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Original Communication

Single nucleotide polymorphisms of hypoxia inducible factor-1 alpha in early-stage oral squamous cell carcinoma: Influence in second primary tumours

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ABSTRACT

Second primary tumours are still a challenge in patients treated for oral squamous cell carcinomas (OSCC). Hypoxia Inducible Factor-1 Alpha (HIF-1∝) polymorphisms have been correlated with susceptibility to head and neck carcinomas in a previous study of the group, however their influences in these second neoplasms need to be studied. Genotypic distribution of C1772T and G1790A polymorphisms is analyzed in a series of 124 patients with early-stage OSCC (pT1 and pT2) and in 26 patients of this group with second primary tumours. Frequencies of polymorphic T and A alleles between patients and a sample of healthy subjects were compared using the Chi square test. Survival analysis of second primary tumours was also studied with Kaplan-Meier curves and Long-rank test. G1790A polymorphism was associated with second primary tumours as A allele was most frequent in patients than in healthy subjects (50% and 6.5% respectively; $p<0.01$). This polymorphism was also correlated with the development of intraoral carcinomas (frequency of A allele of 39.7% in patients and 6.5% in healthy subjects; $p<0.01$). Survival rate for patients with secondary neoplasms was lower than patients without this condition (78.9% 5-year cumulative survival rate and 92.4% respectively; $p<0.01$). G1790A is a factor of susceptibility to intraoral carcinomas and to second primary tumours in patients treated for OSCC. Patients with secondary neoplasms have poorer prognosis, so they may need more aggressive therapies and long-term follow-up.

KEYWORDS: second primary tumours, HIF-1∝ polymorphisms, early-stage OSCC, oral squamous cell carcinoma, G1790A polymorphism

INTRODUCTION

Treatment of OSCC is still a challenge for Head and Neck surgeons and oncologists. Mortality rates have not decreased significantly in the last decades although there are better diagnostic techniques and new oncologic therapies. Early-stage OSCC presents a high rate of loco-regional recurrences (range: 14% to 48%) that decreases significantly the survival rate of this small tumour with a theoretically good prognosis [1-3]. A better understanding of the mechanisms of tumour progression and predictive factors is essential for improvement of survival.

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Second primary tumours represent a cause of failure in patients treated for head and neck carcinomas. The incidence of secondary neoplasms ranges from 12% to 28% among the different series, with a yearly incidence of 2% to 4% [4-8]. In OSCC, second primary tumours are mainly squamous cell carcinomas located in the upper aerodigestive tract [8, 9]. The same carcinogens probably act as inductors of these multiple neoplasms in all the aerodigestive mucosa. However, other secondary cancers different from head and neck location may appear associated to these tumours. Thus, other factors such as genetic predisposition are likely implicated in the development of second primary tumours [10].

In human tumours, it becomes more evident that microenvironment plays an important role in tumour development and treatment response. Tumoral hypoxia is present in a high percentage of solid tumours, due to a misbalance between the high rate of cellular proliferation and the insufficient vascular support [11, 12]. In this hypoxic condition, the activation of several molecular pathways as angiogenesis, apoptosis inhibition and the expression of different cell-cycle proteins occur. Hypoxia-inducible factor-1 (HIF-1) is the key regulator that controls cellular responses to hypoxia [13, 14]. This molecule is a heterodimer with a double helix structure formed by two subunits of 120 and 80 KDa respectively, *HIF-1α* and *HIF-1β* [15]. However, HIF-1 activity depends on HIF-1α subunit that activates the expression of more than 70 genes in hypoxic conditions.

Over-expression of HIF-1α has been correlated with the prognosis of head and neck carcinomas and other carcinomas as colon, breast, prostate, kidney, oesophagus, cervix, endometrium, pancreas and stomach [14-22]. Recently, polymorphisms associated to HIF-1α gene have been related to enhanced expression of this molecule in normoxia conditions [23]. Two polymorphisms, C1772T and G1790A, located in exon 12 of HIF-1α gene and related to the oxygen-dependent degradation dominie have been described. In C1772T polymorphism, a base change of a citosine for a timidine in exon 12 results in an amino acid change of a proline for a serine in codon 582 while in G1790A a change of a guanine for an adenine results in a change of an alanine for a threonine in codon 588 [23-27]. These polymorphisms have been implicated in susceptibility and tumoral progression in head and neck carcinomas and different solid tumours [23-28]. However, their implication in second primary neoplasms associated to head and neck cancers has not been previously studied.

In this study, we analyzed the influence of HIF-1α polymorphisms in OSCC and second primary tumours as well as the impact in prognosis of these secondary neoplasms in patients with intraoral carcinomas.

PATIENTS AND METHOD

Patients

A series of 124 patients with early-stage oral and oropharyngeal carcinomas (pT1N0M0 and pT2N0M0) were selected from the Database of Oncology of the Department of Oral and Maxillofacial Surgery between the period of 1980 to 2002 and from the Samples Registry of the Department of Pathology of the University Hospital La Princesa (Madrid, Spain). The selection criteria for patient's inclusion in the study were:

- All the patients were surgically treated in the Department of Oral and Maxillofacial Surgery.

- None of the patients received previous chemotherapy or radiotherapy.

- All the patients had early tumours (pT1 or pT2), tumours less than 4 cm.

- Patients that received neck dissection in the same surgery of tumour resection had postoperative negative necks (pN0).

The series included 90 men (72.6%) and 34 women (27.4%), with a median age of 57 years (range: 18 to 83 years). Sixty-two tumours were pT1 (stage I of TNM) and 62 tumours were pT2 (stage II). Median follow-up period was of 93 months (range 5 to 242 months). A total of 14 patients (11.5%) received postoperative local irradiation therapy due to different causes: poor differentiated carcinomas, perineural invasion or affected surgical margins. Surgical margins were considered as affected if there was macroscopic or microscopic infiltration in the margin of the

tumour sample or less than 5 mm of normal mucosa. Dates of premalignant lesions, tobacco or alcohol consumption, loco-regional recurrences, histological grade, tumour thickness, perineural invasion and peritumoral inflammatory response were also recorded.

Second primary tumours

The presence of second primary neoplasms, in the group of patients with intraoral early-stage carcinomas, was recorded. These second tumours were classified as previous, synchronous and metachronous, depending on the time of appearance to the index intraoral tumour.

To define a secondary neoplasm, modified Warren and Gates criteria were used [29]:

- The second primary tumour must be histologically malignant.

- The second primary tumour must be a different mass from the index tumour, with at least 2 cm of normal tissue between them.

- Metastasis must be histologically excluded.

- Pulmonary masses must be solitary and histologically different from the index tumour.

Two modifications to classic criteria for intraoral carcinomas were included for this study, as previous reports of head and neck carcinomas talked about intraoral secondary neoplasms as the most frequent location of these tumours:

- The intraoral second primary tumour must appear 5 or more years previous or after the index tumour.

- Tumoral stage of the index tumour must be N0 at diagnosis to exclude extended tumoral disease.

Dates of number of secondary neoplasms, second primary tumour location, histology, premalignant lesions, tobacco or alcohol consumption, index tumour characteristics, index tumour recurrences, death by index tumour or by secondary neoplasms and HIF-1∝ polymorphisms genotype in this group of patients were recorded.

To evaluate mortality rates, cumulative survival was considered, with death by index tumour or second primary tumours as censored events.

Polymorphism of HIF-1∝

For the study of polymorphisms of HIF-1∝, paraffin embedded samples of the index intraoral tumour were taken out from the registry of the Department of Pathology. The DNA was extracted from previously deparaffinised tumours and submitted to PCR-RFLP technique *(Polymerase chain reaction-Restriction fragment length polymorphism)*. Data processing was carried out so that patients' confidentiality was warranted.

To study genotypic differences between patients and healthy population, a cohort of 148 volunteers were enrolled in the study. All of them signed an informed consent form prior to recruitment.

DNA extraction

Deparaffinised tissue sections of 4 µm thick were incubated at 100ºC for 2 hours in Tris 50 mM and Chelex. These sections were incubated again with 400 µg/ml proteinase K at 55ºC during 2 hours. After heat-inactivation of proteinase K (100ºC, 15 min), the cellular debris was pelleted by centrifugation, and the supernatant transferred to a clean tube.

Blood samples of healthy subjects were processed with a kit, (PureGene Kit. Gentra Systems, MN. USA) according to the manufacturer's instructions.

PCR

Polymerase-chain-reaction (PCR) was performed using the specific primers described by Fransen *et al.* [30]. PCR for the identification of C1772T Single Nucleotide Polymorphism (SNP) was performed as follows: briefly, 500 ng of DNA were amplified in a final reaction volume of 50 µl containing 7.5 pmol of each primer, 2.5 U of Biotools DNA Polymerase (Biotools B&M Labs., S. A. Madrid. Spain), 1x reaction Buffer, 3 mM MgCl2, and 200 µM dNTPs. Forward and reverse specific primers were provided by Metabion International AG. (Martinsried. Germany). The remaining PCR reagents were supplied by Biotools B&M Labs. PCR was carried out in a GeneAmp PCR System 2700 (Applied Biosystems. Foster City. CA. USA). An initial denaturising step of 94ºC for 5 min was followed by 35 cycles of denaturising (94ºC for 1 min), annealing (55ºC for 1 min) and primer extension (72ºC for 1 min), and a final elongation step (72ºC for 7 min). PCR for the detection of the G1790A polymorphism was carried out by reproducing the conditions reported by Fransen *et al.* [30]. Amplification products of 147 bp (C1772T) and

255 bp (G1790A) were visualized on 2.5% and 2% agarose gel electrophoresis respectively (Conda Laboratories, Madrid, Spain).

RFLP

Ten microlitres (10 µl) of PCR products were digested overnight with an appropriate restriction endonuclease. 4U of *Bsc4I* restriction endonuclease (New England Biolabs/ Biotools B&M Labs., S. A. Madrid, Spain) was used for the identification of C1772T. Digestion temperature was 55ºC and the fragments generated were 131 and 19 bp (none visualized on the gel), for the homozygous wild-type, 147 bp (non-cleaved) for the homozygous polymorphic variant and 131 and 147 bp for the heterozygous polymorphic genotype (Figure 1A). The generated products were visualized on 2.5% agarose gel. G1790A genotype was determined with *AciI* restriction endonuclease (New England Biolabs/IZASA., S. A. Madrid, Spain). Digestion was carried out at 37ºC with 8U of AciI and the fragments obtained were: 143 and 114 bp (homozygous wild-type), non-cleaved 255 pb (homozygous polymorphic variant) and all of them (heterozygous polymorphic variant) (Figure 1B). These restriction products were visualized on 2% agarose gel.

Hardy-Weinberg equilibrium

To study the genotypic distribution in the sample of healthy subjects, Hardy-Weinberg Equilibrium was assessed. It was determined by the $\chi 2$ test comparing frequencies observed and frequencies expected in the healthy subjects sample as it was determined by Hardy and Weinberg in their mathematical model [31, 32]. We proved that the cohort of healthy subjects was homogeneous and genotypation was correct. Therefore, disequilibrium might be attributed to insufficient sample size.

Statistical analysis

To evaluate polymorphisms of HIF-1∝, a dominant model was used due to the low frequency of homozygous variant genotypes of both G1790A and C1772T. For G1790A polymorphism, we grouped subjects in two categories: homozygous wild-type or G/G genotype and the presence of A allele, combining homozygous and heterozygous polymorphic variants in a single group (G/A and A/A). For C1772T polymorphism, the distribution of genotypes was similar: C/T and T/T in a single group and C/C in another group.

Differences in genotypic distributions between healthy subjects and patients were analyzed by the

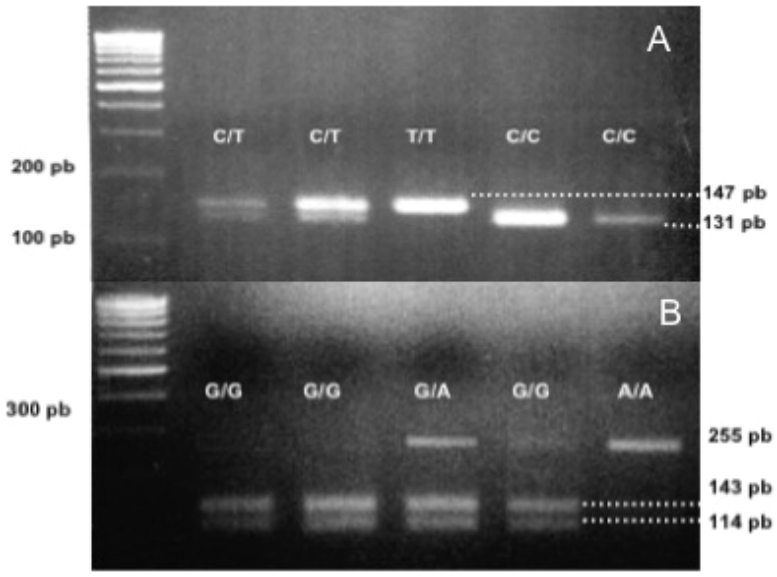

Figure 1. Polymorphisms of HIF-1∝. A: Products generated for C1772T after digestion with Bsc4I restriction endonuclease and visualized in agarose gel 2.5%. B: Products generated for G1790A after digestion with AciI and visualized in agarose gel 2%.

$\chi 2$ test. To evaluate the influence of polymorphisms of HIF-1∝ in susceptibility to secondary neoplasm, we also analyzed differences between second primary tumours group and healthy subjects. In this group of second primary tumours, association with other variables of the study was analyzed. When the assumption of the $\chi 2$ was violated, Fisher's exact test was performed.

Survival analysis for secondary neoplasms was performed by Kaplan Meier curves and Log-rank test. Cumulative survival was defined as the period from surgery of the index tumour to death of the patient by this primary oral carcinoma, death by second primary tumour or last contact. All statistics were calculated using SPSS (version 15.0; SPSS Inc., Chicago, IL) with a two-sided significance level of 0.05.

RESULTS

Descriptive analysis of index tumour group

In the series of 124 patients, 81 cases (65.3%) presented tobacco or alcohol consumption, while 43 cases (34.7%) presented none of these habits. Premalignant lesions were observed in 37 cases (29.8%) and absent in 87 cases (70.2%). Dates of tobacco or alcohol consumption and type of premalignant lesion are shown in Table 1. Location of index OSCC tumour is also shown in Table 1.

There were 47 well-differentiated carcinomas (38.2%), 64 moderate differentiated carcinomas (52%), 12 poor differentiated carcinomas (9.8%) and non-defined in 1 case. Surgical margins were affected in 16 cases (13%) and depth of invasion was more than 2 mm in 96 cases (82.8%). Perineural invasion was found in 17 tumours (18.1%) and peritumoral inflammatory cells infiltration was present in 60 tumours (80%).

Of this 124 patients, 33 patients (26.6%) developed loco-regional relapse in the follow-up period (18 local recurrences, 7 homolateral regional recurrences, 4 contralateral regional recurrences and 4 loco-cervical recurrences). Most of the loco-regional recurrences (27 cases; 81.8%) appeared during the first 3 years of the follow-up period, 5 cases (15.2%) between the third and the fifth year of the follow-up period and only one case (3%) around the fifth year. At the end of the follow-up period, 92 patients (74.2%) were alive, 12 patients (9.7%) were dead due to the index tumour and 20 patients (16.1%) were dead due to other causes different from index tumour.

Table 1. Index OSCC tumour characteristics.

Index OSCC tumour characteristics	
Tobacco/Alcohol consumption	Cases (%)
No consumption	43 (34.7)
- Tobacco	34 (27.4)
- Alcohol	1 (0.8)
- Tobacco + alcohol	46 (37.1)
Total	81 (65.3)
Premalignant lesions	Cases (%)
No premalignant lesions	87 (70.2)
- Leucoplakia	8 (6.5)
- Erythroplakia	5 (4)
- Lichen planus	23 (18.5)
- Syphilis	1 (0.8)
Total	37 (29.8)
Tumour location	Cases (%)
Mobile tongue	77 (62.2)
Floor of mouth	32 (25.8)
Buccal mucosa	1 (0.8)
Gum	3(2.4)
Tongue base	3(2.4)
Retromolar area	3(2.4)
Oropharynx	3(2.4)
Soft palate	2 (1.6)

Descriptive analysis of second primary tumours

Twenty six patients (21%) presented second primary tumours, with two patients with double tumours (28 tumours). There were 21 men (80.8%) and 5 women (19.2%) with a median age of 62 years (range: 39 to 81 years). In 10 cases (53.8%) the secondary neoplasm was previous to the index tumour, in 2 cases (7.1%) synchronous and in 16 cases (57.2%) metachronous. Histological type of second primary tumours and location is shown in Table 2. The most frequent histological type was squamous cell carcinoma with 22 cases (78.5%) and intraoral location was the predominant site for these tumours with 10 cases (35.7%). In this group of patients, 19 cases (73.1%) presented tobacco or alcohol consumption habits (Table 2).

Table 2. Second primary tumour characteristics.

Second primary tumour characteristics	
Tumour location	Cases (%) (n = 28 tumours in 26 patients)
Intraoral Esophagus Larynx Lung Lip Breast Kidney Vesical Colorectal Prostate Hematologic Penis Osseous	10 (35.7) 4 (14.2) 3 (10.6) 2 (7.1) 1 (3.6) 1 (3.6) 1 (3.6) 1 (3.6) 1 (3.6) 1 (3.6) 1 (3.6) 1 (3.6) 1 (3.6)
Histology	Cases (%) (n = 28 tumours in 26 patients)
Squamous cell carcinoma Adenocarcinoma Urothelial carcinoma Lymphoma	22 (78.5) 4 (14.3) 1 (3.6) 1 (3.6)
Tobacco/Alcohol consumption	Cases (%)
No consumption - Tobacco - Tobacco + alcohol Total	7 (26.9) 9 (34.6) 10 (38.5) 19 (73.1)
Premalignant lesions	Cases (%)
No premalignant lesions - Leucoplakia - Erythroplakia - Lichen planus Total	20 (77) 3 (11.5) 2 (7.7) 1 (3.8) 6 (33)

Therefore, only 6 cases (23%) presented premalignant lesions and only 2 of them were intraoral second primary tumours and 1 case a lower lip carcinoma.

In this group, at the end of the follow-up period 12 patients (46.2%) were alive, 3 patients (11.5%) were dead due to the index tumour, 7 patients (26.9%) were dead due to the secondary neoplasm and 4 patients (15.3%) were dead due to other causes different than the tumour. Twelve patients (46.2%) presented loco-regional recurrences of the index tumour in the follow-up period.

Regarding index tumour characteristics in this group of second primary tumours, the most frequent location were the tongue (16 cases; 61.5%) and the floor of the mouth (5 cases; 19.2%), followed by tumours of the buccal mucosa, gum, oropharynx, base of the tongue and

soft palate (1 case; 3.8% respectively). Moderate differentiated carcinomas were more frequent (15 cases; 57.7%), followed by well-differentiated carcinomas (9 cases; 34.6%) and poor differentiated carcinomas (2 cases; 7.7%). Surgical margins of the index tumour were affected in 7 cases (28%), while perineural invasion was present in the index tumour in 3 cases (16.7%) and peritumoral inflammatory response in 13 cases (76.5%). The thickness of the index intraoral tumour was more than 2 mm in 91.7% of them (22 cases).

Genotypic distributions of polymorphisms of HIF-1∝

C1772T polymorphism was analyzed in 69 patients of the series and in 148 healthy subjects. T/T genotype was observed in 5.4% of healthy subjects and in 10.1% of OSCC patients while C/T was presented in 18.2% of healthy subjects and in 8.7% of OSCC patients. G1790A polymorphism was analyzed in 63 patients and in 138 healthy subjects. G/A genotype was detected in 6.5% of healthy subjects and in 34.9% of OSCC patients, A/A was presented in 4.8% of patients but none of healthy subjects presented this genotype.

In the group of second primary tumours, C1772T was analyzed in 14 cases and T allele was detected in 2 cases (14.2%). G1790 A was analyzed in 12 cases and A allele was detected in 6 cases (50%). Four of these 6 cases were patients with intraoral second primary tumours. Distributions of C1772T and G1790A polymorphisms are shown in Table 3.

Hardy-Weinberg Equilibrium in healthy subjects

We have observed that genotypic distributions of G1790A polymorphism were in Hardy-Weinberg Equilibrium in healthy subjects (Table 4).

The genotypic distributions of C1772T polymorphism were not in Hardy-Weinberg Equilibrium in healthy subjects. This was attributed to insufficient sample size that might be of 191 subjects as calculated by mathematical model (n = (z∝/2) 2pq/ d2) (Table 5). However, frequencies observed of C1772T did not differ from frequencies described in Hap Map Project for a Caucasian healthy population (Population, Hap Map Project. CEPH. 2010. http://www.ncbi.nih.gov/SNP/snp_ref.cgi?rs=11549465).

Comparative analysis of polymorphism of HIF-1∝

Differences in genotypic distribution of C1772T and G1790A between healthy subjects and OSCC patients were analyzed. In the study of C1772T, no differences were found between C/C genotype and carriers of T allele between cancer patients (index intraoral tumours) and healthy subjects ($p>0.05$) (Table 6). However, G1790A polymorphism showed statistical differences between G/G genotype and carriers of A allele, since frequency in A allele was highly increased in patients compared with healthy subjects (39.7% and 6.5% respectively; $p<0.01$). The odds ratio calculated for A carriers to suffer OSCC was OR = 9.5 (range: 4.08 to 22.08) (Table 6).

The susceptibility due to polymorphism of HIF-1∝ to present a second primary tumour was also analyzed in this study. In the analysis of C1772T between second primary tumour patients and healthy subjects, no statistical differences were found in genotypic distributions of T allele, as there were only 2 patients that carried this allele. However, frequency of A allele was increased in second primary tumour patients, as carriers of A allele were 50% in second primary tumour group and 6.5% in healthy subjects ($p<0.01$). The odds

Table 3. Genotypic distributions of HIF-1∝ polymorphisms.

GROUPS	C1772T (%)			G1790A (%)		
	C/C	C/T	T/T	G/G	G/A	A/A
Healthy subjects	113 (76.4)	27 (18.2)	8 (5.4)	130 (93.5)	9 (6.5)	0
Index OSCC tumours	56 (81.2)	6 (8.7)	7 (10.1)	38 (60.3)	22 (34.9)	3 (4.8)
Second primary tumours	12 (85.8)	1 (7.1)	1 (7.1)	6 (50)	5 (41.7)	1 (8.3)

Table 4. Hardy-Weinberg Equilibrium for G1790A polymorphism in healthy subjects.

GG	**GA**	**AA**	**N**
130	9	0	139
DN	HN	RN	
F(T)=	0.9676259	=p	
F(C)=	0.0323741	=q	
	1		
Predictors	**P**	**N**	
GG	0.93629988	130.145683	
GA	0.06265204	8.70863309	
AA	0.00104808	0.14568345	
Sample in Hardy-Weinberg Equilibrium			

Table 5. Hardy-Weinberg Equilibrium for C1772T polymorphism in healthy subjects.

TT	**TC**	**CC**	**N**
8	27	113	148
DN	HN	RN	
F(T)=	0.14527027	=p	
F(C)=	0.85472973	=q	
	1		
Predictors	**P**	**N**	
TT	0.02110345	3.123310811	
TC	0.24833364	36.75337838	
CC	0.73056291	108.1233108	
Optimal sample size (95%) 190.79			

ratio for second primary tumours in A allele carriers was OR = 14.4 (range: 3.8 to 53.9) (Table 7).

Association of clinico-pathologic variables in second primary tumours group and survival analysis

We analyzed association of different clinico-pathologic variables between patients with and without second primary tumours in the series of patients. No statistical differences were observed in sex, age, tobacco and alcohol consumption, premalignant lesions, and characteristics of the index tumour. A statistical difference was observed in loco-regional recurrences of the index tumour, as patients with secondary neoplasms presented higher rates of recurrences of these index tumour (42.3%) than patients without secondary neoplasms (22.4%); p = 0.04 (Table 8).

Survival analysis was performed considering death by tumoral disease (index tumour and second primary tumours). Five-year cumulative survival was lower in the group of secondary neoplasms (78.9%) than in the group of no secondary neoplasms (92.4%) ($p<0.01$) (Figure 2).

DISCUSSION

The mortality and morbidity from second primary neoplasms in head and neck squamous cell carcinomas remains one of the most challenging problems for successful treatment in this group of patients. These second primary tumours in OSCC are mainly located in the upper aerodigestive tract [8, 9]. According to the literature, in this study we have observed a rate of second primary tumours of 21% with intraoral location as the most frequent with a 35.7% [9]. The theory of "field of cancerization" proposed by Slaughter *et al.* [33] may be the explanation for this event: the same carcinogen may act modifying a monoclonal cell population that spread throughout the mucosa or modifying independent cell groups that develop different lesions in this mucosa [8, 9]. In this way, agents as tobacco or alcohol are related to multiple intraoral neoplasms. However, recent studies did not find differences in incidence of second tumours after smoking cessation [34]. Wiseman *et al.* found a rate of 25% of second primary neoplasms in nonsmoker patients and nondrinkers with head and neck squamous cell carcinomas [35]. Factors other than tobacco and alcohol, such as tumour genetics and environmental exposures must be implicated in the development of these second cancers [10].

In this study, Warren and Gates classic criteria for secondary neoplasms have been employed [29]. However, two modifications have been introduced for the purpose of this analysis as intraoral squamous cell carcinoma is the most frequent second tumour presented in these patients and it is

Table 6. Association between HIF-1∝ polymorphisms and healthy subjects/ OSCC patients.

Control/Patients	**C1772T**		
	Genotype (%)		**p-Value**
	C/C	**T carriers**	
Healthy subjects	113 (76.4)	35 (23.6)	0.4
Patient (n = 124)	56 (81.2)	13 (18.8)	
Control/Patients	**G1790A**		
	Genotype (%)		**p-Value**
	G/G	**A carriers**	
Healthy subjects	130 (93.5)	9 (6.5)	<0.01 (*)
Patient (n = 124)	38 (60.3)	25 (39.7)	

(*) OR: 9.5 (4.08-22.08).

Table 7. Association between HIF-1∝ polymorphisms and healthy subjects/ second primary tumour patients.

Control/Patients	**C1772T**		
	Genotype (%)		**p-Value**
	C/C	**T carriers**	
Healthy subjects	113 (76.4)	35 (23.6)	0.3
Patient (n = 14)	12 (85.7)	2 (14.3)	
Control/Patients	**G1790A**		
	Genotype (%)		**p-Value**
	G/G	**A carriers**	
Healthy subjects	130 (93.5)	9 (6.5)	<0.01(*)
Patient (n = 12)	6 (50)	6 (50)	

(*) OR: 14.4 (3.8-53.9).

Table 8. Association between second primary tumours and loco-regional recurrences of index OSCC tumours.

Second primary tumours	**Loco-regional recurrences (%)**		**p-Value**
	No recurrences	**Recurrences**	
Absent	76 (77.6)	22 (22.4)	0.04
Present	15 (57.7)	11 (42.3)	

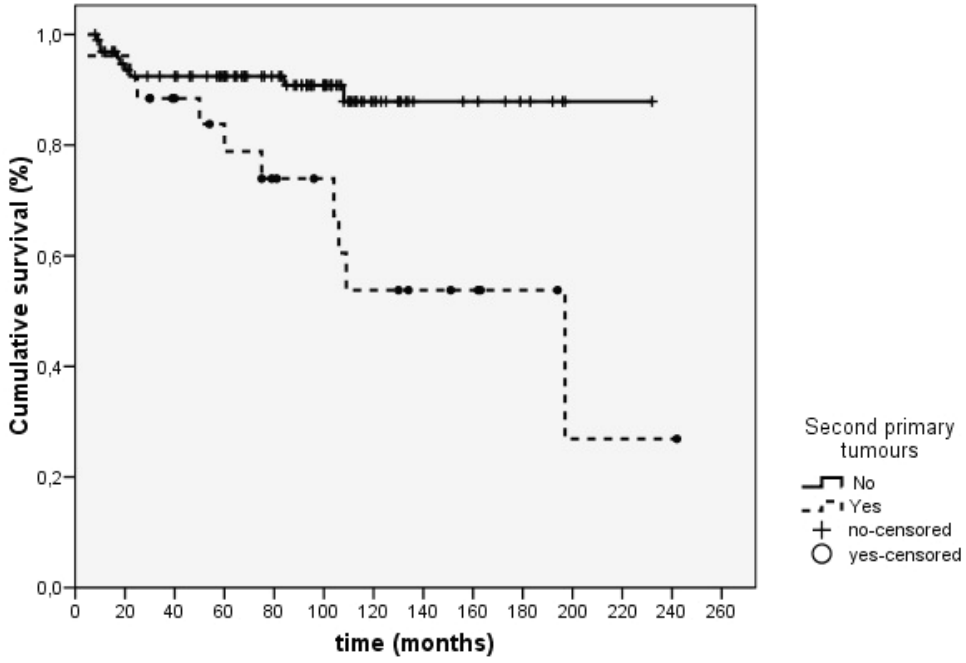

Figure 2. Cumulative survival for second primary tumours ($p<0.01$).

difficult to find a different histological type than the index tumour. We introduced time criteria of 5 years to consider a second independent tumour, as most loco-regional recurrences of the index tumour occur during the first 3 years. In this series the index tumour must also be N0 at diagnosis to discharge advanced disease, criteria that is presented in all the cases.

We analyzed the impact of polymorphisms of HIF-1α in susceptibility of OSCC and second primary tumours. HIF-1α is a transcriptional factor that plays an important role in several processes under hypoxic conditions as angiogenesis and carcinogenesis. In most carcinomas, over-expression of HIF-1α is associated with rise of mortality rates and worse treatment response [14, 16, 19-22]. However, the studies performed in head and neck carcinomas are contradictory. Some studies showed that expression of HIF-1α correlated with worse prognosis [18, 36, 37], but others showed a positive relation with this prognosis [38]. The mechanisms of HIF-1α expression in head and neck carcinomas may be more complex and other factors may determine HIF-1α actions.

In this way, polymorphisms of HIF-1α influence levels of this factor in different carcinomas under normoxia and hypoxic conditions. In the present study, genetic distributions of both C1772T and G1790A polymorphisms and the differences with healthy population are analyzed. C1772T did not show differences between OSCC patients and healthy subjects. However, statistical differences were found in G1790A as A carriers were most frequent in OSCC patients (38.1%) than in healthy subjects (6.5%). We could affirm that the presence of the polymorphic A allele confers susceptibility to OSCC. These preliminary results have been recently published by our group and confirmed afterwards by other authors [39, 40]. In a recent meta-analysis by Zhao *et al.*, C1772T polymorphism was determined as a susceptibility factor for cancer [41]. This study analyzed all the works of polymorphisms of HIF-1α in different solid tumours published at date. Although G1790A polymorphism was not a factor for susceptibility in this meta-analysis, it may confer predisposition to suffer some types of carcinomas as head and neck carcinomas [41].

Related to second primary tumours, no previous studies have analyzed the influence of these polymorphisms. We have observed that G1790A is again a factor of susceptibility for secondary neoplasms in patients with an index intraoral

carcinoma, as polymorphic allele A is more frequent in patients with this condition (50%) than in healthy subjects (6.5%). C1772T polymorphism did not show differences in this group, but due to the small sample of patients, studies with a bigger group are necessary to determine the influence of this polymorphism in second primary tumours.

We have also studied the influence of second primary tumours in OSCC prognosis. In this analysis a high rate of recurrences of the index tumour is observed in the group of secondary neoplasms (42.3% of loco-regional relapse in this group and 22.4% in the group without secondary neoplasms; $p = 0.04$). This suggests that this group of patients develops more aggressive tumours with poorer prognosis. Cumulative survival was also low in second primary tumour group ($p<0.01$). Five-year cumulative survival rate was 78.9% in the group of second primary tumour against 92.4% in the group without secondary neoplasms. Other authors demonstrated more aggressiveness and reduced survival in these patients with multiple neoplasms [9]. Therefore, these patients must be submitted to a long-term and careful follow-up, as screening of those sites with increased risk of second primary tumours will improve early diagnosis and prognosis. Knowledge of high-risk patients also allows early detection of recurrences of OSCC.

In conclusion, G1790A polymorphism confers susceptibility to OSCC and secondary neoplasms in these patients with intraoral carcinomas. Patients with second primary tumours may have worse prognosis due to this second cancer or due to the index OSCC as they present high risk of recurrences. Detection of HIF-1α polymorphism allows selection of high-risk patients, making necessary long-term and careful follow-up and more aggressive therapies.

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