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A functional polymorphism in the promoter region of leptin gene increases susceptibility for non-small cell lung cancer

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ABSTRACT

Leptin hormone and receptor have been associated to cancer development and were identified in lung tissue. In this study, a functional polymorphism in the 5′ flanking region of the leptin gene (*LEP* −2548 G/A) was found to increase susceptibility for non-small cell lung cancer [odds ratio (OR), 1.97; 95% confidence interval (CI), 1.13–3.43]. Age-adjusted logistic regression analysis in men indicated an association of AA genotype with adenocarcinoma (OR, 4.29; CI, 1.64–11.72) and squamous cell carcinoma (OR, 3.19; CI, 1.26–8.13). Logistic regression analysis confirmed the AA genotype as an independent risk factor for lung cancer after adjustment for age and gender (OR, 2.57; CI, 1.34–4.92). The AA genotype was overrepresented only in patients with non-metastatic disease (OR, 1.86; CI, 1.13–3.04). Kaplan–Meier analysis demonstrated an earlier age of onset for lung cancer in AA carriers ($P = 0.023$). Results suggest the existence of genetic susceptibility for lung cancer in carriers of this *LEP* functional polymorphism. Further studies are warranted to extend knowledge of leptin involvement in lung cancer.

1. Introduction

Lung cancer is a widespread disease, with high incidence rates and leading mortality worldwide. In Europe the number of incident cases and cancer deaths from lung cancer in 2002 was 199,844 and 191,301, respectively.[1]

Carcinogenesis in the lung is a multi-step process of progressive disorganization characterized by events over latent periods of time, resulting from exposure to environmental insults.[2] Cigarette smoking is a major risk factor for 85% of lung cancers, although only 1 in 10 life-smokers will develop lung cancer,[3] suggesting individual differences in susceptibility that may be explained by the genetic profile. Accordingly, genetic polymorphisms have been analysed to ascertain their role in lung cancer susceptibility, prognostic and predictive value.[4,5]

Leptin has relevant roles in cell growth, differentiation and angiogenesis[6–8] supporting its involvement in cancer development and progression. The long isoform leptin receptor (LEPRb) was identified in normal human lung tissue and in lung squamous cell cancer derived cell line (SQ-5),[7] suggesting that the lung is a peripheral site of action for leptin. Cumulatively, Tsuchiya and co-workers[7] observed a stimulatory action for human recombinant leptin on SQ-5 cell proliferation mediated through MAP kinase signalling.

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Increased circulating levels of leptin and/or overexpression of leptin mRNA in adipose tissue may be partially explained by a genetic variation in *LEP* gene. The *LEP* gene is located on chromosome 7 (7q31.3), and the single nucleotide polymorphism (SNP) in 5′ flanking region at −2548 loci is believed to have an effect on *LEP* regulation. Higher mRNA expression and serum leptin levels were observed in AA genotype carriers.[9] This genotype has been associated with increased risk for breast and prostate cancers and non-Hodgkin lymphoma.[10–12]

In this case–control study, we sought to determine if this functional polymorphism in *LEP* might be involved in genetic predisposition for lung cancer and advanced disease.

2. Materials and methods

2.1. Study subjects

In this case–control study 482 individuals were analysed, including 140 cases (124 men and 16 women) with histopathologically confirmed lung cancer newly diagnosed from 1999 to 2002 at Oncology Portuguese Institute, Porto, Portugal. Seventy-nine patients had non-metastatic disease (stage Ia–IIIb) and 41 had distant metastasis (stage IV). Smoking status information was obtained from cases' medical records ($n = 102$). The healthy controls (139 males and 202 females) were randomly recruited from the Institute's Blood Donors Bank, all of them unrelated. The median age for cases was 63 years (range 38–89) and for controls 51 years (range 30–89). Written informed consent was obtained from all subjects prior to their inclusion in the study.

2.2. PCR-RFLP assay for detection of LEP −2548 G/A polymorphism

A venous blood sample (8 ml) was obtained from each subject by forearm venipuncture. White cell fraction was used to extract DNA according to salting out procedure.[13]

Genotyping of *LEP* was performed by PCR amplification using *LEP* polymorphism-specific primers: 5′-TTTCCTGTAATTTTCCCGTGAG (forward), 5′-AAAGCAAAGACAGG CATAAA (reverse), according to a previous report.[14] The PCR product was electrophoresed in 2% agarose, stained in 0.5% ethilium bromide and photographed under UV illumination. The resulting 242 bp DNA fragment was incubated at 37 °C/24 h with *Hin*6I, an isosquizomer of *Cfo*I. The polymorphism was defined by the presence (G) or absence (A) of a restriction site. Quality control procedures implemented for the *LEP* genotype analyses included double sampling in about 10% of the samples to assess reliability and the use of negative controls to step-away false-positives. Two authors obtained the results independently, and the ambiguous results were reanalysed.

2.3. Data analysis

We compared frequencies of each of the groups by genotype status. Patients with information about smoking status were stratified as current smokers and former/non-smokers (former smokers quit smoking for over than a decade at the time of diagnosis). Odds ratio (OR) and 95% confidence interval (CI) were calculated as a measure of association between *LEP* genotypes and lung cancer risk. Unconditional logistic regression analysis was used to compute ORs and CIs estimating the association of genotypes to lung cancer histological subtypes in males, while adjusting for age and with risk for overall lung cancer after adjusting for other potential risk factors, including age and gender. Data on smoking status among cases was only available from 102 patients (28 former/non-smokers and 74 current smokers). Cumulative hazard function plots were estimated by the Kaplan–Meier method with the log-rank test in order to compare groups. Hardy–Weinberg equilibrium in patients and disease-free subjects was tested using the Pearson χ^2-test. All statistical analyses were carried out with SPSS software (version 11.0). Statistical significance was set at the 0.05 level.

3. Results

The observed allelic distributions were in Hardy–Weinberg equilibrium in cases ($P = 0.583$) and controls ($P = 0.192$). Frequencies distribution for homozygous AA and GG genotypes were, respectively, 0.21 and 0.35 for lung cancer patients and 0.12 and 0.33 in controls. Heterozygous AG frequencies were 0.44 among patients and 0.54 in normal controls. The allelic distribution among patients and controls was for allele A 0.43 and 0.40, and for allele G 0.57 and 0.61, respectively.

In the recessive genetic model of patients stratified according to main histological types, the AA genotype is overrepresented in overall lung cancer cases (OR, 1.95; CI, 1.16–3.27) and non-small cell lung cancer (NSCLC) patients (OR, 1.97; CI, 1.13–3.43). In males, carriers of AA genotype have a threefold increased age-adjusted risk for lung cancer in all cases (OR,

Table 1 – Recessive model analysis of genotypes distribution and adjusted ORs in males according to the histological subtypes

	Genotype		OR[a]	CI	P
	AG/GG *n* (%)	AA *n* (%)			
Controls	128 (92.1)	11 (7.9)	Referent		
All lung cancer	97 (78.2)	27 (21.8)	3.23	1.49–7.02	0.003
Non-small cell lung cancer	79 (77.5)	23 (22.5)	3.41	1.54–7.59	0.003
Squamous cell carcinoma	50 (79.4)	13 (20.6)	3.19	1.26–8.13	0.015
Adenocarcinoma	29 (74.4)	10 (25.6)	4.29	1.64–11.72	0.003

a Adjusted for age (logistic regression analysis).

Table 2 – Logistic regression analyses for the recessive model, regarding the role of AA genotype, age and gender in the susceptibility to lung cancer

	OR	95% CI	P
Risk genotype (AA)	2.57	1.34–4.92	0.005
Age ⩾ 63 years	2.27	1.41–3.66	0.001
Male gender	13.16	6.86–25.24	<0.0001

3.23; CI, 1.49–7.02), NSCLC patients (OR, 3.41; 1.54–7.59) and in its histological subtypes, adenocarcinoma (OR, 4.29; CI, 1.64–11.72) and squamous cell carcinoma (OR, 3.19; CI, 1.26–8.13), respectively (Table 1).

Genotype frequencies among patients with metastatic disease are 0.27 for GG, 0.54 for AG and 0.20 for homozygous A, while in patients without distant metastasis the frequencies are 0.35 and 0.23 for homozygous G and A, respectively, and 0.42 for heterozygous. Results show that the AA genotype is overrepresented in lung cancer patients with non-metastatic disease (OR, 1.86; CI, 1.13–3.04), while in patients with metastatic disease no significant risk was found.

Logistic regression analysis in genetic recessive model supports an independent risk for the AA genetic variant of *LEP* after adjustment for age and gender (OR, 2.57; CI, 1.34–4.92) (Table 2).

There is a higher frequency of lung cancer patients who are current smokers, carrying the AA genotype, compared with former/non-smokers' patients or normal controls (Table 3). Patients carriers of AA genotype and simultaneously smokers are at increased risk for lung cancer, using as reference group the normal controls (OR, 2.65; CI, 1.44–4.85) or patients former/non-smokers (OR, 4.82; CI, 1.05–22.17).

Fig. 1 shows the cumulative probabilities for lung cancer occurrence and the waiting time to onset of disease. The time to onset of disease is significantly lower in AA versus AG/GG carriers ($P = 0.023$).

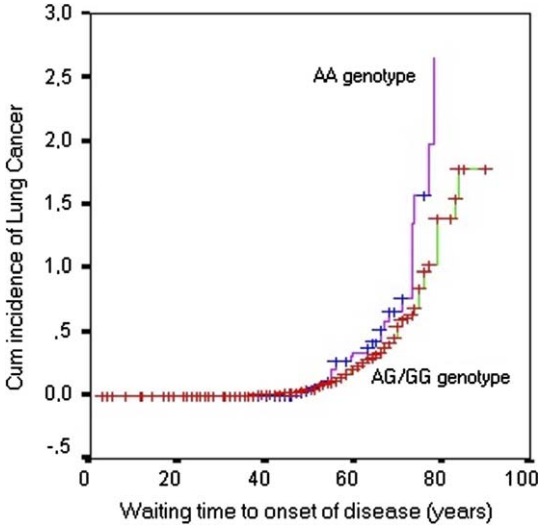

Fig. 1 – Association of *LEP* −2548 G/A and the waiting time to onset of disease. Cumulative hazard function plots by the Kaplan–Meier methodology and log rank test ($P = 0.023$).

4. Discussion

A role for leptin in lung cancer etiology is proposed by observations from the present study, in which an increased risk of lung cancer was associated with the overexpressing genetic variant in *LEP*, as well as from studies demonstrating leptin's proliferative potential in normal and tumoural lung cells.[7]

Leptin has been associated with cancers of the breast, prostate, ovary, and with non-Hodgkin lymphoma.[12,15–17] A rationale exists for leptin involvement in lung cancer development, albeit its mechanisms are not thoroughly studied.

Lung carcinoma develops through a multi-step process, from cells involved in the bronchial epithelium generation and capable of differentiation along several pathways.[2] Carcinogenesis is characterized by the occurrence of initiation, promotion and progression events, happening over latent periods of a decade or more.[18]

Non-small cell lung cancer (NSCLC) comprises about 75–80% of all lung cancers and represents a heterogeneous group of cancers, consisting mainly of squamous cell, adeno- and large cell carcinoma. Our results in NSCLC patients report to the histological sub-types adeno- and squamous cell carcinoma, which were found to be associated with the functional polymorphism in *LEP*. Result of the association between *LEP* polymorphism with small-cell lung cancer was not computed due to small number of cases ($n = 12$).

Our results suggest that carriers of *LEP* functional polymorphism may be at increased risk of initiation and promotion phases of lung tumour development. We found increased risk in AA carriers for localized lung cancer and earlier occurrence of lung cancer among carriers of AA genotype, suggesting that *LEP* polymorphism, which represents an all-

Table 3 – Study of smoking status and *LEP* genotypes among NSCLC patients and frequencies in normal controls

	Controls	Lung cancer patients		OR (CI)[a]	OR (CI)[b]
		Former/non-smokers	Current smokers		
AG/GG	300 (87.7)	26 (92.9)	54 (73.0)	Referent	Referent
AA	42 (12.3)	2 (7.1)	20 (27.0)	2.65 (1.44–4.85)	4.82 (1.05–22.17)

a Controls versus current smokers ($P = 0.001$).
b Former/non-smokers versus current smokers ($P = 0.03$).

life exposure to higher leptin expression, may influence tumour spurt. NSCLC usually disseminates later and has a slow clinical course,[19] agreeing with our findings. Carriers of *LEP* variant that overexpresses leptin may accelerate the course or NSCLC initiation due to exposure to the mitogenic and inflammatory role of leptin. Alternatively, NSCLC natural history seems to be less influenced by smoking and to acquire significantly less genetic damage from smoke, than small-cell lung cancer.[20] Leptin may be more relevant in the NSCLC due to minor influence of smoking habits.

There are studies supporting biological plausibility for a role of leptin in lung development since early fetal life until older age. Leptin and LEPR mRNA expression was detected in fetal lung, suggesting its involvement in lung development since in uterus.[21] In the human adult lung, Tsuchiya and colleagues[7] reported the presence of LEPRb either in normal and squamous cancer cells, and demonstrated in vitro leptin's proliferative stimulus in tumoural SQ-5 cell line, while studies in animals showed high levels of the putative functional LEPR as well as its splice variants in lung.[22] Furthermore, it was shown that the lung itself expresses leptin mRNA[23] suggesting that an autocrine mechanism for leptin may occur in lung. These features support paracrine and/or autocrine pathways for leptin action in lung.

Our results seem to suggest that carriers of AA genotype who are concomitantly smokers may have higher risk for lung cancer. However, no smoking data were available from controls and the association between smoking and *LEP* −2548 G/A was only tested among cases. The observed relationship could equally exist among controls, albeit we hypothesize that normal control carriers of AA genotype may be less prone to smoke, since they will produce more leptin and are less susceptible to weight gain.

Cigarette smoking induces in lung a vigorous inflammatory activation, through the recruitment and activation of pro-inflammatory cells.[24] It is well described that leptin is an important up-regulator of the inflammatory system.[25] It seems plausible that carriers of the overexpressing genotype (*LEP* −2548 AA), may have an exacerbated effect of smoke-induced lung inflammation, cooperating to an increased amplitude and duration, which is known to have a major role in lung cancer pathophysiology.[26]

Cumulatively, acute exposure to tobacco smoke induces, locally, in the lung hypoxia and CO exposure,[27,28] which up-regulates *LEP* transactivation through *HIF-1α*-mediated activation.[23,29] Carriers of the *LEP* variant at locus −2548 will have an up-regulated autocrine mechanism in lung after hypoxia-like stimulus.

Conversely, a large majority of lung cancer patients are smokers and tobacco consumption is a well-established factor.[1] Leptin circulating levels are influenced by cigarette smoking.[30–32] While long-term smokers have higher serum leptin levels,[31] acute exposure to smoke seems to reduce serum leptin through enhanced catecholamine effect and/or via down-regulation of lipoprotein lipase.[33,34]

Taken together, these findings suggest that leptin may have an important role in tumour cells growth, since it has mitogenic potential, and favouring a carcinogenic environment in lung. Further studies on this topic are required in order to clarify leptin and smoke interaction in lung cancer etiopathogenesis.

In the present study, we observed that the overexpressing *LEP* variant is not overrepresented in advanced NSCLC patients, thus supporting lack of contribution from serum leptin for metastatic lung cancer. Our findings agree with those reported in studies on plasma leptin levels,[35–37] since the overexpressing variant is not associated with advanced disease. However, care should be taken when interpreting these results, since the risk estimate for metastatic disease is of similar magnitude to that for non-metastatic disease is based upon smaller numbers and the confidence intervals overlap. Reduced levels of serum leptin have been reported in weight losing advanced stage NSCLC[36,37] and in a quite heterogeneous sample of lung cancer patients at different stages of disease and with variable histological types.[35] Therefore, decrease in plasma leptin concentration may be related to decreased body fat mass which developed secondary to weight loss in these patients. The positive association of *LEP* −2548 G/A with lung cancer shown in our study suggests that the polymorphism may be a proxy for leptin levels prior to the onset of lung cancer. Longitudinal studies assessing continuously circulating leptin levels from early life to adulthood are required to confirm this hypothesis.

Studies in other oncologic models also observed low leptin serum levels in advanced disease patients.[38–40] These findings suggest that plasma leptin levels are not relevant for specific tumour progression although it may be a biomarker of tumour-related nutritional status due to tumour progression.

Leptin is a key hormone in obesity and its levels are highly correlated with the amount of body fat and body mass index (BMI).[41,42] Apparently, BMI is inversely associated with lung cancer mortality.[43] However, this seemingly protective effect of BMI is attenuated or disappears with statistical control for smoking or in populations of lung cancer patients who never smoked.[44]

In this report, we show for the first time that a polymorphism in the promoter region of *LEP* gene at locus −2548 is associated with increased susceptibility for non-small cell lung cancer and earlier onset of disease. These results should be thoroughly confirmed while further studies should evaluate leptin and tobacco interaction in lung cancer development, allowing more accurate chemoprevention and lung cancer-screening programs.

Conflict of interest statement

None declared.

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