at high risk for developing further genetic changes, leading to invasive cancer, especially if it is used together with other accredited markers in defining the carcinogenesis risk.

Furthermore, the present study demonstrates that great caution is required in diagnosis of pre-cancerous lesions or cancer based only on a molecular biology approach even if a high-sensitivity assay such as real-time PCR and an 'hallmark' of cancer such telomerase are employed, since a considerable number of false negatives are known to exist.

Our data, in line with previous studies (37), demonstrate that there is no significant relationship between hTERT expression and several clinico-pathological parameters, such as tumor stage, size, and histological grade. Several authors, however, have suggested that telomerase activation is an early event during neoplastic transformation *in vivo* and is frequently related to the proliferation rate of cancer cells (38,39). At later stages, many solid malignant tumors, most probably as a consequence of a critical size increase and insufficient vascularization, become necrotic in their central region and are associated to a marked down-regulation of hTERT gene expression (39).

It is on the basis of these data and considerations that we decided to evaluate the survival rate only in stage I (T1N0) patients by comparing this vital parameter in individuals with high levels of hTERT expression vs. those endowed with low levels of expression. The statistically significant differences observed suggest the critical value of our findings in the clinico-therapeutical decisions concerning these patients. It is well known, in fact, that whilst in T2-T4 patients neck dissection is indicated as elective or therapeutical, in the T1N0 patient, even though the diagnostic procedures of clinical staging are widely improved, elective neck dissection is still controversial due to the high morbidity and mortality risks related to this type of surgical intervention (40-45).

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