ORAL DISEASES

Oral Diseases (2010) 16, 61–67. doi:10.1111/j.1601-0825.2009.01614.x

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ORIGINAL ARTICLE

Detection of survivin mRNA in healthy oral mucosa, oral leucoplakia and oral cancer

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BACKGROUND: Survivin is involved in modulation of cell death and cell division processes. Survivin expression in normal adult tissues has not been fully understood, although it is markedly lower than in cancer, where it is over-expressed.

OBJECTIVE: To investigate survivin expression in normal, potentially malignant and cancerous oral mucosa.

METHODS: We measured survivin mRNA levels by real-time RT-PCR in specimens of oral mucosa (15 from normal mucosa, 17 from potentially malignant lesions, 17 from neoplasms). Scores were compared using Kruskal–Wallis test and *post hoc* according to Conover. Chi-squared test was used for dichotomous data.

RESULTS: The median relative levels of survivin mRNA resulted six for normal mucosa, eight for potentially malignant lesions, 13 for cancers: differences among these three groups were statistically significant, as between cancer and potentially malignant lesions. Expression in normal mucosa and potentially lesions group showed no significant difference. Low, but not marginal expression of survivin in normal mucosa is a new finding, and it could be explained with the higher sensibility of our methods.

CONCLUSIONS: Survivin expression in oral potentially malignant lesions might indicate a progressive deregulation of expression paralleling oncogenesis, particularly during the first stages of process, suggesting a putative predictive role for survivin.

Oral Diseases (2010) **16**, 61–67

Keywords: survivin; oral cancer; leucoplakia; oral; potentially malignant lesions

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Received 19 January 2009; revised 3 June 2009; accepted 24 June 2009

Introduction

The balance between cellular survival and death represents an essential crossroad to preserve a normal homeostasis and to avoid potentially dangerous cells replication. Survivin is a protein involved both in the cell death regulation and in different aspects of cell division (Li *et al*, 1998; Tamm *et al*, 1998; Li and Altieri, 1999; Chen *et al*, 2000; Grossman *et al*, 2001; Jiang *et al*, 2001; Fortugno *et al*, 2002; Salvesen and Duckett, 2002; Li and Ling, 2006).

Survivin is a member of the IAP family (Ambrosini *et al*, 1997; Salvesen and Duckett, 2002), a cluster of genes playing an important role in apoptosis regulation. Survivin is widely expressed in embryonic and foetal organs, while its expression in adult differentiated tissues is not well clarified: high expression has been shown in haematopoietic and immune system, and in tissues characterized by high self renewal and proliferation (Fukuda and Pelus, 2006; Li and Brattain, 2006). We also know that expression levels in normal tissues are significantly lower than in cancer cells (Adida *et al*, 2000; Tanaka *et al*, 2000; Kamihira *et al*, 2001; Kato *et al*, 2001; Satoh *et al*, 2001; Koch *et al*, 2002; Kuttler *et al*, 2002; Wurl *et al*, 2002; Badran *et al*, 2003; Gazzaniga *et al*, 2003; Grabowski and Scherubl, 2003; Kajiwara *et al*, 2003). For this reason, survivin has been proposed as a diagnostic and therapeutic target in human cancer (Chen *et al*, 2000; O'Connor *et al*, 2000; Mesri *et al*, 2001; Yagihashi *et al*, 2001; Andersen and Thor, 2002; Pennati *et al*, 2002; Schwartz *et al*, 2003), and its possible role as a negative prognostic marker has been suggested for many cancers, including tumours of lung, breast, bladder, gastrointestinal tract and haematological malignancies (Adida *et al*, 2000; Sarela *et al*, 2000; Tanaka *et al*, 2000; Kappler *et al*, 2001; Kato *et al*, 2001; Ikeguchi and Kaibara, 2002; Wurl *et al*, 2002; Zaffaroni *et al*, 2002).

Survivin expression has been investigated in potentially malignant lesions of the mouth by immunohisto-

chemical methods (Tanaka *et al*, 2003; Liu *et al*, 2005; Jane *et al*, 2006), and high expression has been demonstrated in lesions undergoing malignant transformation (Lo Muzio *et al*, 2003a; Lin *et al*, 2005). Survivin is also highly expressed in oral squamous cell carcinoma (SCC), and as for others cancers, it might predict poorer prognosis (Lo Muzio *et al*, 2001, 2003b, 2005b; Tanaka *et al*, 2003; Kim *et al*, 2005; Lin *et al*, 2005; Marioni *et al*, 2005; Jane *et al*, 2006). On the other hand, it has been recently suggested that high expression of survivin could be used to identify patients oral SCC who could benefit from radiation therapy (Freier *et al*, 2007).

Aims of the study

The aim of this study was to evaluate mRNA survivin expression in clinically normal, potentially malignant and cancerous oral mucosa. We have also investigated putative correlation between survivin expression and characteristics of patient (gender, age, smoking and alcohol habits) and lesion (site). To address this issue, survivin mRNA expression was measured by real-time RT-PCR. We would expect that survivin mRNA levels are increased in oral cancer specimens; besides we assume that in oral potentially malignant lesions survivin already shows expression abnormalities. These eventual findings could improve our knowledge in oral tumourigenesis process and help clinicians to better manage oral oncological pathology.

Materials and methods

Samples collection

Samples of oral mucosa of subjects attending the Oral Medicine services of the Dental School of Milan and Parma have been collected between March 2003 and February 2005.

Subjects enrolled in the study included (i) patients with a clinical diagnosis of oral SCC and (ii) patients with a clinical diagnosis of leucoplakia (Axell *et al*, 1996), the most common potentially malignant oral lesion. Samples were obtained from diagnostic biopsy specimens. When clinical diagnosis was not confirmed by histopathological examination, the subjects were excluded from the study.

Samples of clinically normal mucosa were obtained from patients undergoing minor oral surgery procedures, such as impacted tooth extraction or cyst removal. Immediately after surgery fresh tissue samples were placed into a vial containing 1 ml of RNA later™ (Ambion, Austin, TX, USA) a solution able to preserve tissue RNA, and then frozen at −80° until RNA extraction.

Informed consent of the donors was always obtained. Study protocol, conducted following the rules established in the Declaration of Helsinky, has obtained the approval of the local ethics committee.

RNA extraction and cDNA synthesis

Total RNA was isolated from tissue samples using a commercial kit, RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically, and 500 ng was reverse transcribed (RT) using High-Capacity cDNA Archive Kit in a final volume of 100 μl (Applied Biosystems, Foster City, CA, USA), as described by the manufacturer. The RT thermal profile was 25°C for 10 min, 37°C for 120 min.

Real-time RT-PCR

Real-time RT-PCR is considered the gold standard for mRNA quantitative evaluation. Survivin mRNA levels were measured by real-time RT-PCR based on TaqMan methodology, using ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Real-time data analysis was performed with geNorm software (Vandesompele *et al*, 2002). According to geNorm, the following three reference genes were identified for their stable expression: succinate dehydrogenase complex, subunit A (SDHA), TATA box binding protein (TBP) and ubiquitin C (UBC). GeNorm algorithm, at first, calculate the geometric average of the multiple reference genes chosen (Normalization Factor, NF) and then calculate the normalized expression of the gene of interest (GOI) by dividing the raw GOI quantity by the appropriate NF. Our results are expressed as relative levels of survivin mRNA referred to the sample, called calibrator that showed the lowest survinin mRNA expression value. The calibrator is the 1x sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator. For the quantification of the reference genes, we used Assay-on-Demand™ FAM-MGB-labelled probes (Applied Biosystems). The assay identification numbers are: Hs00188166_m1 (SDHA), Hs00427620_m1 (TBP), Hs00824723_m1 (UBC). Primers and probe for survivin mRNA were designed using Primer Express software (Applied Biosystems), and their sequences have been previously reported (Falleni *et al*, 2003).

Amplification reactions were performed with TaqMan Universal PCR master mix (Applied Biosystems), using 5 μl of cDNA in a final volume of 25 μl. SDHA, TBP, UBC, primers and probes were added to the reaction mixture according to the manufacturer's directions, while survivin primers and probe were present at 0.3 and 0.1 μM, respectively. All reactions were performed in duplicate. The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistical analysis

Scores of the three groups were compared using Kruskal–Wallis test and *post hoc* according to Conover, using MedCalc. Dichotomous data were analysed by chi-squared test.

Results

Patient characteristics

Samples from 64 patients were collected in the study period and sent to the Unità di Anatomia Patologica dell'Azienda Ospedaliera S. Paolo. Fifteen samples were excluded as mRNA quality was not sufficient, nine from

normal mucosa and six from potentially malignant and cancerous lesions. Ultimately, survivin expression was quantified in samples from 49 patients: 15 samples were from normal gingival mucosa, 17 from neoplasm and 17 from oral leucoplakia.

The group of patients comprised 21 females and 28 males; the mean age was 56.1 (s.d. 17.6 range 18–84). Tables 1 and 2 summarize characteristics of patients and lesions included in the study.

Two cases of leucoplakia showed signs of mild dysplasia (patients 39 and 41) and one of severe dysplasia (patient 18). All oral cancers were SCC.

Table 1 Characteristics of the patients included in the study

	Normal mucosa	*Leucoplakia*	*Cancer*
Male/female	8/7	9/8	11/6
Mean age (s.d.; range)	42.1 (20.2; 18–71)	62.1 (12.2; 42–79)	62.4 (12.7; 33–84)
Smoker	6	9	6
Ex smoker	0	0	3
Never smoke	9	8	8
Drinker	0	3	4
Light drinker	1	3	2
Never drunk	14	11	11

Table 2 High and low survivin expression in the oral sample included in the study

Patient	*Gender*	*Age (years)*	*Smoking*	*Alcohol*	*Diagnosis*	*Localization*	*mRNA survivin expression levels*	
1	M	54	S	ND	Potentially malignant	Tongue	1	Low
2	M	62	NS	ND	Potentially malignant	Tongue	2	Low
3	F	21	NS	ND	Normal	Gum	3	Low
4	F	29	NS	ND	Normal	Gum	3	Low
5	F	71	S	ND	Normal	Gum	3	Low
6	F	75	NS	ND	Potentially malignant	Cheek	4	Low
7	M	18	NS	ND	Normal	Gum	4	Low
8	F	74	NS	ND	Potentially malignant	Gum	4	Low
9	F	72	NS	D	Potentially malignant	Tongue	4	Low
10	F	22	NS	ND	Normal	Gum	4	Low
11	F	71	S	ND	Normal	Gum	5	Low
12	F	50	NS	ND	Cancer	Tongue	5	Low
13	F	70	NS	ND	Normal	Gum	5	Low
14	F	64	NS	ND	Cancer	Tongue	5	Low
15	M	64	NS	ND	Normal	Gum	6	Low
16	M	34	S	ND	Normal	Gum	6	Low
17	M	23	S	ND	Normal	Gum	6	Low
18	M	42	NS	D	Potentially malignant	Cheek	6	Low
19	M	62	NS	ND	Normal	Gum	6	Low
20	M	40	S	D	Normal	Gum	6	Low
21	F	40	NS	ND	Normal	Gum	7	Low
22	M	56	NS	ND	Potentially malignant	Cheek	7	Low
23	F	74	NS	ND	Potentially malignant	Mouth floor	7	Low
24	F	64	S	D	Potentially malignant	Cheek	8	High
25	M	24	NS	ND	Normal	Gum	8	High
26	M	51	S	ND	Potentially malignant	Cheek	8	High
27	M	50	ES	D	Cancer	Tongue	8	High
28	F	79	NS	ND	Potentially malignant	Gum	8	High
29	M	50	S	D	Potentially malignant	Tongue	9	High
30	F	75	S	ND	Potentially malignant	Cheek	10	High
31	M	51	S	D	Potentially malignant	Cheek	11	High
32	M	66	S	D	Cancer	Palate	11	High
33	M	59	S	ND	Cancer	Tongue	11	High
34	F	79	S	ND	Cancer	Tongue	12	High
35	M	61	S	D	Potentially malignant	Cheek	13	High
36	F	61	NS	D	Cancer	Tongue	13	High
37	M	70	ES	ND	Cancer	Cheek	13	High
38	M	84	ES	ND	Cancer	Tongue	13	High
39	M	43	S	ND	Potentially malignant	Cheek	14	High
40	F	82	NS	ND	Cancer	Gum	15	High
41	F	72	S	ND	Potentially malignant	Gum	15	High
42	M	72	S	ND	Cancer	Tongue	16	High
43	M	33	NS	ND	Cancer	Tongue	17	High
44	M	57	NS	D	Cancer	Tongue	17	High
45	M	42	S	ND	Normal	Gum	20	High
46	M	59	NS	D	Cancer	Tongue	24	High
47	M	54	S	D	Cancer	Trigon	25	High
48	M	61	NS	ND	Cancer	Tongue	26	High
49	F	60	S	ND	Cancer	Gum	29	High

M, male; F, female; S, smoker; ES, ex smoker; NS, non-smoker; D, drinker; ND, never drunk; low, white background; high, grey background.

Survivin expression

All samples of normal oral mucosa showed survivin expression comprised between 3 and 8, except one whose expression was 20 (patient 45). The median survivin expression of this group was 6 (range = 3–20). Samples from potentially malignant lesions had higher median survivin expression (8, range 1–15), and two of the three dysplastic lesions had the highest value of this group. The highest survivin expression values were seen in the cancer group whose median survivin expression was 13 (range 5–29) (Figure 1). The differences among the three groups were statistically significant ($P = 0.0001$). *Post hoc* analysis showed a significant difference between cancer and normal mucosa and between cancer and potentially malignant lesions, while the comparison between normal mucosa and potentially malignant samples showed no statistically significant difference. Repeating the statistical analysis excluding sample from patient 45, a normal oral mucosa that showed an out-of-range survivin expression, did not affect the results (sensitivity analysis).

Low expression and high expression samples

The samples were ordered according to survivin expression and then divided into two groups. The 'low expression group', i.e. with expression <8, comprised 23 samples, while the 'high expression group', i.e. with expression ≥ 8, comprised 26 samples (see Table 2). As shown in Figure 2, low expression lesions predominated in the normal group (86%) and high expression lesions in the cancer group (83%), while in the leucoplakia group they were evenly distributed. The difference in distribution was statistically significant as calculated by chi-squared ($P < 0.001$).

We analysed risk factors distribution in groups stratified according to survivin expression. Smoking and drinking have a very similar distribution between low and high expression samples, with the exception of smoking in patients with leucoplakias, that is significantly more common in lesions with high survivin expression ($P = 0.0049$).

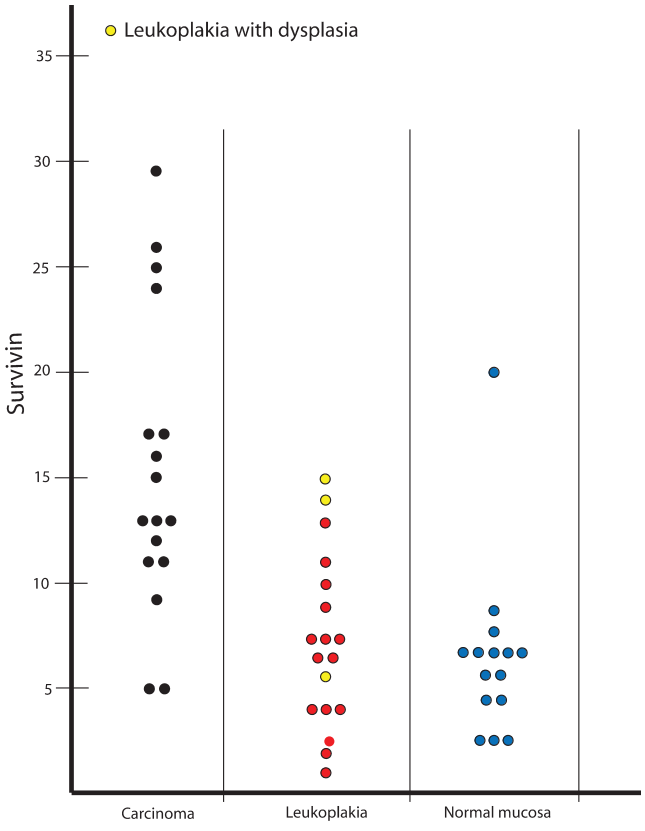

Figure 1 Survivin expression in the group of oral samples

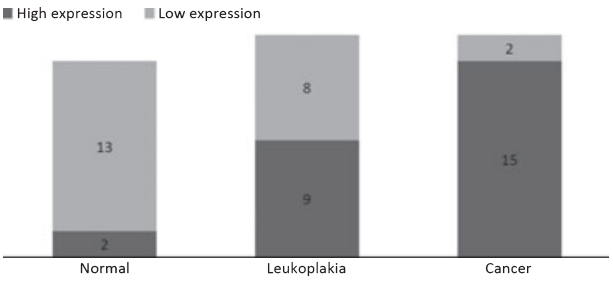

Figure 2 Distribution of high and low expression samples in the three groups of lesions considered

Discussion

Most studies investigating survivin expression in oral lesions employed immunochemistry (Lo Muzio *et al*, 2001, 2003a,b, 2004, 2005a,b; Campisi *et al*, 2005; Lin *et al*, 2005; Marioni *et al*, 2005), a technique that can provide results difficult to measure and highly subjective. Real-time RT-PCR has become the most popular method of objectively quantitating steady state mRNA levels: by using such technique, it was possible to quantify levels of survivin expression, making the assessment less subjective and easier to analyse.

The finding of survivin expression in normal tissue is not completely surprising. Although most studies employing immunochemistry techniques reported no survivin expression in clinically normal mucosa, several studies employing this technique demonstrated survivin expression in basal and parabasal cells of oral mucosa (Weinman *et al*, 2003; Lo Muzio *et al*, 2005a; Marioni *et al*, 2005). Survivin has also been demonstrated in a number of normal tissues, particularly in those with high proliferation rate, as it is critical for mitotic cell division and is upregulated in mitosis (Lechler *et al*, 2007). In our work, survivin expression has been demonstrated in all samples of normal mucosa, although at a low rate (13 out of 15 samples were in the 'low expression' group), this result is probably due to the highly sensible method we employed and to the relatively high proliferation rate of oral mucosa.

Upregulation of survivin in cancer specimens (80% in 'high expression group') confirms the putative role that this protein plays in carcinogenesis processes. However, we believe that the most interesting results are those

from potentially malignant lesions. Whether confirmed, survivin predictive value would represent a clinically relevant finding; in fact, at the moment it is very difficult to predict behaviour of oral potentially malignant lesions. Interpretation of survivin expression in our sample of oral potentially malignant lesions is not easy. In fact, low and high expression lesions are equally represented among leucoplakias (47% and 53% respectively); a possible explanation of such distribution is a progressive deregulation of survivin expression paralleling oncogenesis.

Post hoc test showed no significant difference in survivin expression between normal mucosa and potentially malignant lesions, confirming previous results showing significant difference between potentially malignant lesions and cancer classes, but not between potentially malignant and normal mucosa (Liu *et al*, 2005). However former studies reported also opposite results with significant difference between potentially malignant and normal mucosa, but not between cancer and potentially malignant lesions (Tanaka *et al*, 2003). Such differences can be due to different techniques or, more likely, to different selection and/or nature of the potentially malignant lesions included in the study. In our study, only three out of 17 leucoplakia had histological features of dysplasia, a risk factor for malignant transformation, and two of them showed the highest survivin expression of the group, very close to cancer values. Lin *et al* (2005) have detected survivin positivity staining in 60 of 62 epithelial dysplastic specimens, and mean labelling index of survivin expression in cases undergoing malignant transformation was significantly higher than those which did not transform. Putative predictive value of survivin was also reported by Lo Muzio *et al* (2003a,b) who showed a higher rate of positive samples among lesions evolving into SCC compared with lesions not evolving; in addition they found no significant correlation between survivin expression and the degree of dysplasia. One limit of our study is the small number of samples, particularly of dysplastic lesions. This is due just to chance, as we included all consecutive patients meeting criteria and providing consent. In fact, larger groups of dysplastic leucoplakias would have allowed a more powerful analysis of relationship between a well known risk factor of malignant transformation (dysplasia) and a putative one (survivin expression).

In our study, smoking seems to affect suvivin expression among subject with potentially malignant lesions only; in fact, high expression lesions were significantly more common among smokers, but not among alcohol drinkers. These results need further confirmation, particularly as they seem to be in contrast with a previous study suggesting that survivin may be less expressed in human papillomavirus-positive oral SCCs affecting smokers (Lo Muzio *et al*, 2005a).

Better understanding of survivin significance during oral oncogenesis will be possible when role of survivin different isoforms and subcellular localizations will be clarified (Engels *et al*, 2007). However, to confirm such preliminary results and recommending survivin as a prognostic marker for potentially malignant oral lesions, prospective long-term follow-up studies are needed. Besides, it is likely that, even if reliable biomarkers will be available, an accurate assessment of risk of malignant transformation of leucoplakia and similar lesions will require a multidimensional appraisal, incorporating clinical, histological and molecular features.

Author contributions

All authors contributed extensively to the work presented in this paper. In particular, G Lodi, L Moneghini and S Bosari: study conception and design; R Franchini, C Bez, M Manfredi and P Vescovi: clinical work and data collection; L Moneghini and C Pellegrini: laboratory analysis; G Lodi, R Franchini and A Sardella: statistical analysis; all authors were extensively contributed in manuscript preparation; S Bosari and A Carrassi: study coordination.

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