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BRIEF COMMUNICATION

LEPR polymorphisms and haplotypes in Mexican patients with colorectal cancer

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Introduction: Obesity and colorectal cancer could be linked by adipocytokines, which are proteins associated with cell proliferation. High levels of the adipocytokine leptin promote the development of colorectal cancer through its receptor.
Objective: To determine the association between c.326A>G and c.668A>G *LEPR* gene polymorphisms and colorectal cancer.
Materials and methods: DNA was extracted from the peripheral blood of 147 patients with sporadic colorectal cancer and 134 healthy people. Genotypes were obtained by PCR-RFLP and the association was determined by the odds ratio (OR) test using the SPSS™, version 10.0, program. Haplotype frequencies and linkage disequilibrium were estimated by the Arlequin, version 3.5, software.
Results: Both polymorphisms were in Hardy-Weinberg equilibrium. Only the c.326A>G heterozygous genotype revealed an increased risk for colorectal cancer development (OR=1.81, 95% CI=1.04-3.16, p=0.04). The AG haplotype showed a significant association with colorectal cancer (OR=0.58, 95% CI=0.35-0.96, p<0.03). Linkage disequilibrium between the variants was only evident for the patients group (r^2=0.36).
Conclusion: Our results suggest that AG individuals heterozygous for the c.326A>G *LEPR* variant have a higher risk of colorectal cancer development whereas the AG haplotype (c.326A/c.668G) has a protective effect in the Mexican population.

Key words: Receptors, leptin; colorectal neoplasms; polymorphism, genetic; haplotypes; odds ratio; Mexico.

Polimorfismos y haplotipos del gen *LEPR* en pacientes mexicanos con cáncer colorrectal

Introducción. La relación entre la obesidad y el cáncer colorrectal podría estar dada por las adipocitocinas, proteínas asociadas con la proliferación celular. Los niveles elevados de la adipocitocina leptina promueven el desarrollo del cáncer colorrectal a través de su receptor.
Objetivo. Determinar la asociación de los polimorfismos c.326A>G y c.668A>G del gen *LEPR* con el cáncer colorrectal.
Materiales y métodos. A partir de sangre periférica, se extrajo el ADN de 147 pacientes con cáncer colorrectal esporádico y de 134 personas sanas. La genotipificación se hizo mediante PCR-RFLP y la asociación se determinó por la *odds ratio* (OR) en el programa SPSS™, versión 10.0. Las frecuencias haplotípicas y el desequilibrio de ligamiento se estimaron utilizando el programa Arlequin, versión 3.5.
Resultados. Ambos polimorfismos estaban en equilibrio de Hardy-Weinberg. Solo el genotipo heterocigoto c.326A>G reveló un mayor riesgo de desarrollar cáncer colorrectal (OR=1,81; $IC_{95\%}$ 1,04-3,16; p=0,04). El haplotipo AG mostró una asociación significativa con este cáncer (OR=0,58; $IC_{95\%}$ 0,35-0,96; p≤0,03) y el desequilibrio de ligamiento entre las variantes fue evidente únicamente en el grupo de pacientes (r^2=0,36).
Conclusión. Los resultados sugieren que los individuos heterocigotos con el haplotipo AG para la variante c.326A>G en el gen *LEPR* tenían un mayor riesgo de desarrollar cáncer colorrectal, en tanto que el haplotipo AG (c.326A/c.668G) tenía un efecto protector en la población mexicana.

Palabras clave: receptores de leptina; neoplasias colorrectales; polimorfismo genético; haplotipos; oportunidad relativa; México.

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Conflict of interest
The authors declare no conflict of interest.

Colorectal cancer is the second (in women) or third (in men) most common neoplasm worldwide. In México, colorectal cancer ranks fourth in both sexes (1). Environmental, genetic and epigenetic factors have been related to colorectal cancer development (2-4). Although obesity increases the risk of colorectal cancer by 1.5- to 2-fold (5) and 17.7% of the cases are ascribed to excess body mass (6), the cause of this association is not yet clear.

The relationship between colorectal cancer and obesity may reflect the impaired structure or function of adipocytokines, which are proteins that regulate cell proliferation, angiogenesis, and apoptosis (7). For instance, an increase in leptin appears to promote the development and progression of colorectal cancer (8).

The LEPR (*leptin receptor*) protein is encoded by a gene in the 1p31.3 locus (9). The *LEPR* polymorphisms c.326A>G (rs1137100) and c.668A>G (rs1137101) are located in exons 4 and 6, respectively. The former results in a conservative substitution of arginine for lysine in codon 109 (p.K109R) while the latter produces a non-conservative change of glutamine to arginine in codon 223 (p.Q223R) (10). This p.Q223R substitution occurs inside the region coding for the extracellular domain and could affect the interaction with leptin (11). Even if the impact of the LEPR pathway in C colorectal cancer is not yet clear, increased expression in tumor tissue has been related to proliferation and angiogenesis (12).

The objective of this study was to determine the association between c.326A>G and c.668A>G *LEPR* polymorphisms and colorectal cancer in Mexican patients.

Material and methods

Patients and controls

The analysis included 147 patients diagnosed by histopathological and clinical criteria with sporadic colorectal cancer at the *Hospital Civil de Guadalajara "Dr. Juan I. Menchaca"*. The group included 87 males and 60 females with an average age of 57 years (range: 20 to 69 years). The control group was integrated by 134 blood donors from the same hospital without a colorectal cancer diagnosis.

All subjects signed the informed consent. This study was approved by the ethics committee of *Centro Universitario de Los Altos, Universidad de Guadalajara* (CUA/CINV/PCIL-011/2009).

DNA extraction

We used the Miller method (13) combined with the DTAB-CTAB protocol (14) on each 5-ml sample of peripheral blood with EDTA added as an anticoagulant. DNA purity and concentration were determined by spectrophotometry.

PCR-RFLP

We searched for *LEPR* variants with a PCR-RFLP protocol according to predetermined conditions (15,16). For the c.326A>G variant, we used 5'-TTTCCACTGTTGCTTTCGGA-3' forward and 5'-AAACTAAAGAATTTACTGTTGAAACAAATGGC-3' reverse primers under the following conditions: DNA initial denaturation for 5 min at 94°C; 40

cycles of 94°C for 45 s, annealing at 61.8°C for 45 s, extension at 72°C for 90s, and a final extension at 72°C for 10 min. We looked for the c.668A>G *LEPR* polymorphism using 5'-AAACTCAACGACACTCTCCTT–3' forward and 5'-TGAACTGACATTAGAGGTGA-3' reverse primers and amplification conditions similar to those described above except for an annealing temperature of 55°C.

For the single nucleotide polymorphism analysis, we used the restriction enzymes *Hae*III for c.326A>G and *Hpa*II for c.668A>G (both of which recognize the polymorphic G allele) for 16 hours. The assays were done in duplicate.

Electrophoresis

DNA fragments were identified in 6% polyacrylamide gels stained with silver nitrate. Their sizes were 101, 70 and 31 bp for c.326A>G and 80, 58 and 22 bp for c.668A>G.

Statistical analysis

Allelic and genotypic frequencies were determined by direct counting. The distribution of genotypes was tested for Hardy-Weinberg equilibrium using the c^2 test. Haplotype frequencies and linkage disequilibrium values were calculated by the Arlequin, version 3.5, software (17). Linkage disequilibrium with $r^2>0.3$ was considered to be significant (18). Intergroup differences were established by c^2 or Fisher's exact test and the association was determined by the odds ratio (OR) in SPSS™, version 10.0.

Results

LEPR polymorphisms

Demographic and clinical data of CRC patients are listed in table 1. In the control group, both *LEPR* polymorphisms were in Hardy-Weinberg equilibrium ($p>0.05$). For methodological reasons, not all individuals were included in the analysis. The comparison between controls and colorectal cancer patients associated the AG genotype of c.326A>G to an increased risk for colorectal cancer (OR=1.81, 95% CI=1.04-3.16, p=0.04) while no association was found for the c.668A>G polymorphism (table 2).

Table 1. Demographic data of colorectal cancer patients (n=147)

Variable	n (%)
Gender	
Female	60 (41)
Male	87 (59)
Diabetes	
Yes	28 (19)
No	102 (69)
Missing data	17 (12)
Smoking	
Yes	75 (51)
No	59 (40)
Missing data	13 (9)
Alcohol consumption	
Yes	70 (47.6)
No	60 (40.8)
Missing data	17 (11.6)

Table 2. Genotypes and allele frequencies of c.326 A>G and c.668A>G *LEPR* polymorphisms in controls and colorectal cancer patients

Gene *LEPR* SNP c.326 A>G	Control (N=100)		CRC (N=127)		OR (95% CI)	p*
	n	%	n	%		
Genotype						
AA	54	54	53	42	1.0 (Reference)	
AG	36	36	64	50	1.81 (1.04-3.16)	**0.04**
GG	10	10	10	8	1.02 (0.39-2.65)	0.97
Allele						
A	144	72	170	67	1.0 (Reference)	
G	56	28	84	33	1.27 (0.85-1.90)	0.25
SNP c (%).668 A>G	**Control n**	**CRC (%)**	**Control n**	**CRC (%)**		
Genotype						
A	39	29	43	29	1.0 (Reference)	
AG	69	52	79	54	1.04 (0.61-1.78)	0.89
GG	26	19	25	17	0.87 (0.43-1.76)	0.70
Allele						
A	147	55	165	56	1.0 (Reference)	
G	121	45	129	44	0.95 (0.68-1.33)	0.76

CRC: Colorectal cancer; OR: Odds ratio; CI: Confidence interval.
*Chi-square or Fisher´s exact test; figure in bold corresponds to p<0.05

LEPR haplotypes

The haplotype AA was the most frequent in controls (93/194; 48%) and colorectal cancer patients (132/254; 52%). LD was only evident among the latter (r^2=0.36). The AG haplotype showed a significant association with colorectal cancer (OR=0.58, CI=0.35-0.96, p<0.03) (table 3).

Table 3. Haplotype frequencies for c.326 A>G/c.668 A>G *LEPR* polymorphisms

Haplotype *LEPR* (c.326A>G/ c.668A>G)	Chromosomes				OR (95% CI)	p*
	Control (N=100)		CRC (N=127)			
	n	%	n	%		
AA	93	48	132	52	Reference	
AG	46	23.7	38	15	0.58 (0.35-0.96)	**0.03**
GA	11	5.6	13	5	0.83 (0.36-1.94)	0.6
GG	44	22.7	71	28	1.14 (0.72-1.80)	0.6

CRC: Colorectal cancer; OR: Odds ratio; CI: Confidence interval
*Chi-square or Fisher´s exact test; figure in bold corresponds to p<0.05

Discussion

Our finding of an increased risk of colorectal cancer in heterozygous AG individuals for the *LEPR* c.326A>G polymorphism compares with a similar association documented in Korean patients with gastric cancer (19). Regarding the same single nucleotide polymorphism (SNP), an increased risk (OR=1.64; 95%CI=1.10-2.45; p=0.016) for luminal A breast cancer was observed in GG patients (20) whereas the G allele was associated with prostate cancer mortality (hazard ratio=0.82: 95%CI=0.67-1.00; p=0.027) in another study (21). Moreover, this missense c.326A>G (p.K109R) variant was associated with increased birth weight in the Human Gene Mutation Database (HGMD-public: CM032948) (22). However, no association was found in other cancer studies (16,23,24).

Inasmuch as lysine (K) and arginine (R) are basic large-size amino acids (25), the Ensembl project— as predicted by SIFT and Polyphen— classified this variant as tolerable and benign (26). Yet, the higher propensity of lysine to post- translation modifications (methylation, ubiquitination, acetylation, phosphorylation, and sumoylation) indicates that its substitution could lead to protein structure alterations and increase colorectal cancer susceptibility (27).

The lack of association of the *LEPR* c.668A>G polymorphism with colorectal cancer in this and previous studies (11,28-31), and also documented in other neoplasms (16,23,32-37), probably reflects that the change of glutamine (Q), a polar amino acid of medium size, to arginine (R), a large-sized and basic amino acid, is harmless.

However, opposite results have been reported. In Caucasian women with breast cancer, the G allele was related to differentiation grade (OR=2.45, 95%CI 1.40–4.31) (38). Likewise, an increased risk for breast (39), oral (40), and lung (41) cancer development ascribed to the same variant has been proposed. Additionally, two meta-analyses revealed no association with cancer (42,43). These controversial results could be explained by the genetic structure of populations or the presence of additional variants modifying the *LEPR* gene.

The apparent protective effect of the AG haplotype disclosed in the present analysis (c.326A>G/c.668A>G) is consistent with the view that the accumulation of *LEPR* variants and gene interactions modulates colorectal cancer risk and development.

In conclusion, our results indicate that in the Mexican population, the AG genotype of the c.326A>G *LEPR* variant increases the risk of CRC, whereas the AG haplotype (c.326A/c.668G) protects against such tumors.

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