Leptin receptor expression and Gln223Arg polymorphism as prognostic markers in oral and oropharyngeal cancer

P.R.S. Rodrigues[1], L.L. Maia[2], M. Santos[3], G.T. Peterle[2], L.U. Alves[1], J.T. Takamori[1], R.P. Souza[4], W.M. Barbosa[2], A.M.C. Mercante[1], F.D. Nunes[5], M.B. Carvalho[1], E.H. Tajara[6], I.D. Louro[2] and A.M.A. Silva-Conforti[2,7]

[1]Laboratório de Biologia Molecular, Hospital Heliópolis, São Paulo, SP, Brasil
[2]Programa de Pós-Graduação em Biotecnologia, Universidade Federal do Espírito Santo, Vitória, ES, Brasil
[3]Escola Multicampi de Ciências Médicas do Rio Grande do Norte, Universidade Federal do Rio Grande do Norte, Caicó, RN, Brasil
[4]Instituto do Câncer, Arnaldo Vieira de Carvalho, São Paulo, SP, Brasil
[5]Departamento de Patologia Bucal, Faculdade de Odontologia, Universidade de São Paulo, São Paulo, SP, Brasil
[6]Departamento de Biologia Molecular, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, SP, Brasil
[7]Departamento de Biologia, Universidade Federal do Espírito Santo, Alegre, ES, Brasil

Corresponding author: A.M.A. Silva-Conforti
E-mail: adriana.biomol@gmail.com

ABSTRACT. The leptin gene product is released into the blood stream, passes through the blood-brain barrier, and finds the leptin receptor (LEPR) in the central nervous system. This hormone regulates food

intake, hematopoiesis, inflammation, immunity, differentiation, and cell proliferation. The LEPR Gln223Arg polymorphism has been reported to alter receptor function and expression, both of which have been related with prognostics in several tumor types. Furthermore, several studies have shown a relationship between the Gln223Arg polymorphism and tumor development, and its role in oral and oropharyngeal squamous cell carcinoma is now well understood. In this study, 315 DNA samples were used for *LEPR* Gln223Arg genotyping and 87 primary oral and oropharyngeal squamous cell carcinomas were used for immunohistochemical expression analysis, such that a relationship between these and tumor development and prognosis could be established. Homozygous *LEPR* Arg223 was found to be associated with a 2-fold reduction in oral and oropharyngeal cancer risk. In contrast, the presence of the Arg223 allele in tumors was associated with worse disease-free and disease-specific survival. Low LEPR expression was found to be an independent risk factor, increasing the risk for lymph node metastasis 4-fold. In conclusion, the Gln223Arg polymorphism and LEPR expression might be valuable markers for oral and oropharyngeal cancer, suggesting that LEPR might serve as a potential target for future therapies.

Key words: LEPR expression; Gln223Arg; Prognostic marker; Head and neck cancer

INTRODUCTION

Head and neck cancer (HNC) is a significant cause of morbidity and mortality world-wide, and an estimated 600,000 new cases and 300,000 deaths each year can be attributed to this disease (Bauman et al., 2012). The 5-year survival rate for HNC is approximately 50% and treatment is mainly based on radiotherapy in combination with surgery and/or chemotherapy (Ferlay et al., 2008; Leemans et al., 2011). HNC has a multifactorial etiology involving smoking and drinking habits, human papillomavirus infection, and genetic factors (Gallì et al., 2009).

Leptin is a hormone released into the bloodstream and transferred through the blood-brain barrier, eventually reaching the leptin receptor LEPR (also known as OBR) located at the plasma membrane of hypothalamic cells. Leptin regulates food intake, hematopoiesis, inflammation, immunity, cell differentiation, and proliferation (Lavens et al., 2006; Snoussi et al., 2006; Andò and Catalano, 2011).

LEPR is a member of a family of cytokine class 1 receptors (Vaisse et al., 1996). Upon ligand binding, LEPR undergoes structural changes, which tethers Janus kinase (JAK) molecules thus enabling cross phosphorylation. This step is needed for the phosphorylation of signal transducer and activator of transcription (STAT) molecules at the cytoplasmic region of the receptor. STAT3 autodimerizes after phosphorylation of JAK2 and translocates into the nucleus, where it activates transcription at specific gene promoters. Among the various genes induced by STAT3 are those encoding enzymes involved in the development of many tumors (Frühbeck, 2006; Lavens et al., 2006; Anuradha et al., 2012; Liu et al., 2013).

Several *LEPR* polymorphisms have been described in humans. An A/G transition at nucleotide 668 converts a glutamine to an arginine at codon 223 (Gln223Arg) of the protein. This transition results in an exchange of a neutral amino acid for one with a positive charge, altering receptor function and signaling capacity and modifying circulating leptin levels (Anuradha et al., 2012). This polymorphic variant is located inside the extracellular domain coding region and affects the entire structure of the receptor (Li et al., 2012).

Previous studies have correlated the Gln223Arg polymorphism and LEPR expression with the development and prognosis of several tumors such as breast (Park and Scherer, 2011; Anuradha et al., 2012; He and Chen, 2012; Wazir et al., 2012), lung (Li et al., 2012), stomach (Kim et al., 2012), and prostate (Hoon et al., 2008) tumors. However, conclusive data are not yet available for HNC and studies on this area are scarce. Therefore, the present study aimed to analyze the Gln223Arg polymorphism and LEPR expression and their association with the development, prognosis, and survival of patients with oral and oropharyngeal cancer.

MATERIAL AND METHODS

Ethical approval

This study was approved by the Ethics Committee in Research of the Heliópolis Hospital (CEP #446) and a written informed consent was obtained from all patients enrolled.

Samples

Samples were collected by the Head and Neck Genome Project (GENCAPO), a collaborative consortium created in 2002 with more than 50 researchers from institutions in Brazil. In this study, 315 DNA samples were obtained and used for *LEPR* Gln223Arg polymorphism genotyping, consisting of samples from 186 (59.0%) individual controls with no history of cancer and 129 (41.0%) patients with oral and oropharyngeal squamous cell carcinomas. LEPR immunohistochemical analysis was performed on tumor tissue samples from 87 patients who had oral and oropharyngeal squamous cell carcinomas and had been surgically treated at the Head and Neck Surgery Department of Heliópolis Hospital and Arnaldo Vieira de Carvalho Cancer Hospital, São Paulo, Brazil, during the period of January 2002 to December 2009. The clinical follow-up was at least 48 months after surgery. Previous surgical or chemotherapeutic treatment, distant metastasis, no removal of cervical lymph nodes, and positive surgical margins were exclusion criteria (UICC, 2009).

Among the individuals analyzed, the mean (SD) age was 54.2 years (±11.1 years), with 173 (93.0%) men and 13 (7.0%) women in the control group. For patients with HNC, the mean age was 54.9 years (±10.7 years), including 104 (80.6%) men and 25 (19.4%) women (Table 1). Regarding the anatomical localization of the tumor, 95 (73.6%) were on the oral cavity and 34 (26.4%) on the oropharynx. The clinical-pathological characteristics of the tumors are described in Table 2.

Genotyping

Genomic DNA was extracted from peripheral blood samples of 309 individuals

(patients with cancer and controls) as previously described (Miller et al., 1988). Genotypes were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism. *LEPR* exon 6 was amplified using primers described by Snoussi et al. (2006) and analyzed for the Gln223Arg polymorphism (rs1137101). Selected amplification primers were 5'-AAA CTC AAC GAC ACT CTC CTT-3' and 5'-TGA ACT GAC ATT AGA GGT GAC-3', which produced an 80-bp fragment. PCR conditions were as follows: a 25-µL reaction mixture containing 200 ng genomic DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM of each deoxyribonucleoside 5'-triphosphate, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Life Technologies Inc.®, Rockville, MD, USA), and 25 pmol of each primer (Biobrás®, São Paulo, SP, Brasil). PCR amplification was initiated with a melting step of 5 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 52°C, and 45 s at 72°C; with a 5-min extension at 72ºC. PCR products were digested overnight with *Msp*I (New England Biolabs®, Beverly, MA, USA) following the manufacturer instructions. Restriction fragments were resolved on a 12% non-denaturing polyacrylamide gel. The single nucleotide polymorphism Arg223 in the *LEPR* gene was characterized by two distinctive fragments of 58 and 22 bp, whereas the *LEPR* Gln223 wild-type allele was identified by a single 80-bp fragment.

Tissue microarray

Tissue microarrays generated using buffered formalin-fixed paraffin-embedded tissue sections from samples from 87 patients with primary oral and oropharyngeal squamous cell carcinomas treated at the Head and Neck Surgery Department of Heliópolis Hospital, São Paulo, SP, were used for immunohistochemistry (IHC) analysis. Histological characterization of all samples was done by hematoxylin and eosin staining, followed by IHC analysis of tissue microarrays (TMA). Each sample was represented by 2 x 1 mm cylinders in the TMA slide (Beecher Instruments®, Silver Spring, MD, USA).

IHC

An anti-LEPR polyclonal antibody (Santa Cruz Biotechnology®, Dallas, TX, USA) was used in the IHC reaction at 1:50 dilution (Rimm et al., 2001; Hedvat et al., 2002; Hsu et al., 2002). Positive and negative controls (absence of primary antibody) were used for reaction quality control. Sample scoring was performed by semi-quantitative microscopic analysis, considering the number of stained tumor cells and signal intensity. Two spots were evaluated for each sample and a mean score was calculated. Considering the percentage of LEPR immune-positive tumor cells, a score of 1 was given when ≤10% cells were positive, 2 when 10-50% cells were positive, and 3 when ≥50% cells were positive. Signal intensity was scored as weak (1), moderate (2), or strong (3). Both scores were multiplied (Soini et al., 2000; Campos et al., 2009) and the resulting score was used to categorize LEPR expression as low (<4) or high (≥4).

Statistical analysis

Chi-square and Fisher exact tests were used for association analysis and confirmation was obtained by the Lilliefors test (significance was considered when P < 0.05). Multivariate-

logistic regression was used to obtain odds ratios (ORs) and 95% confidence intervals (CIs). Survival was calculated by the number of months between surgery and death for each patient or the last appointment when the patient was alive. In order to calculate disease-free survival, the time endpoint was the date of disease relapse. The Kaplan-Meier model was used for survival analysis, using the Wilcoxon P value and the Cox proportional hazards to adjust P values and obtain hazard ratios (HRs). Statistical calculations were performed using the Epi-Info® v3.4.3, 2007 and Statsoft Statistica® v7.0.61.0 softwares.

RESULTS

LEPR Gln223Arg polymorphism

The *LEPR* Gln223Arg polymorphism frequencies were determined to be as follows: 60 (46.5%) patients with cancer were genotyped as carrying the homozygous wild-type allele Gln/Gln, 61 (47.3%) as Gln/Arg, and 8 (6.2%) as Arg/Arg. In the control group, 68 (36.5%) individuals were genotyped as Gln/Gln, 92 (49.5%) as Gln/Arg, and 26 (14.0%) as Arg/Arg. The observed genotype frequencies of the *LEPR* Gln223Arg polymorphism were at Hardy-Weinberg equilibrium in the controls (P = 0.563). The Arg223 allele in the homozygote state was more frequent in control individuals than in patients, with a significant association (P = 0.045). Multivariate analysis showed that the Arg/Arg genotype was an independent factor for HNC development, representing a decreased risk of approximately 2.5 times in relation to the wild-type genotype (Gln/Gln) (OR = 0.40, 95%CI = 0.16-0.97; Table 1). However, the genotype Gln/Arg showed no relation to risk.

Table 1. Epidemiological features and *LEPR* Gln223Arg polymorphism association with oral and oropharyngeal cancer risk.

Features	Patients		Controls		P	Multivariate analysis	
	No.	(%)	No.	(%)		OR (95%CI)[a]	P[b]
Gender							
Female	25	(19.4)	13	(7.0)	0.001	1	
Male	104	(80.6)	173	(93.0)		0.12 (0.04-0.35)	<0.001
Age in years							
(mean, 54.5, d.f. ± 10.9)							
≤55	66	(51.2)	105	(56.5)	0.497	-	-
>55	63	(48.8)	81	(43.5)		-	-
Smoker							
No	59	(45.7)	103	(55.4)	0.039	1	
Yes	70	(54.3)	83	(44.6)		3.57 (1.48-8.61)	0.005
Drinking habit							
No	34	(26.4)	96	(51.6)	<0.001	1	
Yes	95	(73.6)	90	(48.4)		2.42 (0.98-5.96)	0.055
LEPR genotype Gln223Arg							
Gln/Gln	60	(46.5)	68	(36.5)	0.045	1	
Gln/Arg	61	(47.3)	92	(49.5)		0.78 (0.47-1.28)	0.317
Arg/Arg	8	(6.2)	26	(14.0)		0.40 (0.16-0.97)	0.042
Total	129	(41.0)	186	(59.0)			

OR = odds ratio; CI = confidence interval; d.f. = degrees of freedom. [a,b]Values adjusted by multivariate-logistic regression.

The Gln223Arg polymorphism did not show a significant association with tumor size (P = 0.406), lymph node metastases (P = 0.430), lymphatic invasion (P = 0.824), or perineural

invasion (P = 0.621), but was significantly associated with differentiation grade (P = 0.038; Table 2). It was also significantly associated with disease relapse (P = 0.003) and disease-specific death (P = 0.039; Table 2).

Table 2. Clinical and pathological tumor features and their associations with the *LEPR* Gln223Arg polymorphism and LEPR expression.

Features	Genotype Gln223Arg					Expression level			
	Total		Gln/Gln	Gln/Arg	P	Total		Low	High	P
	N	(%)		+Arg/Arg		N	(%)			
Tumor size (T)[a]										
T1+T2	50	(38.8)	21	29	0.406	34	(39.1)	15	19	0.844
T3	31	(24.0)	13	18		21	(24.1)	9	12	
T4	48	(37.2)	26	22		32	(36.8)	16	16	
Lymph node (N)[a]										
Absent (N-)	64	(49.6)	32	32	0.430	33	(37.9)	9	24	0.006
Present (N+)	65	(50.4)	28	37		54	(62.1)	31	23	
Differentiation										
Well differentiated	52	(40.3)	21	31	0.038	38	(43.7)	18	20	0.761
Moderately differentiated	69	(53.5)	38	31		42	(48.3)	18	24	
Poorly differentiated	8	(6.2)	1	7		7	(8.0)	4	3	
Lymphatic invasion										
Negative	61	(47.3)	29	32	0.824	28	(32.2)	14	14	0.604
Positive	68	(52.7)	31	37		59	(67.8)	26	33	
Perineural invasion										
Negative	93	(72.1)	42	51	0.621	65	(74.7)	26	39	0.054
Positive	36	(27.9)	18	18		22	(25.3)	14	8	
Disease relapse										
No	50	(38.8)	30	20	0.003	35	(40.2)	13	22	0.149
Yes	52	(40.3)	16	36		45	(51.7)	24	21	
Not available*	27	(20.9)	14	13		7	(8.0)	3	4	
Disease specific death										
No	59	(45.7)	31	28	0.039	42	(48.3)	16	26	0.123
Yes	41	(31.8)	13	28		36	(41.4)	20	16	
Not available*	29	(22.5)	16	13		9	(10.3)	4	5	
Total	129	(100.0)	60	69		87	(100.0)	40	47	

*Not available (not considered in the statistical calculations); [a]TNM classification 7th edn. (UICC, 2009).

Multivariate analysis showed that presence of the Arg223 allele was an independent marker of disease relapse representing an increased risk of over 3-fold when compared to the absence of this allele (OR = 3.30, 95%CI = 1.37-7.93), but the same association was not found for disease-specific death (OR = 2.40, 95%CI = 0.95-6.08; Table 3).

Disease-free and disease-specific survival were significantly correlated with the Gln223Arg polymorphism (P = 0.001 and P = 0.012, respectively). According to a 24-month after-surgery follow-up, approximately 60% of patients with at least one Arg223 allele presented disease relapse, as compared to approximately 30% of recurrence in patients without the Arg223 allele (Figure 1A). Additionally, according to a 36-month after-surgery follow-up, approximately 60% of patients with at least one Arg223 allele died of disease-specific causes, as compared to approximately 20% of deaths in patients without the Arg223 allele (Figure 1B). Multivariate analysis revealed that the presence of the Arg223 allele in the *LEPR* gene was an independent marker for early disease relapse and disease-specific death, with a 2-fold increased risk when compared to absence of this allele (respectively, HR = 2.38, 95%CI = 1.29-4.39 and HR = 2.11, 95%CI = 1.05-4.24; Table 4).

Table 3. Multivariate analysis of the relationships between clinical and pathological tumor features with the LEPR gene polymorphism and protein expression.

Features	Lymph node (N)[c]		Disease relapse		Disease-specific death	
	OR (95%CI)[a]	P[b]	OR (95%CI)[a]	P[b]	OR (95%CI)[a]	P[b]
LEPR expression						
High	1	-	-	-	-	
Low	3.75 (1.40-10.04)	0.009	-	-	-	-
LEPR genotype Gln223Arg						
Gln/Gln	-	-	1	1		
Gln/Arg+Arg/Arg	-	-	3.30 (1.37-7.93)	0.008	2.40 (0.95-6.08)	0.065
Tumor size (T)[c]						
T1+T2	1	1	1			
T3	1.13 (0.36-3.56)	0.833	1.04 (0.34-3.15)	0.946	1.22 (0.37-4.02)	0.740
T4	4.56 (1.43-14.62)	0.011	1.24 (0.44-3.45)	0.682	2.73 (0.96-7.80)	0.060
Differentiation						
Well differentiated	1	-	-	-	-	
Moderately differentiated	2.33 (0.79-6.91)	0.126	-	-	-	-
Poorly differentiated	3.61 (0.31-42.76)	0.309	-	-	-	-
Lymph node (N)[c]						
Absent (N-)	-	-	1	1		
Present (N+)	-	-	4.67 (1.61-13.59)	0.005	6.67 (2.24-19.87)	<0.001
Irradiated						
No	-	-	1	1		
Yes	-	-	0.23 (0.08-0.68)	0.008	0.56 (0.18-1.70)	0.304

OR = odds ratio; CI = confidence interval. [a,b]Values adjusted by multivariate-logistic regression; [c]TNM classification 7th edn (UICC, 2009).
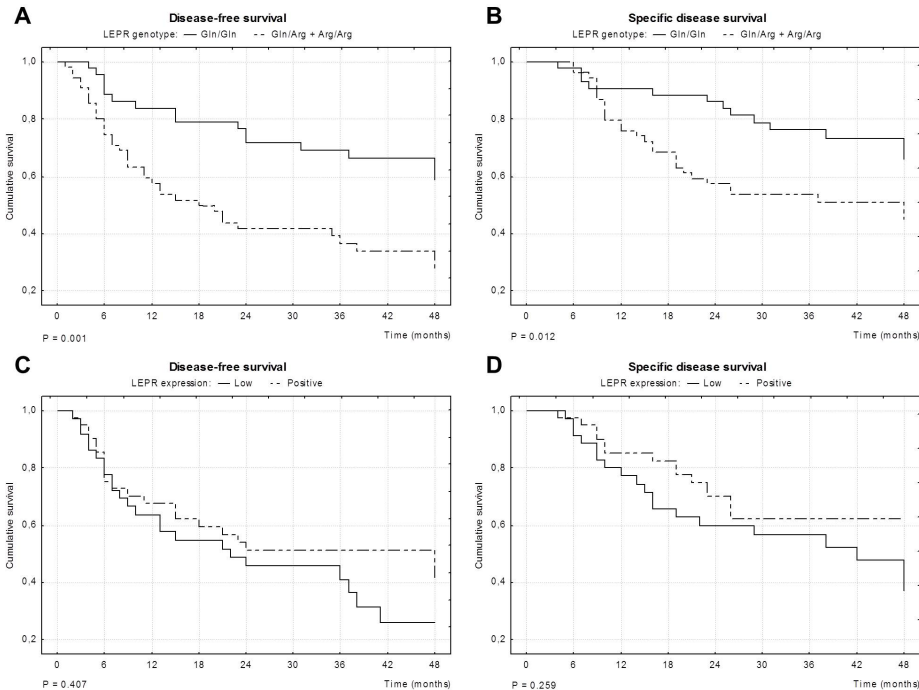
Figure 1. Survival plots. **A.** and **B.** Disease-free survival and disease-specific survival according to the LEPR Gln223Arg polymorphism. **C.** and **D.** Disease-free survival and disease-specific survival according to LEPR expression.

Table 4. Multivariate analysis of disease-specific survival.

Features	Disease-free survival		Disease-specific survival	
	HR (95%CI)[a]	P[b]	HR (95%CI)[a]	P[b]
LEPR genotype Gln223Arg				
Gln/Gln	1		1	
Gln/Arg+Arg/Arg	2.38 (1.29-4.39)	0.006	2.11 (1.05-4.24)	0.036
Tumor size (T)[c]				
T1+T2	1		1	
T3	1.22 (0.58-2.54)	0.598	1.27 (0.52-3.11)	0.604
T4	1.37 (0.71-2.63)	0.343	1.62 (0.80-3.29)	0.184
Lymph-node (N)[c]				
Absent	1		1	
Present	2.52 (1.27-4.99)	0.008	3.28 (1.44-7.47)	0.005
Irradiated				
No	1		1	
Yes	0.39 (0.20-0.74)	0.004	0.81 (0.37-1.76)	0.592

HR = hazards ratio; CI = confidence interval. [a,b]Values adjusted by Cox proportional hazards; [c]TNM classification 7th edn (UICC, 2009).

LEPR expression

LEPR expression was evaluated in 87 tumors, which were classified as low (40, 46.0%) and high (47, 54.0%). LEPR expression did not show a significant association with tumor characteristics such as size (P = 0.844), differentiation grade (P = 0.761), lymphatic invasion (P = 0.604), or perineural invasion (P = 0.054), but was significantly associated with lymph node metastases (P = 0.006; Table 2). Multivariate analysis showed that low LEPR expression was an independent marker for lymph node metastases (OR = 3.75, 95%CI = 1.40-10.04; Table 3).

In contrast, LEPR expression did not significantly correlate with disease relapse (P = 0.149) or disease-specific death (P = 0.123; Table 2), nor with disease-free or disease-specific survival (P = 0.407 and P = 0.259, respectively; Figure 1C and D).

DISCUSSION

Leptin is a cell growth factor hormone that has been associated with tumor cell migration and invasion, as well as with angiogenesis in some tumors (Cleveland et al., 2010). LEPR is involved with several signaling pathways such as JAK/STAT, phosphatidylinositol 3-kinase, protein kinase B, and mitogen activated protein kinase. These pathways, under leptin-LEPR control, are strongly related to cell survival and differentiation (Bracho-Riquelme et al., 2011).

The amino acid exchange caused by the *LEPR* Gln223Arg gene polymorphism yields a weaker interaction between leptin and its receptor, and as a consequence, reduction in cell growth signaling (Shuai and Liu, 2003). Our results show that the Arg223 homozygous genotype was associated with a 2.5-times smaller risk of developing cancer than the Gln223 homozygous variant. Similar results have been reported in prostate cancer, suggesting that this allele increases tumor development risk (Monteiro et al., 2009). Therefore, Gln223Arg might play an important role in the inhibition of cell division (Yapijakis et al., 2009). In contrast, in breast cancer, it appears that the Arg223 allele increases cancer risk (Snoussi et al., 2006), shedding uncertainty regarding through which mechanism this allele impacts oncogenesis.

In comparison, our results suggest that the presence of Arg223 is associated with re-

lapse risk and with worse disease-free and disease-specific survival. Similar studies in breast (Snoussi et al., 2006) and prostate cancer (Monteiro et al., 2009) have shown that patients carrying Arg223 have a significantly increased risk of relapse, and consequently worse prognosis. Reports that high levels of circulating leptin are related to decreased global survival of patients with breast cancer (Goodwin et al., 2005) and that weak interaction between leptin and LEPR favors angiogenesis and tumor growth (Kurahara et al., 1999; Ishikawa et al., 2006) support the hypothesis that the presence of Arg223 might result in worse prognosis in these patients. In contrast with these results, studies in lung (Li et al., 2012) and colorectal cancer (Liu et al., 2013) present the Gln223 allele as that related with worse prognosis.

According to our results, low LEPR expression increased the risk of lymph node metastasis by 4-fold when compared with that seen for tumors exhibiting strong expression. Weak LEPR expression had a similar effect as the Gln223Arg polymorphism, weak leptin-LEPR interaction, and increased circulating leptin (Kurahara et al., 1999; Gröschl et al., 2005); these effects have been previously suggested as facilitators of tumor invasion and metastasis (Hohberger et al., 2008; Liu et al., 2013).

In conclusion, the present study attributed a protective role to the homozygous *LEPR* Arg223 allele in oral and oropharyngeal cancer. Arg223 was shown to be an independent marker of worse prognosis, as illustrated by a lower disease-free and disease-specific survival. Low LEPR expression was found to be an independent marker of metastasis risk, showing a 4-fold increased risk of lymph node metastasis. These results suggest a tumor marker role for LEPR, as well as the potential for it to serve as a target for molecular therapies in patients with oral and oropharyngeal cancer.

Conflicts of interest

The authors declare no conflict of interest.

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