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RESEARCH ARTICLE

EVALUATION OF GENETIC VARIATION IN LEPTIN RECEPTOR GENE AND RISK OF URINARY BLADDER CANCER

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ABSTRACT

The aim of the study was to investigate genetic polymorphism in leptin receptor gene, (LEPR) and the risk of bladder cancer through a retrospective case-control analysis that consisted of 270 cases of bladder cancer and 252 controls. For the *LEPR Gln223Arg* polymorphism, no significant differences were observed in the genotype frequencies with regard to bladder cancer. The interaction of *LEPR Gln223Arg* polymorphism also did not show any significant associations either with environmental factors or with histopathology. This is the first case-control analysis carried out on Single Nucleotide Polymorphism in leptin receptor gene and carcinoma of urinary bladder so far. No other studies pertaining to same are available in the literature.

INTRODUCTION

Carcinoma of the urinary bladder is one of the leading causes of deaths in India (Vaish *et al.,* 2005) and it certainly appears that the incidence of bladder cancer is increasing in India (Kekre, 2008). A significant role in initiation of bladder cancer is played by environmental chemicals. Carcinogens derived from occupational exposures, cigarette smoking, inflammatory conditions and schistosomal infections are important factors. All of these conditions may lead to genetic changes, which irreversibly convert a normal urothelial cell to one with the malignant phenotype (Jung and Messing, 2000). Leptin exerts its physiological action through the leptin receptor (LEPR), a member of the class 1 cytokine receptor family (Snoussi *et al.,* 2006). Leptin receptor is the product of the 1p31 gene (Mendez-Sanchez *et al.,* 2006). It spans over 70 kb and includes 20 exons which encode a 1,165 amino acid protein (Mendez-Sanchez *et al.,* 2006). It is a single transmembrane protein (Tartaglia, 1997). Leptin binding to LEPR initiates a cascade of signalling events beginning with the activation of the constitutively receptor-associated Janus Kinase (Jak2), a tyrosine kinase (Kloek *et al.,* 2002). In addition to promoting the autophosphorylation of Jak2, the activation of Jak2 stimulates the phosphorylation of multiple residues on the intracellular domain of LEPR-Tyr985, Tyr1077 and Tyr1138 (Gong *et al.,* 2007). Each of these phosphorylation sites lies in a unique amino acid motif, and thus, recruits a distinct set of downstream signalling proteins when phosphorylated. Several variants commonly occur in the human leptin receptor gene, which cause 2 non-conservative changes, from glutamine to arginine at codon 223 (*CAG* to *CGG*) in exon 6 (*Q223R*) and from lysine to asparagine at codon 656 (*AAG* to *AAC*) in exon 14 (*K656N*); a conservative change from lysine to arginine at codon 109 (*AAG* to *AGG*) in exon 4 (*K109R*); 2 silent changes from proline to proline at codon 1019 (*CGT* to *CAT*) (*Pro1019Pro*) and from serine to serine at codon 343 (*GTG* to *GCG*) in exon 1 (*Ser343Ser*) (Gotoda *et al.,* 1997).

The glutamine to arginine substitution (*Q223R*, rs1137101) in the *LEPR* gene lies within the first cytokine domain in the region encoding the extracellular domain of the leptin receptor activity (Quinton *et al.,* 2001). Therefore, the amino acid change affects all forms of the receptor. It has been shown that the *LEPRGln223Arg* polymorphism is associated with variation in ligand binding; higher levels of ligand binding activity has been demonstrated in individuals homozygous for the *G* (*223Arg*) allele than for carriers of the *A* (*223Gln*) allele (Quinton *et al.,* 2001).

MATERIALS AND METHODS

Study design and study subjects

This retrospective case-control study comprised 270 histopathologically proven cases of urinary bladder cancer and 252 cancer-free controls. Peripheral blood samples from patients with urinary bladder cancer, treated at Advanced

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Urology Centre (AUC) of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, were collected during routine investigations. The ethical clearance for the present study was obtained from the Institute's Ethics Committee. Cases having HIV, allergies and other cancers, or patients having received chemotherapy were excluded. Informed consents were obtained from all the participants. Data with respect to their age, gender, smoking status, alcohol consumption, occupational status, area inhabited and eating habits were recorded. In patients with bladder tumour, the stage and grade of the tumour were noted.

Genotype analysis

Peripheral blood samples (2-4 ml) were collected from cases and controls in EDTA-coated vials. Genomic DNA was subsequently extracted from peripheral blood lymphocytes by the standard phenol-chloroform method. The LEPR Gln223Arg polymorphism was determined by the PCR-RFLP assay as per the conditions given in Table 1 (Fig. 1). Independent repetition of genotyping in randomly selected samples produced the same results and hence, proved concordance.

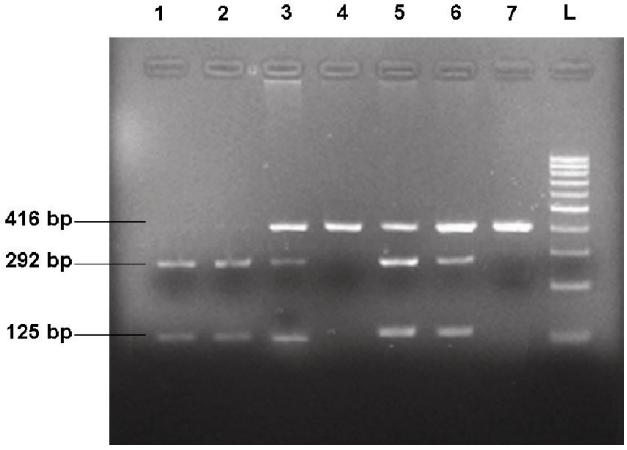

Fig. 1. A representative 2% agarose gel showing RFLP product of *LEPR* after digestion with *Msp*I: lanes 1, 2 = Arg/Arg (292 and 125 bp); lanes 4, 7 = Gln/Gln (416 bp); lanes 3, 5, 6 = Gln/Arg (416, 292 and 125 bp); and lane L = 100 bp DNA marker

Statistical Analysis

The power calculations were conducted at 80% with a significance level of 0.05. The sample size used for the present study was adequate. The data showed normal distribution on applying one-sample Kolmogorov-Smirnov Z test when age was taken as the test variable. The data were age-matched, as confirmed by T-test. The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained using χ^2 test and Fisher-Exact test for categorical variables. The odds ratios were calculated without adjustment for potential confounders, i.e., sex, area, job status, smoking, alcohol consumption and diet. Based on previous studies, occupations related to auto mechanics (Manju *et al.,* 2009), agricultural production, livestock and animal specialities; electrical assembly, installation and repair; and health services (Cassidy *et al.,* 2009); printing industry, transportation equipment industry, electrical/gas/sanitary services (Samanic *et al.,* 2008); were altogether taken under the category of high-risk occupations and the rest under low-risk occupations. To achieve an adequate sample size with power of study at 80%, the various tumour stages were clubbed together and merged into two groups, i.e., superficial (Ta + T1) and muscle-invasive (T2 + T3 + T4).

Both additive and dominant modes of inheritance were considered. The *p*-values were two-sided. Values less than 0.05 were considered as significant. All analyses were performed using SPSS, version 15.0 and Epi Info, version 3.4.3.

RESULTS

The distribution of the genotype frequencies of *LEPR Q223R (Gln223Arg)* polymorphism among cases and controls is summarized in Table 2. The allele frequencies were 69.82% for A (Gln) allele and 30.18% for G (Arg) allele in the cases. In the control group, the allele frequencies for A (Gln) allele were 71.82% and for G (Arg) allele were 28.18%. No significant differences were observed in the genotype frequencies with regard to bladder cancer for the LEPR polymorphism on considering either of the models. The interaction of *LEPR Gln223Arg (Q223R)* polymorphism with various environmental factors has been summarized in Table 3. No significant associations were observed for this particular polymorphism with any of the stratified variables.

Table 1. Conditions of genotyping assays for the polymorphism in *LEPR* gene

Single Nucleotide Polymorphism	Primers	PCR product	Enzyme	Gel band pattern
LEPR Gln223Arg (Ni *et al.,* 2009)	5'-ACCCTTTAAGCTGGGTGTCCCAAATAG-3'	416 bp	*Msp*1 (5 U, 4 hr, 40°C)	Arg (G) allele: 292 bp, 125 bp
	5'-AGCTAGCAAATATTTTTGTAAGCAATT-3'			Gln (A) allele: 416 bp (Figure I)

Table 2. Distribution of *LEPR 223 A/G (Gln223Arg)* genotype frequencies among cases and controls

Genotype	Cases (%) (n = 270)	Controls (%) (n = 252)	OR (95% CI)	*p*-value
AA (Gln/Gln)	151 (55.93)	142 (56.35)	1 (Ref.)	--
AG (Gln/Arg)	75 (27.78)	78 (30.95)	0.90 (0.60-1.36)	0.614
GG (Arg/Arg)	44 (16.29)	32 (12.7)	1.29 (0.75-2.22)	0.322
AG + GG (Gln/Arg + Arg/Arg)	119 (44.07)	110 (43.65)	1.02 (0.71-1.46)	0.922

*Significant *p*-values are in bold ($p < 0.05$); OR, odds ratio; CI, confidence interval

Table 3. Stratification analysis of the *LEPR 223 A/G (Gln223Arg)* genotype frequencies in cases and controls

Variable	Genotype	Cases (%) (n = 270)	Controls (%) (n = 252)	OR (95% CI)	*p*-value
Gender					
Males	AA	135 (50.00)	120 (47.62)	1 (Ref.)	-
	AG	64 (23.70)	66 (26.19)	0.86 (0.55-1.34)	0.491
	GG	37 (13.70)	26 (10.32)	1.26 (0.70-2.30)	0.409
	AG + GG	101 (37.41)	92 (36.51)	0.98 (0.66-1.44)	0.898
Females	AA	16 (5.93)	22 (8.73)	1 (Ref.)	-
	AG	11 (4.07)	12 (4.76)	1.26 (0.39-4.06)	0.663
	GG	7 (2.59)	6 (2.38)	1.60 (0.38-6.82)	0.463
	AG + GG	18 (6.67)	18 (7.14)	1.38 (0.50-3.82)	0.496
Inhabitation					
Rural	AA	75 (27.78)	51 (20.24)	1 (Ref.)	-
	AG	43 (15.93)	33 (13.09)	0.89 (0.48-1.64)	0.681
	GG	20 (7.41)	15 (5.95)	0.91 (0.70-2.07)	0.800
	AG + GG	63 (23.33)	48 (19.05)	0.89 (0.51-1.55)	0.666
Urban	AA	76 (28.15)	91 (36.11)	1 (Ref.)	-
	AG	32 (11.85)	45 (17.86)	0.85 (0.48-1.52)	0.564
	GG	24 (8.89)	17 (6.75)	1.69 (0.80-3.58)	0.135
	AG + GG	56 (20.74)	62 (24.60)	1.08 (0.66-1.78)	0.745
Occupation					
High Risk	AA	58 (21.48)	27 (10.71)	1 (Ref.)	-
	AG	28 (10.37)	18 (7.14)	0.72 (0.32-1.64)	0.397
	GG	21 (7.78)	8 (3.17)	1.22 (0.44-3.46)	0.674
	AG + GG	49 (18.15)	26 (10.32)	0.88 (0.43-1.79)	0.697
Low risk	AA	93 (34.44)	115 (45.63)	1 (Ref.)	-
	AG	47 (17.41)	60 (23.81)	0.97 (0.59-1.59)	0.894
	GG	23 (8.52)	24 (9.52)	1.19 (0.60-2.34)	0.599
	AG + GG	70 (25.93)	84 (33.33)	1.03 (0.66-1.60)	0.888
Smoking					
Smokers	AA	67 (24.81)	25 (9.92)	1 (Ref.)	-
	AG	33 (12.22)	14 (5.56)	0.88 (0.38-2.05)	0.746
	GG	26 (9.63)	13 (5.16)	0.75 (0.31-1.81)	0.477
	AG + GG	59 (21.85)	27 (10.71)	0.82 (0.41-1.64)	0.536
Non-smokers	AA	84 (31.11)	117 (46.43)	1 (Ref.)	-
	AG	42 (15.56)	64 (25.40)	0.91 (0.55-1.52)	0.713
	GG	18 (6.67)	19 (7.54)	1.32 (0.62-2.82)	0.439
	AG + GG	60 (22.22)	83 (32.94)	1.01 (0.64-1.59)	0.975
Alcohol consumption					
Alcoholic	AA	56 (20.74)	49 (19.44)	1 (Ref.)	-
	AG	34 (12.59)	23 (9.13)	1.29 (0.64-2.62)	0.440
	GG	23 (8.52)	13 (5.16)	1.55 (0.66-3.64)	0.271
	AG + GG	57 (21.11)	36 (14.29)	1.39 (0.76-2.54)	0.259
Non-alcoholic	AA	95 (35.18)	93 (36.91)	1 (Ref.)	-
	AG	41 (15.18)	55 (21.82)	0.73 (0.43-1.23)	0.212
	GG	21 (7.78)	19 (7.54)	1.08 (0.52-2.26)	0.821
	AG + GG	62 (22.96)	74 (29.36)	0.82 (0.51-1.31)	0.379
Eating habits					
Vegetarian	AA	79 (29.30)	77 (30.56)	1 (Ref.)	-
	AG	38 (14.07)	45 (17.86)	0.82 (0.47-1.45)	0.474
	GG	24 (8.89)	13 (5.16)	1.80 (0.81-4.05)	0.119
	AG + GG	62 (22.96)	58 (23.02)	1.04 (0.63-1.73)	0.866
Non-vegetarian	AA	72 (26.67)	65 (25.79)	1 (Ref.)	-
	AG	37 (13.70)	33 (13.09)	1.01 (0.55-1.88)	0.967
	GG	20 (7.41)	19 (7.54)	0.95 (0.44-2.05)	0.888
	AG + GG	57 (21.11)	52 (20.63)	0.99 (0.58-1.69)	0.967

* Significant *p*-values are in bold ($p < 0.05$); OR, odds ratio; CI, confidence interval

Table 4. Distribution of the *LEPR 223 A/G (Gln223Arg)* genotypes according to stages and histo-pathological grades

STAGES	Genotype	Cases (%) (n = 270)	OR (95% CI)	*p*-value
Superficial	AA	119 (44.07)	1 (Ref.)	-
	AG	57 (21.11)	0.87 (0.56-1.36)	0.522
	GG	36 (13.33)	1.34 (0.76-2.37)	0.280
	AG + GG	93 (34.44)	1.01 (0.69-1.48)	0.962
Muscle-invasive	AA	32 (11.85)	1 (Ref.)	-
	AG	18 (6.67)	1.02 (0.51-2.03)	0.942
	GG	8 (2.96)	1.11 (0.43-2.82)	0.814
	AG + GG	26 (9.63)	1.05 (0.57-1.93)	0.871
GRADES				
G1	AA	43 (15.56)	1 (Ref.)	-
	AG	24 (8.89)	1.02 (0.55-1.87)	0.956
	GG	7 (2.59)	0.72 (0.27-1.87)	0.470
	AG + GG	31 (11.48)	0.93 (0.53-1.62)	0.788
G2	AA	81 (30.00)	1 (Ref.)	-
	AG	41 (15.18)	0.92 (0.56-1.51)	0.731
	GG	29 (10.74)	1.59 (0.86-2.93)	0.111
	AG + GG	70 (25.93)	1.12 (0.73-1.71)	0.597
G3	AA	27 (10.00)	1 (Ref.)	-
	AG	10 (3.70)	0.67 (0.29-1.55)	0.317
	GG	8 (2.96)	1.31 (0.50-3.39)	0.540
	AG + GG	18 (6.67)	0.86 (0.43-1.72)	0.649

* Significant *p*-values are in bold ($p < 0.05$); OR, odds ratio; CI, confidence interval

The interaction of *LEPR Gln223Arg (Q223R)* polymorphism with each histological subcategory is summarized in Table 4. No significant associations were observed for this polymorphism among any of the stages or grades.

DISCUSSION

Gln223Arg polymorphism is the *A* to *G* substitution at codon 223 of *LEPR* gene. This results in a change of amino acid glutamine to arginine (Ni *et al.,* 2009). The *Gln223Arg* polymorphism is within the region encoding the extracellular domain of the leptin receptor and, therefore, the amino acid change affects all forms of the receptor. It has been suggested that the single amino acid change in the *LEPR* gene, a glutamine for an arginine with a change from neutral to positive, could affect the functionality of the receptor and alter its signalling capacity (Chung *et al.,* 1997; Chagnon *et al.,* 1999; Chagnon *et al.,* 2000). The finding of higher leptin binding activity (LBA) levels in homozygous carriers of the *G* allele (*LEPR Arg223Arg*) and higher levels of leptin in the *LEPR Arg223Arg* homozygotes provides supportive evidence for the former (Quinton *et al.,* 2001).

Yuan *et al.* (2004) suggested that leptin receptor is aberrantly expressed in bladder cancer tissue and is possibly involved in the carcinogenesis of bladder in a population of Taiwan. In the current study, no significant differences were observed in the genotype frequencies with regard to bladder cancer for the *LEPR* polymorphism on considering additive and dominant models. These results were in accord with many other studies of *LEPR Gln223Arg* polymorphism which were carried out on cancers other than that of urinary bladder. To our knowledge, this is the first assay of *LEPR Gln223Arg* SNP in patients having bladder cancer.

Earlier studies did not show any significant associations of this polymorphism with breast cancer. No significant association emerged for *LEPR Gln223Arg* genotypes in breast cancer development or prognosis from a population of New York (Cleveland *et al.,* 2010). Another small Korean study found no association for the leptin receptor variant (Woo *et al.,* 2006). A Nigerian population was not able to show a relationship between the *LEPR Gln223Arg* polymorphism and either breast cancer risk or prognosis (Okobia *et al.,* 2008). An increased risk of breast cancer and worse prognosis among women carrying the *Arg* variant of the *LEPR Gln223Arg* polymorphism was found in Tunisian population (Snoussi *et al.,* 2006). A significantly increased risk in both premenopausal and postmenopausal women in a dose dependent manner for *LEPR Gln223Arg* and *LEPR Arg223Arg* genotypes was found. In addition, the presence of *LEPR 223Arg* allele was seen to be associated with poorer overall survival (Snoussi *et al.,* 2006). The *LEPR Gln223Arg* genotypes have also been analyzed in prostate and colorectal cancers. A case-control study from UK population showed no significant association between leptin receptor gene polymorphisms and the risk of young-onset prostate cancer (Kote-Jarai *et al.,* 2003). No significant associations emerged with *Gln223Arg* genotypes and colorectal adenoma risk (Chia *et al.,* 2007).

In another study, the association analysis indicated an increased colorectal carcinoma risk for carriers of the *Arg/Arg (GG)* genotype of the *LEPR* gene (Pechlivanis *et al.,* 2009). The *LEPR* SNP *Gln223Arg* has been shown to affect serum leptin-binding affinity, with individuals homozygous for the *Arg (G)* allele having higher serum leptin-binding affinity than carriers of the *Gln (A)* allele (Quinton *et al.,* 2001). No study is available on polymorphism in leptin receptor gene with regard to bladder cancer till date. There was only one study on leptin receptor on bladder cancer which stated for the first time that the leptin receptor was aberrantly expressed in bladder cancer tissue and was possibly involved in the carcinogenesis of bladder cancer (Yuan *et al.,* 2004).

Conclusion

Although of significant interest, further work is required to determine the frequency of these polymorphisms in larger cohorts of cancer patients and normal individuals, not only in bladder cancer but also in various other cancers, as the literature pertaining to LEP and LEPR polymorphisms is very less.

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