RESEARCH ARTICLE

Association of Leptin Receptor Lys109Arg and Gln223Arg Polymorphisms with Increased Risk of Clear Cell Renal Cell Carcinoma

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Abstract

Background: Although roles of genetic polymorphisms of leptin receptor (LEPR) gene in several cancers have been documented, the association between polymorphisms of LEPR and clear cell renal cell carcinoma (CC-RCC) remains unknown. The aim of this study was to explore any relation. Materials and Methods: The study population consisted of 77 patients with CC-RCC and 161 healthy control subjects. Polymorphism analyses of Lys109Arg and Gln223Arg were performed by direct DNA sequencing and PCR-restriction fragment length polymorphism approaches respectively. Results: Comparisons of allelic and genotypic frequencies in Lys109Arg and Gln223Arg showed no significant difference between the cases and controls. However, when evaluating the combined genotype of Lys109Arg and Gln223Arg, risk with GG/GG was increased (OR=1.85, 95%CI=1.04-3.30) and with GA/GG or GG/GA was decreased (OR=0.07, 95%CI=0.01-0.54; OR and 95%CI of the latter could not be calculated for a value of zero) . Furthermore, the G-G haplotype frequency of Lys109Arg and Gln223Arg in the cases was higher (OR=1.68; 95%CI=1.02-2.76). In contrast, the A-G and G-A haplotype frequencies in the cases were lower than those in the controls (OR=0.06; 95%CI=0.01 to 0.47; OR and 95%CI of the latter could not be calculated for a value of zero). In addition, the Lys109Arg A allele was in LD with the Gln223Arg A allele (d'=0.9399) in the CC-RCC subjects, but not in the controls. Conclusions: Our data suggest that the GG/GG combined genotype and G-G haplotype of Lys109Arg and Gln223Arg can act as evaluating factors for CC-RCC risk.

Keywords: Leptin receptor - polymorphism - clear cell renal cell carcinoma

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Introduction

Leptin is secreted by adipocytes and interacts with leptin receptor (LEPR) in the hypothalamus, which plays an important role in the regulation of food intake and energy expenditure (Zhang et al., 1994; Shimizu et al., 2001). Leptin also has a growth factor function, Leptin/LEPR signaling promotes the proliferation, angiogenesis and inhibits the apoptosis of epithelial cells (Jarde et al., 2011; Zhou et al., 2011; Alegre et al., 2013). The LEPR, a member of the cytokine receptor super-family, is a single transmembrane protein (Fruhbeck, 2006). The LEPR has six alternatively spliced isoforms, which are classified according to the length of their cytoplasmic domain to four short, one long form, and a soluble form (Oswal et al., 2010). The long isoform is believed to have biologically action and is essential for signal transduction through JAK-STAT pathway (Wauman et al., 2011). There are several common variants of the LEPR genes, and four allelic variants are associated with amino acid changes (Lys109Arg, Gln223Arg, Ser343Ser, and Lys656Asn). The variants in the LEPR gene may cause abnormal signal transduction. The potential associations of these variants with cancer have been evaluated in different cancers, included cancers of breast (Wang et al., 2012; Dallal et al., 2013), colorectum (Drew, 2012; Liu et al., 2013), gastric (Kim et al., 2012), lung (Li et al., 2012) and oral (Yapijakis et al., 2009). However, the relationship between the polymorphisms of LEPR and CC-RCC is not well studied.

RCC is the second largest and the most lethal cancer in the urinary system. RCC accounts for about 2.1% of malignant tumors in adult, and the incidence rate of male and female is about 3:2 (Ferlay et al., 2010). RCC is divided into four main types according to Heidelberg classification: clear cell carcinoma, papillary renal cell carcinoma, chromophobe cell carcinoma and renal oncocytoma (Kovacs et al., 1997). CC-RCC is the most common type among them, constituting >80% of cases. In RCC patients, leptin levels in blood were higher and associated with venous invasion (Horiguchi et al., 2006a). Leptin mediates proliferation of RCC Caki-2 cell lines

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(Li et al., 2008). Furthermore, the leptin receptor was expressed in renal tissue and six human RCC cell lines (Horiguchi et al., 2006b). These support a role for leptin signaling in RCC carcinogenesis.

The aim of this study was to determine whether there are potential polymorphisms in LEPR, which associated with CC-RCC. To manifest this hypnosis, genotyping Lys109Arg and Gln223Arg in CC-RCC subjects and controls were performed, and the relation was analyzed.

Materials and Methods

Patients

In the current study, 77 patients who were histologically diagnosed as CC-RCC between December 2005 and September 2012 were enrolled. The patients consisted of 14 females and 63 males, with age from 17 to 85 years (56.22±12.27). One hundred and sixty one controls were selected from the Health Screening Center of Wuxi People's Hospital. Controls were matched to cases by ethnicity, age and sex. Subjects with underlying diseases or abnormal laboratory data were excluded from the study.

All protocols were approved by the ethics committee of the institution before the study began, and the protocols conformed to the ethical guidelines of the 1975 Helsinki Declaration.

DNA extraction

Tissue samples from 77 patients with CC-RCC were obtained at initial diagnosis. Peripheral blood samples from healthy individuals were collected in tubes containing EDTA-K2 as the anticoagulant. DNA was extracted with Wizard® genomic DNA purification kit (Promega, USA) following the manufacturer's instruction.

PCR-direct sequence analysis of Lys109Arg (A/G)

The genotyping of Lys109Arg polymorphism was performed using PCR-direct sequence analysis. The primers for PCR and sequencing were sense: 5'-CCTGCTGGACTCTCAAAGAA-3', and antisense: 5'-TGTTAAAATCATAGCCATAAGACATCT-3'. PCR was performed in 50 μL volume, the mixture include 2.5 mM $MgCl_2$, 0.5 μM of both forward and reverse primer, 200 μM of each dNTP, 1 U Taq DNA polymerase (Promega, USA) and approximately 150 ng DNA. The amplification conditions were the follows: 5 minute initial denaturation at 94°C followed by 35 cycles of amplification, each including 30 s denaturation at 94°C, 45 s annealing at 58°C, and 45 s extension at 72°C. The PCR products were sequenced by Sangon Biotech (Shanghai) Co., Ltd on the ABI Genetic Analyzer 3730 XL (Applied Biosystems, Foster City, California, US).

PCR-RFLP analysis of Gln223Arg (A/G)

The Gln223Arg polymorphism was determined by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The PCR primers were sense: 5'-ACCCTTTAAGCTGGGTGTCCCAAATAG-3' and antisense: 5'-AGCTAGCAAATATTTTTGTAAGCA ATT-3'. The 25 μL reaction mixtures consisted of 100 ng of DNA, 1×PCR buffer, 1.5 mM/L $MgCl_2$, 0.5 μM of both forward and reverse primer, 200 μM/L of each dNTP and 0.5U of Taq (Promega, USA). The Gln223Arg cycling protocol was initiated by one cycle at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec. The PCR products were digested with Msp I (Fermentas, Lithuania) restriction enzyme following the manufacturer's instruction. The digested products then underwent electrophoresis in 2% agarose gel. Results were interpreted based on the positive amplification band (Gln/Gln (A/A): 416bp; Arg/Arg (G/G): 291bp, 125bp; Gln/Arg (A/G): 416bp, 291bp, 125bp).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) version 15 (SPSS Inc, USA). Allele, genotype and haplotype frequencies, Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated using the Arlequin program, version 3.5 (Excoffier et al., 2010). The frequencies of alleles, genotypes and haplotypes were compared in cases and controls by chi-square test, Fisher's exact test. Odds ratios [OR] and 95% confidence intervals (CIs) for relative risk of CC-RCC were calculated. A probability value of $p<0.05$ was considered as statistically significant and all the reported p values were two-tailed.

Results

Hardy-Weinberg equilibrium test for Lys109Arg and Gln223Arg in the CC-RCC subjects and controls

The HWE tested for Lys109Arg and Gln223Arg revealed no significant deviation (p=1.00 in the CC-RCC subjects and p=0.76 in the controls). Similarly, SNP of Gln223Arg was in Hardy-Weinberg equilibrium in CC-RCC subjects and controls (p=1.00 in CC-RCC subjects and p=1.00 in the controls).

Alleles and genotype of Lys109Arg and Gln223Arg in the CC-RCC subjects and controls

Table 1 showed the frequencies of Lys109Arg and Gln223Arg polymorphisms in the CC-RCC subjects and controls. The data showed there were no significant differences in allelic frequencies and genotype distributions of Lys109Arg and Gln223Arg polymorphisms between the CC-RCC subjects and controls.

Combined genotype of Lys109Arg and Gln223Arg in the CC-RCC subjects and controls

When evaluating the Lys109Arg and Gln223Arg genotype interaction, we observed a significant increase of GG/GG combined genotypes (OR=1.85; 95%CI=1.04-3.30) in the CC-RCC subjects than those in the control. In addition, a significant decrease of GA/GG and GG/GA combined genotypes (OR=0.07; 95%CI=0.01-0.54, OR and 95%CI of the latter can't be calculated for a value of zero) was found in CC-RCC subjects (Table 2).

Haplotype of Lys109Arg and Gln223Arg in the CC-RCC subjects and the controls

Table 3 showed the frequencies of Lys109Arg and

Table 1. Allele and Genotype Frequencies of Lys109Arg and Gln223Arg in the CC-RCC Subjects and Controls

Polymorphism	Genotype	CC-RCC (n=77)		Control (n=161)		χ^2 value	*P*	Odds Ratio	95%CI
		n	AF (%)	n	AF (%)				
	G/A	21	27.27	52	32.3	0.619	0.43	0.79	0.43-1.43
Lys109Arg	A/A	2	2.6	7	4.35	0.439	0.51	0.59	0.12-2.89
	G/G	54	70.13	102	63.35	1.059	0.3	1.36	0.76-2.44
	G allele	129	83.77	256	79.5	1.224	0.27	1.33	0.80-2.21
	A allele	25	16.23	66	20.5				
	G/A	20	25.97	41	25.47	0.007	0.93	1.03	0.55-1.91
	A/A	2	2.6	4	2.48	0.003	0.96	1.05	0.19-5.84
Gln223Arg	G/G	55	71.43	116	72.05	0.01	0.92	0.97	0.53-1.77
	G allele	130	84.42	273	84.78	0.011	0.92	0.97	0.57-1.65
	A allele	24	15.58	49	15.22				

*n, absolute number; CC-RCC, clear cell renal cell carcinoma; CI, confidence interval

Table 2. Combined Genotype Frequencies of Lys109Arg and Gln223Arg in the CC-RCC Subjects and Controls

Gln223Arg/Lys109Arg	CC-RCC (n=77)		Control (n=161)		χ^2 value	*P*	Odds Ratio	95%CI
Combined Genotype	n	AF (%)	n	AF (%)				
GA/GA	20	25.97	26	16.15	3.23	0.07	1.82	0.94-3.53
GA/GG	1	1.3	25	15.53	10.84	0.001[a]	0.07	0.01-0.54
GA/AA	0	0	1	0.62	0.48	0.49	-	-
GG/GA	0	0	12	7.45	6.04	0.01[a]	-	-
GG/GG	54	70.13	90	55.9	4.41	0.04[a]	1.85	1.04-3.30
GG/AA	0	0	0	0	-	-	-	-
AA/GA	0	0	3	1.86	1.45	0.23	-	-
AA/GG	0	0	1	0.62	0.48	0.49	-	-
AA/AA	2	2.6	3	1.86	0.14	0.71	1.4	0.23-8.58

*n, absolute number; CC-RCC, clear cell renal cell carcinoma; CI, confidence interval; [a]Considered significant with *p* value threshold of 0.05

Table 3. Haplotype Frequencies of Lys109Arg and Gln223Arg in the CC-RCC Subjects and Controls

Gln223Arg/Lys109Arg	CC-RCC (n =77)		Control (n =161)		χ^2 value	*P*	Odds Ratio	95%CI
Haplotype	n	AF (%)	n	AF (%)				
A-A	24	15.58	36	10.77	1.83	0.18	1.47	0.84-2.56
A-G	1	0.65	30	9.73	12.85	0.001[a]	0.06	0.01-0.47
G-G	129	83.77	243	75.06	4.2	0.04[a]	1.68	1.02-2.76
G-A	0	0	13	4.44	6.39	0.01[a]	-	-

* n, absolute number; CC-RCC, clear cell renal cell carcinoma; CI, confidence interval; [a]Considered significant with *p* value threshold of 0.05

Gln223Arg haplotypes in the CC-RCC and control subjects. Examination of the haplotypes of Lys109Arg and Gln223Arg revealed evidence for association in CC-RCC subjects. We found the G-G haplotype frequency of Lys109Arg and Gln223Arg was significantly higher in the CC-RCC subjects than those in the control (OR=1.68; 95%CI=1.02-2.76). However, the A-G and G-A haplotype frequencies of Lys109Arg and Gln223Arg in the cases were lower than those in the control (OR=0.06; 95%CI=0.01 to 0.47; OR and 95%CI of the latter can't be calculated for a value of zero).

Linkage disequilibrium analysis between Lys109Arg and Gln223Arg alleles

The linkage disequilibrium (LD) for Lys109Arg and Gln223Arg polymorphisms was analyzed. The pairs of Lys109Arg A/G and Gln223Arg A/G alleles displayed a significant ($p<0.05$) LD in the CC-RCC subjects. The Lys109Arg A allele was in LD with the Gln223Arg A alleles (d'=0.9399). Most of the CC-RCC subjects with the Lys109Arg A allele had the Gln223Arg A allele, and only 4.76% (1/21) of the CC-RCC subjects with Lys109Arg A allele had the Gln223Arg G allele. However, in the controls, the Lys109Arg A allele was not in LD with the Gln223Arg A allele (d'=-0.0502).

Discussion

Leptin plays a key role in regulating body-fat homeostasis. Leptin also is an important tumor cell growth factor. Several studies have demonstrated that leptin stimulates endothelial cell proliferation and angiogenesis, inhibits apoptosis gene and protein expression in cell lines (Samuel-Mendelsohn et al., 2011; Ptak et al., 2013; Wu et al., 2013; Yuan et al., 2013). Leptin exerts its biological functions through binding to the extracellular domain of LEPR. There are several variants of the LEPR genes. Among LEPR SNPs, Lys109Arg and Gln223Arg variants are associated with amino acid substitutions in the extracellular region of the leptin receptor and have potential functional consequences. Reports have been suggested the associations of Lys109Arg and Gln223Arg polymorphisms with an increased risk for several cancers (Liu et al., 2007; Han et al., 2008; Yapijakis et al., 2009; Kim et al., 2012). However, to date, few studies have addressed the relationship between LEPR polymorphisms

and CC-RCC risk. Thus, our study was performed to evaluate this association between Lys109Arg and Gln223Arg polymorphisms and CC-RCC.

In this study, the data showed there were no significant differences in allelic frequencies and genotype distributions of the examined LEPR gene polymorphisms between the CC-RCC cases and controls. However, when evaluating the Lys109Arg and Gln223Arg genotype interaction, a significant increase of GG/GG combined genotype frequency was observed in the CC-RCC cases than controls ($p<0.05$). In addition, we found a significant decrease of GA/GG ($p<0.001$) and GG/GA ($p<0.01$) combined genotypes frequencies in the CC-RCC cases. Examination of the haplotypes of Lys109Arg and Gln223Arg revealed greater evidence for association. The G-G haplotype frequency of Lys109Arg and Gln223Arg was found to be significantly higher in the CC-RCC cases than controls ($p<0.05$). However, we found the A-G ($p<0.001$) and G-A ($p<0.01$) haplotypes frequencies of Lys109Arg and Gln223Arg were high in the controls. Our results suggest the GG/GG combined genotype of Lys109Arg and Gln223Arg variants is related to the CC-RCC risk. Haplotype analysis also showed an association between the G-G haplotype of Lys109Arg and Gln223Arg variants and the CC-RCC risk. However, the data suggest the haplotype of A-G or G-A and the combined genotype of GA/GG or GG/GA are negative association with the risk of CC-RCC. The mechanism through which Lys109Arg and Gln223Arg variants may increase the risk of cancer remains unclear. Study has shown the lower leptin binding to the leptin receptor with the A allele of the Gln223Arg (Stefan et al., 2002). Lys109Arg and Gln223Arg lie within the putative leptin binding regions, which may be associated with signaling capacity of the leptin receptor.

The allelic frequency of particular gene polymorphisms may vary significantly in different geographic and ethnic population (Paracchini et al., 2005). The frequencies of LEPR polymorphisms also were different in previous reports. The frequencies of Lys109Arg wild-type (A) were 31.52%, 35.2%, 18.09% and Gln223Arg were 51.94%, 58.6%, 8.9% in whites and African American(Nyante et al., 2011), Caucasian (Teras et al., 2009) and Asian (Woo et al., 2006; Liu et al., 2007) normal population, respectively. In our study, the frequencies of Lys109Arg and Gln223Arg wild-types were 20.5% and 15.22% in controls, which was similar to Asian population. Therefore, further studies are needed to clarify the association between Lys109Arg and Gln223Arg and CC-RCC in population with geographic and racial differences.

Our results add to the limited available data on the association between the LEPR gene polymorphisms and CC-RCC risk. We did observe important interactions between Lys109Arg and Gln223Arg polymorphisms in CC-RCC. Furthermore, our data showed the Lys109Arg A allele was in LD with the Gln223Arg A allele in CC-RCC subjects, but not in the controls. These suggest the interaction of Lys109Arg and Gln223Arg may play an important role in the development of CC-RCC. One of the challenges is defining the association between Lys109Arg and Gln223Arg polymorphisms in CC-RCC. Therefore, research to understand the associations and the functionality between Lys109Arg and Gln223Arg polymorphisms are needed. Moreover, given the limited sample size, additional studies with large sample size are necessary to demonstrate the relationships of these polymorphisms with CC-RCC risk.

In conclusion, this analysis suggests the combination of Lys109Arg and Gln223Arg polymorphisms is significantly correlated with CC-RCC risk. The combination of GG/GG genotype and G-G haplotype are high risk factors for developing CC-RCC. In contrast, the haplotype of A-G or G-A and the combined genotype of GA/GG or GG/GA may confer protective effect. Our study may provide new information on the role of Lys109Arg and Gln223Arg polymorphism combination in CC-RCC risk.

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