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ORIGINAL ARTICLE

Association of PON1, TNF-α and TGF-β gene polymorphisms with prognosis in oral and oropharyngeal squamous cell carcinoma

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ABSTRACT

Objective: The oral and oropharyngeal squamous cell carcinoma (OOSCC) accounts for 90–95% of tumours in the oral cavity. Single nucleotide polymorphism (SNP) in the coding region of PON1, tumour necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) have been associated with to development of different cancers. Our aim was to investigate the prognostic value of PON1 (rs854560 and rs662), TNF-α (rs1800629 and rs361525) and TGF-β (rs1800469) SNPs in OOSCC.

Materials and methods: We genotyped 163 OOSCC patients and 146 patients from group of control for PON1 (rs854560 and rs662), TNF-α (rs1800629 and rs361525) and TGF-β (rs1800469) SNPs by real-time polymerase chain reaction (PCR).

Results: TNF-α (rs1800629) GG genotype was significantly more frequent in intraoral lesions and clinical stages III and IV, while the polymorphic AA genotype in lip lesion and clinical stages I and II. Moreover, TGF-β (rs1800469) AG and AA genotypes were significantly more frequent in larger tumours (T3 e T4). TNF-α (rs1800629) AG genotype had poor survival and patients carrying the PON1 (rs662) TT genotype tended to poor survival.

Conclusions: Results suggest that the rs1800629 and rs1800469 could exert influence in the more aggressive behaviour of OOSCC and the genotypes AG of rs1800629, and TT of rs662 could be markers with prognostic value in OOSCC.

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Introduction

Oral cancer is considered a public health problem and it presents high rates of incidence and morbimortality, being more prevalent in males and developing countries, ranging from 1 to 10 cases each 100,000 habitants in many countries [1–3].

Most carcinogenic factors might cause oxidative reactions in tissues, through the formation of reactive oxygen species (ROS) in cellular events, which are minimized by the cellular antioxidant defence system. But, when this system fails, these molecules when induced into oxidative stress, they might contribute significantly to the development of different diseases [4], such as cancer, through the oxidation of DNA bases and the formation of lesions in its chain, which might result in the activation of highly reactive carcinogens agents [5].

To control the formation and activity of ROS different antioxidant, methods are activated and among them, there is the human serum paraoxonase, the PONs, especially PON1. PON1 is a protein developed mainly in the liver and has been associated as a protective factor to different diseases, due to its antioxidant activity and the reduction in its levels has been associated with increased oxidative stress [4–6].

High levels of lipid peroxidation and low levels of antioxidants were found in the serum of patients with oral and oropharyngeal squamous cell carcinoma (OOSCC). Besides, studies show that the angiogenesis process and the presence of chronic inflammatory processes, with the production of pro-inflammatory cytokines and distribution of immune defence cells, are also factors related to the appearance of malignant tumours [7]. Among the proinflammatory cytokines developed in greater quantity, there are tumour necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β), which act in the process of formation of new blood vessels, but which may also affect endothelial cell proliferation and its differentiation [8].

TNF-α induces a variety of cellular responses and among them, there is the promotion of apoptosis of cancer cells through signalling of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway [7,8]. Increased PON1

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activity was associated with reduced levels of TNF-α in patients with atherosclerosis, speculating that low levels of PON1 promoted an increase in the number of ROS which would sensitize receptors which activate the NF-κB pathway, which in turn would stimulate expression of TNF-α [9].

TNF-α and TGF-β are known as central molecules in the signalling of antitumor mechanisms, however, some cancers may be resistant to signalling of death induced by both molecules [8,10].

A considerable number of studies have demonstrated the presence of important single nucleotide polymorphisms (SNPs) in PON1 [11], TNF-α [12,13] and TGF-β [14]. Functional SNPs may be responsible for alterations in the encoding of amino acid residues and contribute to the diversity of the proteins formed as well as influences their expression and functionality [15].

The presence of SNPs may be related to the development of cancers and could explain why different prognoses of the disease are found among patients who, despite having the same diagnosis and treatment, have different outcomes and outcomes; thus, the identification of biological markers, such as SNPs, could be used to better understand the disease [16].

The SNPs rs854560 and rs662 from PON1, rs1800629 and rs361525 from TNF-α and rs1800469 from TGF-β have been extensively investigated as an associated or protective factor to neoplasia. In cancer, for example, the SNP rs662 or Q192R from PON1 was associated with a reduced risk of cancer in a group of Asian patients, while PON1's rs854560 or L55M was associated with a higher risk of developing cancer, especially breast cancer [11].

In oral squamous cell carcinoma (OSCC) associated with human papillomavirus (HPV), the presence of SNP rs1800629 of TNF-α, in isolation or in combination with other SNPs, it was associated with a possibility of individual susceptibility to OSCC associated with HPV16 [12]; on the other hand, a study by Singh et al. [13] in view of the assessment of SNPs of TNF-α and susceptibility to OSCC, it was found a significant association of rs361525 with OSCC. In addition, the SNP rs1800469 of TGF-β has been associated with a reduced risk of lung cancer compared to non-small cell lung carcinoma (NSCLC) [14].

In this context, this study aims at investigating if the SNPs of the PON1 genes (rs854560 and rs662), TNF-α (rs1800629 and rs361525) and TGF-β (rs1800469), could be associated with the clinical and pathological variables of the disease, as well as the survival of the patients in OOSCC, in order to contribute to further clarification about the influence of SNPs on patient survival.

Materials and methods

Study population

The present analysis was based on a prospective cohort study design. The sample population ($N = 309$) consisted of a case group of 163 patients and a control group of 143 healthy blood donors, between 2011 and 2013, who participated after informed consent. All patients were diagnosed with OOSCC based on histological confirmation. Clinicopathologic characteristics of patients such as gender, age, tumour site, tumour size, clinical staging, radiation and chemotherapy and consumption of tobacco and alcohol were obtained by means of medical records and interview, respectively. The management protocols for all patients were similar. Healthy individuals with a positive history of cancer, as well as the cases of patients with a history of chronic inflammatory disease were all excluded from the study to perform the control. The protocol applied was approved by the ethical committee number CAAE: 58492614.5.0000.5546.

DNA extraction

About 4 ml of peripheral venous blood was collected in ethylenediamine tetraacetic acid (EDTA) from each subject. Genomic DNA was extracted from pelleted white blood cells using Miller's et al. method, described below: Buffy coats of nucleated cells obtained from anticoagulated blood (acid-citrate-dextrose (ACD) or EDTA) were resuspended in 15 ml polypropylene centrifugation tubes with 3 ml of nuclei lysis buffer (10 mM Tris–HCl, 400 mM NaCl and 2 mM Na2EDTA, pH 8.2). The cell lysates were digested overnight at 37 °C with 0.2 ml of 10% sodium dodecyl sulfate (SDS) and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na2EDTA). After digestion was complete, 1 ml of saturated NaCl (approximately 6 M) was added to each tube and shaken vigorously for 15 s, followed by centrifugation at 2500 rpm for 15 min. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 15 ml polypropylene tube. Exactly two volumes of room temperature absolute ethanol were added and the tubes inverted several times until the DNA precipitated. The precipitated DNA strands were removed with a plastic spatula or pipette and transferred to a 1.5 ml microcentrifuge tube containing 100–200 pl TE buffer (10 mM Tris–HCl, 0.2 mM Na2EDTA, pH 7.5). The DNA could dissolve 2 h at 37 °C before quantitating [17]. Concentrations of DNA were measured using NanoVue spectrophotometer (GE Healthcare Life Sciences) and diluted to 20 ng/ml.

Genotyping

All samples (case group and control group) were genotyped. All SNPs were genotyped using the TaqMan 5′-*exonuclease allelic discrimination assays* (Applied Biosystems, Foster City, CA). Polymerase chain reactions (PCRs) were performed in 8 μl reaction volumes in 96-well plates: 3.8 μl of sample DNA [5 ng/μl], 0.2 μl of Taqman probes 40X [TNF-α (rs1800629 e rs361525); TGF-β (rs1800469)] or 0.4 μl of Taqman probes 20X [PON1 (rs854560 e rs662)] and 4 μl of Master Mix Genotyping (Applied Biosystems, Foster City, CA). The standard protocol provided with the kit was followed. Thermocycler conditions followed by 60 °C for 30 s, 95 °C for 10 min, 40 cycles (92 °C for 15 s and 60 °C for 1 min) and 60 °C for 30 s. Reporter fluorescence emission was detected

Table 1. Clinical profile of patients with oral and oropharyngeal squamous cell carcinoma and healthy controls.

	Variables																
	Gender		Age		Smoke		Alcohol consumption		Tumour site			TNM			Clinical stage	
	Male	Female	<52 years	≥52 years	Yes	No	Yes	No	Lip	IO[a]	O[b]	T[c]	N[d]	M[e]	I and II	III and IV
OOSCC[f]	124 (76.1%)[g]	39 (23.9%)[g]	26 (16.0%)[g]	137 (84.0%)[g]	147 (90.2%)[g]	16 (9.8%)[g]	111 (68.1%)[g]	52 (31.9%)[g]	9 (5.5%)	87 (53.4%)	67 (41.1%)	**1 and 2–71 (43.55%) 3 and 4– 92 (56.45%)**	**0–67** (41.1%) **1, 2 and 3–96** (58.9%)	**0–163** (100%) **1–0** (0%)	34 (20.85%)	129 (79.15%)
Healthy controls	59 (40.4%)[g]	87 (59.6%)[g]	104 (71.2%)[g]	42 (28.8%)[g]	18 (12.3%)[g]	128 (87.7%)[g]	73 (50.0%)[g]	73 (50.0%)[g]	–	–	–	–	–	–	–	–

T is primary tumour size that can be T1, T2, T3 or T4. T1 and T2 together are 71 (43.55%); T3 and T4 together are 92 (56.45%). N is regional nodule involvement. N0 totalized 67 (41.1%) and N1, N2 and N3 together are 96 (58.9%). M is Distant metastasis. M0 totalized 163 (100%) and M1 there was no case 0 (0%).
[a]Intraoral; [b]Oropharynx; [c]Primary tumour; [d]Regional nodule; [e]Distant metastasis.
[f]Oral and oropharyngeal squamous cell carcinoma.
[g]χ^2 with $p < .05$.

Table 2. The distribution of clinical stage according to tumour site.

Clinical stage	Lip	Intraoral	Oropharynx
I and II	4 (2.5%)[a]	11 (6.7%)[a]	18 (11%)[a]
III and IV	5 (3.1%)[a]	76 (46.6%)[a]	49 (30.1%)[a]

[a]χ^2 with $p < .05$.

by software *Step One Plus*. Genotyping was performed in duplicate.

Statistical analysis

To check if the polymorphisms were in Hardy–Weinberg equilibrium, the genotypic frequency of each polymorphism and linkage disequilibrium was analysed using the program Haploview version 4.2. The chi-square and Fisher exact tests were used for comparison of differences in genotypes or allele frequencies among groups and to analyse associations between the genotypes of SNPs with gender, age, consumption of tobacco and alcohol, tumour site, tumour size, nodal involvement and clinical staging. Additionally, the additive-dominant-recessive genetic model was performed, considering the influence of the C and T (or A and G) alleles in an additive ('CC' versus 'CT' *versus* 'TT'), dominant CT + TT) and recessive ('CC + CT' *versus* 'TT'), for association among SNP genotypes with tumour location, clinical staging, tumour size and nodal involvement. Then, with the objective to assess the influence of SNP in the development of OOSCC, binary logistic regression analysis was used to measure the association between the dependent variable and independent variables using the odds ratios and 95% CIs. Explanatory variables with p value of $<.1$ in the bivariate analysis were included in the multiple logistic regression model. The relationship between the genotypes of SNPs with overall survival of the disease was analysed by Kaplan–Meier curve to verify the prognostic value. For all statistical tests used, it was considered a significance level of 5% ($p<.05$).

Results

Patient characteristics

Patient characteristics are shown in Table 1. In Table 2, it was found that the majority of intraoral and oropharynx lesions were associated with clinical stages III and IV ($p<.05$).

Distribution of PON1, TNF-α e TGF-β SNPs

In the 309 samples, 15 had no amplification in any of the SNPs. For the rs662, 288 samples were genotyped. For the rs854560, 259 samples were genotyped, for the rs1800469 there were 289 samples, for the rs1800629 there were 294 samples and for the rs351525 there were 291 samples which were genotyped. Table 3 exhibits the evaluation of heterozygous frequency, Hardy–Weinberg equilibrium and minor allele frequency (MAF) in SNPs. All polymorphisms are in Hardy–Weinberg equilibrium, except for SNP PON-1 rs662.

Table 3. Evaluation of heterozygous frequency, Hardy–Weinberg equilibrium and minor allele frequency (MAF) in SNPs.

SNP	Frequency of heterozygotes observed (%)	Frequency of predictive heterozygotes (%)	*p*	Allele	MAF
rs1800469	47.9	47.5	.87	G/a	0.388
rs1800629	19.7	19.4	.1	G/a	0.109
rs351525	8.6	10	.07	G/a	0.053
rs662	53.1	47.1	.043[a]	C/t	0.381
rs854560	39.8	42.5	.367	A/t	0.31

[a]χ^2 with $p< .05$.

Table 4. Association between genotypes in additive-dominant-recessive models with clinical variables: tumour site, clinical stage, tumour size and nodal involvement.

SNP	AA*	Genetic model	Genotypes	L[a]	IO[b]	O[c]	CS[d] I and II	CS III and IV	T1 and T2	T3 and T4	Absent NI[e]	Present NI
rs662	T	Additive	CC	2 (1.3%)	11 (7.3%)	6 (4.0%)	3 (2.0%)	16 (10.7%)	7 (4.7%)	12 (8.0%)	8 (5.3%)	11 (7.3%)
			CT	3 (2.0%)	45 (30.0%)	36 (24.0%)	18 (12%)	66 (44.0%)	34 (22.7%)	50 (33.3%)	36 (24.0%)	48 (32.0%)
			TT	3 (2.0%)	26 (17.3%)	18 (12.0%)	8 (5.3%)	39 (26%)	22 (14.7%)	25 (16.7%)	17 (11.3%)	30 (20.0%)
		Dominant	CC	2 (1.3%)	11 (7.3%)	6 (4.0%)	3 (2.0%)	16 (10.7%)	7 (4.7%)	12 (8.0%)	8 (5.3%)	11 (7.3%)
			CT + TT	6 (4.0%)	71 (47.3%)	54 (36.0%)	26 (17.3%)	105 (70%)	56 (37.3%)	75 (50%)	53 (35.3%)	78 (52%)
		Recessive	CC + CT	5 (3.3%)	56 (37.3%)	42 (28.0%)	21 (14.0%)	82 (54.7%)	41 (27.3%)	62 (41.3%)	44 (29.3%)	59 (39.3%)
			TT	3 (2.0%)	26 (17.3%)	18 (12.0%)	8 (5.3%)	39 (26%)	22 (14.7%)	25 (16.7%)	17 (11.3%)	30 (20.0%)
rs854560	T	Additive	AA	5 (3.7%)	39 (29.1%)	21 (15.7%)	10 (7.5%)	55 (41%)	26 (19.4%)	39 (29.1%)	24 (17.9%)	41 (30.6%)
			AT	1 (0.7%)	28 (20.9%)	27 (20.1%)	8 (6.0%)	48 (35.8%)	22 (16.4%)	34 (25.4%)	20 (14.9%)	36 (26.9%)
			TT	1 (0.7%)	9 (6.7%)	3 (2.2%)	3 (2.2%)	10 (7.5%)	5 (3.7%)	8 (6.0%)	6 (4.5%)	7 (5.2%)
		Dominant	AA	5 (3.7%)	39 (29.1%)	21 (15.7%)	10 (7.5%)	55 (41.0%)	26 (19.4%)	39 (29.1%)	24 (17.9%)	41 (30.6%)
			AT + TT	2 (1.5%)	37 (27.6%)	30 (22.4%)	11 (8.2%)	58 (43.3%)	27 (20.1%)	42 (31.3%)	26 (19.4%)	43 (32.1%)
		Recessive	AA + AT	6 (4.5%)	67 (50.0%)	48 (35.8%)	18 (13.4%)	103 (76.9%)	48 (35.8%)	73 (54.5%)	44 (32.8%)	77 (57.5%)
			TT	1 (0.7%)	9 (6.7%)	3 (2.2%)	3 (2.2%)	10 (7.5%)	5 (3.7%)	8 (6.0%)	6 (4.5%)	7 (5.2%)
rs1800469	A	Additive	GG	3 (2.0%)	31 (20.5%)	26 (17.2%)	16 (10.6%)	44 (29.1%)	33 (21.9%)[f]	27 (17.9%)[f]	24 (15.9%)	36 (23.8%)
			AGAA	5 (3.3%)	43 (28.5%)	28 (18.5%)	11 (7.3)	65 (43.0%)	25 (16.6%)[f]	51 (33.8%)[f]	35 (23.2%)	41 (27.2%)
				0 (0%)	8 (5.3%)	7 (4.6%)	3 (2.0%)	12 (7.9%)	6 (4.0%)[f]	9 (6.0%)[f]	3 (2.0%)	12 (7.9%)
		Dominant	GG	3 (2.0%)	31 (20.5%)	26 (17.2%)	16 (10.6%)	44 (29.1%)	33 (21.9%)[f]	27 (17.9%)[f]	24 (15.9%)	36 (23.8%)
			AG + AA	5 (3.3%)	51 (33.8%)	35 (23.2%)	14 (9.3%)	77 (51%)	31 (20.5%)[f]	60 (39.7%)[f]	38 (25.2%)	53 (35.1%)
		Recessive	GG + AG	8 (5.3%)	74 (49.0%)	54 (35.8%)	27 (17.9%)	109 (72.2%)	58 (38.4%)	78 (51.7%)	59 (39.1%)	77 (51.0%)
			AA	0 (0%)	8 (5.3%)	7 (4.6%)	3 (2.0%)	12 (7.9%)	6 (4.0%)	9 (6.0%)	3 (2.0%)	12 (7.9%)
rs1800629	A	Additive	GG	7 (4.5%)[f]	67 (43.5%)[f]	50 (32.5%)[f]	21 (13.6%) [f]	103 (66.9%)[f]	53 (34.4%)	71 (46.1%)	51 (33.1%)	73 (47.4%)
			AG	1 (0.6%)[f]	17 (11.0%)[f]	11 (7.1%)[f]	8 (5.2%)[f]	21 (13.6%)[f]	11 (7.1%)	18 (11.7%)	11 (7.1%)	18 (11.7%)
			AA	1 (0.6%)[f]	0 (0%)[f]	0 (0%)[f]	1 (0.6%)[f]	0 (0%)[f]	1 (0.6%)	0 (0%)	1 (0.6%)	0 (0%)
		Dominant	GG	7 (4.5%)	67 (43.5%)	50 (32.5%)	21 (13.6%)	103 (66.9%)	53 (34.4%)	71 (46.1%)	51 (33.1%)	73 (47.4%)
			AG + AA	2 (1.3%)	17 (11.0%)	11 (7.1%)	9 (5.8%)	21 (13.6%)	12 (7.8%)	18 (11.7%)	12 (7.8%)	18 (11.7%)
		Recessive	GG + AG	8 (5.2%)[f]	84 (54.5%)[f]	61 (39.6%)[f]	29 (18.8%)	124 (80.5%)	64 (41.6%)	89 (57.8%)	62 (40.3%)	91 (59.1%)
			AA	1 (0.6%)[f]	0 (0%)[f]	0 (0%)[f]	1 (0.6%)	0 (0%)	1 (0.6%)	0 (0%)	1 (0.6%)	0 (0%)
rs361525	A	Additive	GG	8 (5.3%)	76 (50.3%)	55 (36.4%)	26 (17.2%)	113 (74.8%)	60 (39.7%)	79 (52.3%)	54 (35.8%)	85 (56.3%)
			AG	1 (0.7%)	5 (3.3%)	4 (2.6%)	3 (2%)	7 (4.6%)	4 (2.6%)	6 (4.0%)	6 (4.0%)	4 (2.6%)
			AA	0 (0%)	2 (1.3%)	0 (0%)	0 (0%)	2 (1.3%)	0 (0%)	2 (1.3%)	1 (0.7%)	1 (0.7%)
		Dominant	GG	8 (5.3%)	76 (50.3%)	55 (36.4%)	26 (17.2%)	113 (74.8%)	60 (39.7%)	79 (52.3%)	54 (35.8%)	85 (56.3%)
			AG + AA	1 (0.7%)	7 (4.6%)	4 (2.6%)	3 (2%)	9 (6%)	4 (2.6%)	8 (5.3%)	7 (4.6%)	5 (3.3%)
		Recessive	GG + AG	9 (6%)	81 (3.6%)	59 (39.1%)	29 (19.2%)	120 (79.5%)	64 (42.4%)	85 (56.3%)	60 (39.7%)	89 (58.9%)
			AA	0 (0%)	2 (1.3%)	0 (0%)	0 (0%)	2 (1.3%)	0 (0%)	2 (1.3%)	1 (0.7%)	1 (0.7%)

*associated allele.
[a]Lip; [b]Intraoral; [c]Oropharynx; [d]Clinical stage; [e]Nodal involvement.
[f]χ^2 with $p< .05$.

SNPs PON1 (rs662 and rs854560) and TNF-α (rs1800629 and rs361525) were not in linkage disequilibrium (r^2: 20 and r^2: 0, respectively).

The association between genotypes and clinicopathological variables

The relationship between genotypes and clinicopathological variables were evaluated by qui-square test (Table 4). Patients with TNF-α (rs1800629) GG genotype had significantly more frequent intraoral lesions and clinical stages III and IV, while the polymorphic AA genotype was associated with lip lesion and clinical stages I and II ($p<.05$). Moreover, patients with TGF-β (rs1800469) AG and AA genotypes were significantly more frequent in larger tumours (T3 e T4) ($p<.05$).

The association between the presence of SNP and the development of OOSCC

The relationship between presence of SNP as a variable independent and association with smoking and drinking habits in the development of OOSCC was evaluated by binary logistic regression (Table 5). There was no significant relationship between presence of SNP as variable independent and development of OOSCC. The presence of SNP was not considered an isolated risk factor for the development of the disease.

Association of PON1, TNF-α e TGF-β genotypes with overall disease survival

To evaluate the prognostic value of genotypes, the relationship between overall survival of the disease and PON1, TNF-α e TGF-β genotypes were studied. Patients carrying the

Table 5. Result of multivariate logistic regression analysis, final model for smoking and drinking.

SNP	Genetic model	Genotypes	Unadjusted odds ratio	Adjusted odds ratio	*p*
rs662	Additive	CC	1	1	
		CT	0.653 (IC 95% 0.295–1.446)	0.571 (IC 95% 0.162–2.012)	.383
		TT	0.750 (IC 95% 0.453–1.241)	0.535 (IC 95% 0.239–1.199)	.129
	Dominant	CC	1	1	
		CT TT	0.778 (IC 95% 0.374–1.620)	0.836 (IC 95% 0.265–2.636)	.760
	Recessive	CC CT	1	1	
		TT	0.803 (IC 95% 0.492–1.310)	0.641 (IC 95% 0.295- 1.393)	.261
rs854560	Additive	AA	1	1	
		AT	0.871 (IC 95% 0.379–2.002)	0.607 (IC 95% 0.166–2.220)	.451
		TT	0.829 (IC 95% 0.356–1.932)	0.332 (IC 95% 0.085–1.292)	.112
	Dominant	AA	1	1	
		AT TT	1.012 (IC 95% 0.621–1.648)	1.443 (IC 95% 0.664–3.135)	.355
	Recessive	AA AT	1	1	
		TT	0.852 (IC 95% 0.384–1.891)	0.473 (IC 95% 0.137–1.635)	.237
rs1800469	Additive	GG	1	1	
		AG	0.565 (IC 95% 0.267–1.195)	0.414 (IC 95% 0.123–1.400)	.156
		AA	0.541 (IC 95% 0.260–1.123)	0.665 (IC 95% 0.207–2.133)	.492
	Dominant	GG	1	1	
		AG AA	0.917 (IC 95% 0.571–1.473)	0.572 (IC 95% 0.261–1.255)	.164
	Recessive	GG AG	1	1	
		AA	0.551 (IC 95% 0.275–1.106)	0.546 (IC 95% 0.181–1.646)	.282
rs1800629	Additive	GG	1	1	
		AG	0.444 (IC 95% 0.040–4.959)	0.694 (IC 95% 0.017–28.435)	.847
		AA	0.483 (IC 95% 0.041–5.628)	0.500 (IC 95% 0.011–21.930)	.500
	Dominant	GG	1	1	
		AG AA	0.887 (IC 95% 0.503–1.565)	1.340 (IC 95% 0.543–3.310)	.525
	Recessive	GG AG	1	1	
		AA	0.451 (IC 95% 0.040–5.029)	0.650 (IC 95% 0.016–26.036)	.819
rs351525	Additive	GG	1	1	
		AG	1.784 (IC 95% 0.160–19.917)	1.493 (IC 95% 0.035–63.260)	.834
		AA	3.000 (IC 95% 0.239–37.672)	1.957 (IC 95% 0.038–100.373)	.738
	Dominant	GG	1	1	
		AG AA	0.669 (IC 95% 0.305–1.469)	0.820 (IC 95% 0.238–2.832)	.754
	Recessive	GG AG	1	1	
		AA	1.866 (IC 95% 0.167–20.806)	1.524 (IC 95% 0.036–64.777)	.826

TNF-α (rs1800629) AG genotype had poor survival ($p<.05$) and patients carrying the PON1 (rs662) TT genotype had a tendency to poor survival ($p=.05$) (Figure 1).

Discussion

OOSCC mainly affects males with a more advanced age, whose clinical history predominantly shows the consumption of tobacco for a long period of time, which is mostly associated with alcohol [18].

The activity regulation in the TNF-α, PON1 and TGF-β genes is performed through a common activation pathway, NF-kappaB or NF-κB, which are responsible for regulating the expression of many genes during immune response, cell adhesion, differentiation, proliferation, angiogenesis and cellular apoptosis [19]. TNF-α is considered a pleiotropic cytokine which acts on the induction of different cellular responses, such as the process of inflammation and proliferation of cells and apoptosis of tumour cells [7,8].

In patients with atherosclerosis, increased PON-1 activity was associated with reduced levels of TNF-α, with low levels of PON-1 being thought to sensitize NF-κB pathway activation receptors by elevating ROS formation, stimulating expression of TNF-α, which together with the activation of TGF-β represent central mechanisms in the signalling of the antitumor response [8–10]. However, the presence of SNPs in their structures may be associated with changes in their functionality and resistance to antitumor response in different cancers. Moreover, among the SNPs evaluated in cancer, those found in TNF-α, PON1 and TGF-β are those with the most frequent allelic frequency [11–14].

In addition to the high exposure to carcinogens, the chronic inflammation process has been evaluated as one of the aetiological factors of some types of cancers [7,8] and the presence of SNPs in genes of cytokines involved in the inflammation process can favour the development of tumour cells [16]. Moreover, variability in cytokine gene sequences can strongly affect the activity of the immune system and its ability to monitor and eliminate cancer cells [16].

In this study, the GG genotype for rs1800629 in the additive model was more frequent in intra-oral lesions, a more vascularized region that tends to result in poor disease prognosis and less conservative treatments [3,18]. However, the AA genotype in the recessive model was associated with lip lesion, which presents a better prognosis and less aggressive treatments. These findings suggest that the G allele may be negatively influencing the disease evolution.

In addition, when these genotypes were associated with clinical stage of the disease, a significant association was observed. Patients with GG and AG genotypes presented a more advanced clinical stage (III and IV) when compared to the AA genotype (Table 5). Reinforcing these data, it was observed poor survival time for patients with GG and AG genotypes of rs1800629 in the additive model when compared to the AA genotype (Figure 1) ($p<.05$).

It could be suggested that the high expression of the cytokine, induced by the polymorphic A allele among the

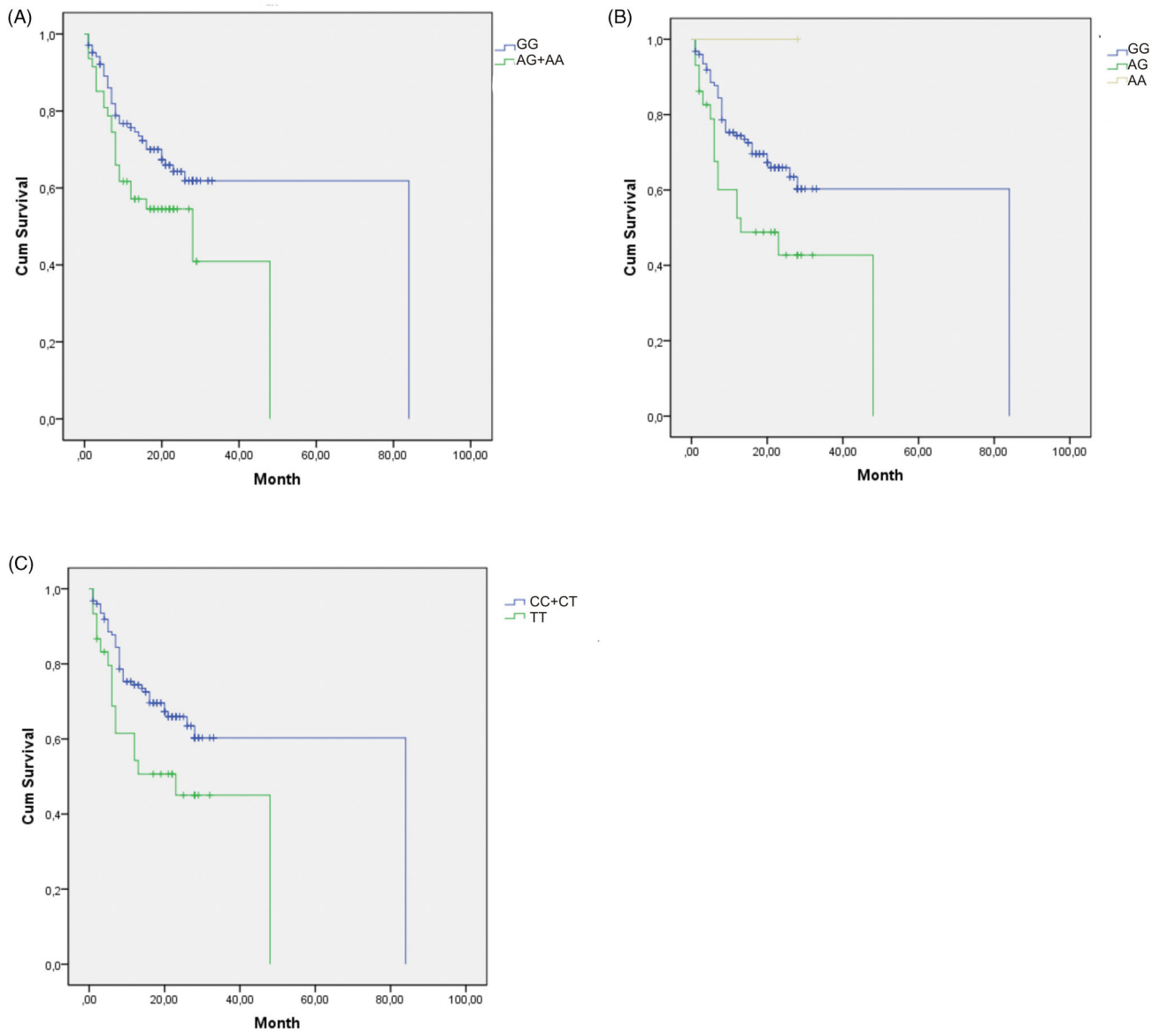

Figure 1. Kaplan–Meier analysis of the overall survival in patients with OOSCC. (A) TNF-α (rs1800629) in dominant model ($p = .031$); (B) TNF-α (rs1800629) in additive model ($p = .046$); (C) PON1 (rs662) in recessive model ($p = .056$).

study patients, is acting positively on the tumour immune response and on the overall survival of the disease. This may be related to increased TNF-α transcription *in vitro* and *in vivo* induced by the SNP, evidenced by the high levels of production of this protein in individuals with G/A and A/A genotypes [12]. Thus, increased production of TNF-α could contribute significantly to the immunity of its T cells, since it is known that IL-10 may play an important role in the induction of recruitment, cytotoxic activity and proliferation of CD8 + T cells, and that TNF-α and IFN-γ are also critical for triggering and maintaining immune responses as described for cytotoxic CD8 + T lymphocytes, which depends on the presence of these cytokines to perform effector mechanisms of elimination by apoptosis of tumour cells [20].

Besides, these lymphocytes, together with natural killer (NK) cells, are the main agents that act on tumour immunity and, after recognition of tumour cells, induce apoptosis by different pathways, such as through the release of cytoplasmic granules containing perforins and granzymes or by the expression of the Fas ligand (FasL) on its surface, which acts on the activation of caspases [21].

Additionally, TNF-α may induce the activation of the NF-κB signalling pathway, favouring the development of different malignancies [13]. Activation of this pathway in pre-neoplastic sites could result in exacerbation of local inflammation (elevated TNF-α, high IL-1, elevated IL-12, low IL-10 and low TGF-β) and favour the uncontrolled cellular replication process [13].

On the other hand, the relationship between the genotypes for rs1800469 and the anatomic location of the tumour showed that for the additive model, patients with the AG genotype presented, in their majority, larger lesions (T3 and T4). In the dominant model, a higher number of T3 and T4 lesions were found in the AG and AA genotypes, which could result in more aggressive lesions with a poor prognosis (Table 5) ($p < .05$).

A possible explanation for this fact is that TGF-β is a cytokine involved in the process of cell growth regulation, differentiation, apoptosis, adhesion and motility and the presence of SNPs in its structure may be associated with changes in its function, favouring progression of different diseases, including differentiation and progression of tumour cells [10].

TGF-β acts as an important tumour suppressor factor by inhibiting proliferation and inducing cellular apoptosis. However, tumour cells may develop mechanisms to bypass this induction and may cause the cells to respond to this cytokine in a way that contributes to tumour progression [22]. In addition to this, TGF-β mediates the production of different mitogenic growth factors, which stimulate proliferation of metastases and tumour survival [10, 23].

Thus, the high expression of TGF-β in patients with the polymorphic A allele, which may result from a negative regulation of the protein that leads to changes in its expression or function [22], would favour a greater susceptibility to the development of larger tumours and a negative influence on overall disease survival when compared to the GG genotype [22].

Finally, the TT genotype in the recessive model for rs662 was associated with poor survival time within threshold significance (p=.05) (Figure 1). This is due to the fact that PON1 is a protein involved in oxidative stress, minimizing the formation of ROS and acting in the formation of less reactive and less harmful compounds to the body of the individual, however, low levels of the protein, as found in patients with the rs662 TT genotype, may exacerbate the production of these molecules by the inefficient antioxidant activity of PON1 in the regulation process, favouring the carcinogenesis process and poor prognosis of the disease [4,5].

The maintenance of the balance between the formation of ROS and the activity of antioxidants, such as PONs, is fundamental for normal cell growth and survival [6]. Increased oxidative stress is associated with a higher risk of development of various cancers [4].

Similar to this study, other authors also evidence the influence of the polymorphic allele on disease survival. Geng et al. [24] in a study about the influence of SNPs on gastric cancer observed that individuals with CT and TT genotypes presented poor survival when compared to the CC genotype, in addition to an increased risk for metastasis formation. Tang et al. [25] found that the T allele may reduce the risk of oesophageal adenocarcinoma in non-smokers, suggesting that SNP may have a protective character for the disease. Kahraman et al. [26] observed that exposure to tobacco increases free radical production and cellular oxidative stress, in addition to decreasing the expression of PON1. The presence of the rs662 C > T SNP in the coding region of PON1 results in an amino acid substitution and determines a substrate-dependent effect on the protein activity [25]. Eom et al. [27] reported that patients with CT and TT genotypes had lower levels of 8-hydroxydeoxyglycine and thiobarbituric acid reactive substances in the urine when compared to the CC genotype in lung cancer patients, reducing the risk of developing the disease.

A systematic review with meta-analysis did not reveal a protective or risk character for the SNP rs662 [28]. A possible explanation for the discrepancy of data found in the literature is that alloenzyme encoded by the T allele hydrolyses different substrates, such as paraoxon, more rapidly than the enzyme associated with the C allele. However, isoenzyme C is more efficient in protection against low-density lipoproteins oxidation [29].

The results of this study suggest that the genotypes AG and polymorphic AA of rs1800469 could be associated with lesion in T3 and T4, while genotype wild type GG of rs1800629 with intra-oral lesions and advanced clinical stage (III and IV). Regarding survival, the genotype AG of rs1800629 and polymorphic TT of rs662 could be associated with a poor survival, suggesting that these genotypes could be markers with prognostic value in OOSCC and could influence on the behaviour of the OOSCC. However, these results should be viewed with caution, since the present work is a pilot study with small sample and the follow-up of these patients is still ongoing, so that future analyses can be performed.

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Disclosure statement

The authors declare that they have no conflict of interest.

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