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European Journal of Cancer 39 (2003) 2306–2309

European Journal of Cancer

www.ejconline.com

Gene promoter hypermethylation in oral rinses of leukoplakia patients—a diagnostic and/or prognostic tool?

M. López[a], J.M. Aguirre[b,*], N. Cuevas[a], M. Anzola[a], J. Videgain[c], J. Aguirregaviria[c], M. Martínez de Pancorbo[a]

[a]*Department of Zoology and Cellular Biology, Faculty of Pharmacy, University of the Basque Country/EHU, Vitoria-Gasteiz 48940, Spain*
[b]*Oral Medicine and Oral Pathology, Department of Stomatology, Faculty of Medicine and Dentistry, University of the Basque Country/EHU, Leioa, 48940 Spain*
[c]*Department of ENT and Maxilofacial Surgery of San Sebastián Oncology Institute, Donostia, Spain*

Received 12 February 2003; accepted 13 February 2003

Abstract

Leukoplakia is the most frequent oral precancerous lesion and shows a variable rate of malignant transformation. We hypothesised that the detection of molecular alterations, like the promoter hypermethylation of DNA, in oral cytological samples from patients at risk of developing primary or recurrent tumours could be a valuable diagnostic and prognostic tool in the management of these lesions. Two groups of patients with differing risks of developing oral squamous cell carcinoma (OSCC) were analysed. DNA was extracted from the oral rinse of each patient. The methylation status of the *p16*, *p14* and *MGMT* gene promoters was determined using a methylation-specific polymerase chain reaction (MSP). Methylation of *p16* and *MGMT* was observed in 44 and 56% of the oral samples, respectively. Only 12% of the cases showed *p14* methylation. DNA hypermethylation was more frequent in patients with previous OSCC. DNA promoter hypermethylation is frequent during early oral carcinogenesis and even more so in the later stages. MSP using oral rinses is a non-invasive and highly sensitive technique which could be used to monitor patients with precancerous and cancerous oral lesions.

Keywords: *p16/INK4a*; *p14/ARF*; *MGMT*; Methylation; Cytological samples; Leukoplakia; Oral squamous cell carcinoma

1. Introduction

The high incidence of oral cancer in the Basque Country (24.1/100 000 in men) compared with other Spanish or European regions (16.4/100 000 in men), means there is a need (in this area in particular) for future preventive strategies [1].

The aetiology of precancerous and cancerous oral lesions is multifactorial, with multiple carcinogenic agents affecting the oral mucosa. The appearance of multiple primary, secondary and recurrent tumours is a major problem in patients with such lesions, affecting both prognosis and treatment strategies [2]. Leukoplakia is the most frequent oral precancerous lesion and is variably associated with malignant transformation; figures ranging from 0.13 to 30% have been reported in the literature [3–5]. The detection of molecular alterations involved in the tumorigenesis of oral squamous cell carcinoma (OSCC) could be prognostic for the evolution of this type of lesion.

Promoter hypermethylation of a growing number of genes that are linked to the development of cancer has been observed in various types of tumours. Examples include tumour suppressor genes ($p16^{INK4a}$, $p14^{ARF}$, $p15^{INK4b}$, *p73, Rb*...), DNA repair genes (O_6-methylguanine-DNA-methyl-transferase (*MGMT*), *hMLH1*, *BRCA-1*...), carcinogen detoxifiers (*GSTP*) and metastasis and angiogenesis inhibitors (*E-cadherin*, *TSP-1*, *DAPK*...) [6]. These epigenetic changes are often associated with the loss of gene expression and appear to be essential for the occurrence of the multiple genetic events that are necessary to drive tumour progression [7].

In OSCC patients, high levels of hypermethylation of the $p16^{INK4a}$ and $p14^{ARF}$ genes [8] and the DNA repair

* Corresponding author. Tel.: +34-946-012924; fax: +34-944-802551.
E-mail address: otpagurj@lg.ehu.es (J.M. Aguirre).

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doi:10.1016/S0959-8049(03)00550-1

gene, *MGMT* [9], have been detected. The presence of epigenetic methylation in these genes might therefore be a useful molecular target for identifying tumour cells in patients at risk of OSCC. Previous studies have demonstrated that tumour-specific alterations could also be detected in the saliva of head and neck cancer patients [10,11]. The aim of our study was to evaluate $p16^{INK4a}$, $p14^{ARF}$ and *MGMT* promoter hypermethylation in the oral rinse of patients with clinically-benign homogeneous leukoplakia.

2. Materials and methods

2.1. Patients

Samples from 34 patients from the Oncological Institute of San Sebastian were collected and classified into two groups. Group L consisted of 19 patients that had a homogeneous oral leukoplakia when the sample was collected. 11 of these patients were men and 8 were women, with a mean age of 54.7 years (range 25–77 years). There were 15 patients in group LCP (9 men and 6 women with a mean age of 59.4 years (range 34–84 years)). This group had a homogeneous oral leukoplakia and had previously had one or more OSCC.

2.2. Sample collection and DNA extraction

An oral rinse made for 1 min with 10 ml of distilled water and a few hairs together with their roots were collected from each patient and stored at −20 °C until use. Oral samples were centrifuged at 2500 rpm for 15 min and washed with phosphate-buffered saline (PBS) to obtain the cellular pellet of each sample. Supernatants were kept to obtain DNA that was in the medium by ethanol precipitation. The cell pellet was digested in a proteinase K solution (0.2 mg/ml) at 56 °C for 3 h. DNA was extracted by the phenol-chloroform method followed by ethanol precipitation. Hair roots were incubated overnight at 37 °C in proteinase K (0.4 mg/ml) solution and then boiled for 10 min to inactivate the proteinase K.

2.3. Methylation-specific polymerase chain reaction (MSP)

1 μg of DNA from each sample was subjected to bisulphite treatment using the DNA CpGenome Modification Kit (Oncor). The modified DNA was used as a template for the MSP. The polymerase chain reaction (PCR) was performed using specific primers for either methylated or the modified unmethylated sequences for the *p16* [12,13], *p14* [14] and *MGMT* [9] genes. PCR conditions were as follows: 94 °C for 5 min; then 35 cycles of 94 °C for 30 s, a specific annealing step for 30 s (annealing temperatures used were 68 °C for *p16*, and 65 °C for the *p14* and *MGMT* genes), 72 °C for 30 s, and a final extension step of 5 min at 72 °C. A hot start was used and the appropriate negative and positive controls (DNA methylated cell line) were included in each PCR reaction.

10 μl of each PCR product was loaded directly onto 8–10% non-denaturing polyacrylamide gels stained with ethidium bromide. Samples were electrophoresed and visualised under ultraviolet (UV) illumination.

2.4. Statistics

Clinical and genetic data were compared and analysed by the χ^2 test and logistic regression analysis.

3. Results

Aberrant promoter methylation of the genes was detected in 82% (28/34) of the oral rinse samples. 15 cases (79%) of group L and 13 cases (87%) of group LCP exhibited aberrant promoter hypermethylation in at least one of the three genes. Simultaneous analysis of constitutive DNA from patients' hair-roots and oral rinses confirmed that gene methylation detected in cytological samples appeared exclusively in the oral mucosa.

The tumour suppressor gene *p16* was hypermethylated in 44% (15/34) of the patients (Table 1). *p16* promoter hypermethylation was considered positive when both pairs of primers were positive for the methylated reactions (Fig. 1). *p14* promoter hypermethylation was observed in only 4 cases (12%), but 19 patients (56%) showed *MGMT* promoter hypermethylation.

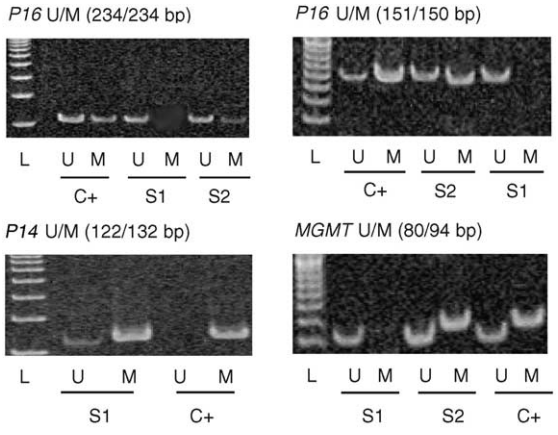

Fig. 1. Representative examples of methylation-specific polymerase chain reactions (MSP) for *p16* (two pairs of primers), *p14* and *MGMT*. In each case, the oral rinse samples (S) of leukoplakia patients are numbered consecutively, C+ represents DNA from a cell line with a methylated promoter of each target gene. Lanes U and M correspond to the unmethylated and methylated reactions, respectively. L represents a DNA ladder.

Table 1
Clinical features of patients who showed promoter hypermethylation of the $p16^{INK4a}$, $p14^{ARF}$ and *MGMT* genes

Patient clinical data	Feature	*N* (%) of $p16^{INK4a}$ methylated	*N* (%) of $p14^{ARF}$ methylated	*N* (%) of *MGMT* methylated
Age (years)	>50	11 (48)	3 (13)	15 (65)
	$\geqslant 50$	4 (36)	1 (9)	4 (37)
Gender	Male	10 (50)	2 (10)	7 (35)
	Female	5 (36)	2 (14)	12 (86)
Leukoplakia localisation	Fom	5 (42)	3 (25)	6 (50)
	Buccal	3 (43)	–	4 (57)
	Palate	3 (75)	1 (25)	1 (25)
	Gingiva	2 (29)	–	5 (72)
	Tongue	2 (40)	–	2 (40)
	Retromolar	–	–	1 (100)
Alcohol–tobbaco habit	Both	9 (53)	3 (18)	10 (59)
	Smoker	3 (75)	–	2 (60)
	Drinker	1 (33)	–	2 (67)
	None	2 (20)	1 (10)	5 (50)
Patients group	L	7 (37)	1 (5)	9 (47)
	LCP	8 (53)	3 (20)	10 (53)
Total		15 (44)	4 (12)	19 (56)

Fom, floor of the mouth; L, patients with leukoplakia; LCP, patients with leukoplakia and previous oral squamous cell carcinoma (OSCC); MGMT, O_6-methylguanine–DNA–methyltransferase.

DNA hypermethylation was partially detected for all of the genes studied (Fig. 1), probably due to the cellular heterogeneity of the samples, but possibly due to a genuine inactivation of these genes.

The DNA hypermethylation detected was independent of the localisation of the leukoplakias. No association was found between overall hypermethylation and the clinical characteristics of the patients. However, the highest frequency of hypermethylation was observed in patients over 50 years of age who were smokers and/or drinkers. The LCP group showed a higher rate of hypermethylation, especially of the *p14* gene, although the numbers were small.

4. Discussion

The study of gene inactivation through promoter hypermethylation in human cancers has been facilitated by the recent development of the MSP technique [12]. The high sensitivity (1:1000) and specificity of the method allow the accurate detection of tumour cells.

Promoter hypermethylation of the *p16* and *MGMT* genes was previously identified in primary tumours and matched salivary samples of head and neck patients [11] but not in patients with oral precancerous lesions. Our study demonstrated, for the first time, abnormal hypermethylation in oral samples from patients at risk of OSCC.

The levels of *p16* and *MGMT* promoter hypermethylation identified in the oral rinses from our leukoplakia patients were similar or higher than those previously observed in tumours from head and neck and OSCC patients [8,15]. Moreover, the high frequency of hypermethylation in the samples of patients with leukoplakia (Group L) suggests that *p16* and *MGMT* promoter hypermethylation might be an early event in oral carcinogenesis.

By contrast, the level of hypermethylation of the *p14* gene was very low in patients in group L and increased in the LCP group suggesting that *p14* promoter hypermethylation may be mainly a late step during oral tumorigenesis. Higher frequencies of hypermethylation of *p14* in OSCC patients have been reported before in Ref. [8].

Hypermethylation of the *p16*, *p14* and *MGMT* genes is an independent event in OSCC [14]. The epigenetic defect in the DNA repair gene *MGMT* could predispose to multiple nucleotide changes in the genomic material [6], thereby becoming a key element in tumorigenesis. Aberrant promoter hypermethylation of the *p16* and *p14* genes may affect the *p16*/*Rb* and *p14*/*p53* tumour suppressor pathways, respectively.

DNA hypermethylation was observed in almost all of the leukoplakia patients. However, not all leukoplakias undergo malignant transformation. Hypermethylation events might lead to a genetic instability, but other genetic alterations involving multiple pathways are necessary for a neoplastic conversion. Long-term follow-up studies are therefore needed in order to reach definitive conclusions regarding the role of DNA hypermethylation in oral tumorigenesis.

Clinical-pathological data, in combination with the use of oral rinses to distinguish DNA hypermethylation, might be a reliable and valuable, diagnostic and prognostic tool in patients who are at risk of developing OSCC. The technique may also enable the risk of recurrences to be determined. To conclude, molecular analysis of oral rinse samples may offer a cost-effective approach for the early detection of OSCC in a population who are at risk.

Acknowledgements

This work has been supported by Grants from the University of the Basque Country (UPV I02-EA134/96, UPV I02.I02-TB 128/97); the Basque Government (PI-97/27) and FIS project/Basque Government Health Department (99/09409). The authors thank Mariana Villarroel for helping with the editing of the manuscript.

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