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Original Article

Association of IL-4 and IL-4 receptor gene polymorphisms with the risk, immunotherapeutic effects and prognosis of advanced renal cell carcinoma

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Abstract: We aimed to investigate the association of IL-4 and IL-4 receptor (IL-4R) gene polymorphisms with the immunotherapeutic effects and prognosis of advanced renal cell carcinoma (RCC) in Chinese population. Genotypes of rs2243250 and rs1805010 in IL-4 and IL-4R genes were detected in 112 advanced RCC patients and 150 healthy controls by PCR-RFLP, and these associations were analyzed by chi-square test and log-rank test. The distributions of genotype and allele of rs2243250 and rs1805010 in patients and controls were significant differences ($P<0.05$). But the clinical baselines, immunotherapeutic effects, and incidence of adverse events were not significant difference in patients with different genotype or allele ($P>0.05$). However, rs2243250 TT genotype carried patients had significantly longer median progression free survival (mPFS) and median overall survival (mOS) than others (Log rank $P<0.05$); rs1805010 carriers AG had significantly longer mPFS, AA had significantly longer mOS than others (Log rank $P<0.05$). Rs1805010 genotypes and adverse events of immunotherapy had prognosis value with a risk ratio of 1.86 and 1.65, respectively. We infer that the polymorphisms rs2243250 in IL-4 and rs1805010 in IL-4R gene could consider to be biomarkers of predicting the risk and survival for advanced RCC patients in Chinese population.

Keywords: Interleukin-4, Interleukin-4 receptor, polymorphism, renal cell carcinoma, immunotherapy, prognosis

Introduction

Renal cell carcinoma (RCC) is a severe and highly malignant cancer with an increasing incidence of appropriately 2% each year [1]. Almost 30% of RCC patients suffer advanced or metastatic carcinoma with 5-year survival rate of lower than 20% in the developing countries [2]. However, RCC as well as other carcinomas are multifactorial diseases involved in the interactions between genetics and environments [3]. Genetic variations such as gene polymorphisms, single nucleotide polymorphisms (SNPs), have been demonstrated to play a key role in the carcinogenesis through affecting the cell apoptosis, inflammatory reaction, cytokine function, and so on [4, 5].

Interleukin-4 (IL-4) is a pleiotropic cytokine produced by basophils, mast cell, and activated $CD4^+$ T cell [6]. It has important effects on the proliferation, differentiation, survival, and gene expression of different immunologically competent cells [7]. Numerous studies have indicated that IL-4 and IL-4 receptor (IL-4R) gene polymorphisms were associated with the increasing risks of oral cancer [8], transitional cell carcinoma [9, 10], and cervical cancer [11]. Furthermore, a meta-analysis involving more than ten thousand individuals suggested that the low frequency allele in the SNP rs2243250 polymorphism in IL-4 gene was significantly associated with the decreased oral cancer risk but increased renal cell cancer risk [12].

For RCC, various SNPs in IL-4 and IL-4R gene have been demonstrated susceptibility to RCC. The CC genotype of IL-4 gene rs2243250 polymorphism could significantly decreased RCC

Table 1. Characteristics of participants

Characteristic	Patients	Controls	*P*
Sample size	112	150	
Mean age (years)	57.52±8.65	55.58±8.33	0.07
Gender			
Male	72 (64.29%)	92 (61.33%)	0.63
Female	40 (35.71%)	58 (38.67%)	
Smoke	33 (29.46%)	38 (25.33%)	0.51
Drink	14 (12.50%)	17 (11.33%)	0.80
KPS (scores)	69.11±10.36		
Clinical stage			
III	78 (69.64%)		
IV	34 (30.36%)		
Distant metastasis			
Yes	21 (18.75%)		
No	91 (81.25%)		
Prior therapy			
INF-α	49 (43.75%)		
IL-2	21 (18.75%)		
INF-α+IL-2	42 (37.50%)		
Range of follow-up (months)	2-52		
Median follow-up (months)	25		

risk in an association analysis of Chinese population [13]. IL-4R polymorphism rs1805010 was associated with increased RCC risk [14]. Meanwhile, the exploration on the association of IL-4 and IL-4R gene polymorphisms with prognosis of RCC has also been launched in Japanese [15] and Australian [16] population, respectively. These studies suggested that polymorphisms in IL4 and IL-4R genes may consider to be the biomarkers for the prognosis of RCC.

Overall, the associations of IL4 and IL-4R gene polymorphisms with RCC risk in Chinese population have been cleared by recent studies; but the relationship between these polymorphisms with immunotherapeutic effects and prognosis of RCC in Chinese population have never been clarified. This lack of information impedes a complete comprehension of the role of IL-4 systems variants in carcinogenesis of RCC. Hence we aimed to determine whether there exists an association of IL-4 and IL-4R gene polymorphisms with the risk, clinical baseline, immunotherapeutic effects, adverse events, and prognosis of advanced RCC in our Chinese Han populated samples.

Materials and methods

Subjects

One hundred and twenty-four patients with advanced RCC were recruited from the Xinxiang Central Hospital and affiliated regional hospitals of Xinxiang and Anyang in North of China. But twelve patients were excluded by the poor quality of DNA amplifications. Finally, one hundred and twelve (72 male, 40 female) advanced RCC (clinical stage diagnostic criteria of AJCC 2010 [17]) patients with pathological diagnosis of clear-cell carcinoma were included. All the patients have never received tumor removed operation or systemic chemotherapy and immunotherapy until they were enrolled. After the strict testing and evaluation by CT or MRI, the patients were received standardized immunotherapy (49 INF-α, 21 IL-2, 42 INF-α and IL-2) and adjuvant therapy under the guidance of NCCN in China (http://www.nccnchina.org/).

Meanwhile, one hundred and fifty healthy controls (92 male, 58 female) with high quality of DNA sample and without complex disorders including hypertension, diabetes, immunological mediated disease, mental disorders, and cancers were selected from located Henan province. All participants in the study were Chinese Hans. The mean age of the patients and controls were 57.52 ± 8.65 and 55.58 ± 8.33 years, respectively. All subjects consented to participate in the study after reviewing the informed consent. The study was approved by the Institutional Review Board of the Xinxiang Central Hospital and affiliated regional hospitals. The demographic characteristics of the participants were showed in **Table 1**. No significant differences in age, gender, smoking and drinking histories between the two groups.

Genotyping

The global genomic DNAs were extracted from peripheral blood mononuclear cells using standard protocols then stored at -20°C. The IL-4 SNP rs2243250 and IL-4R SNP rs1805010 polymorphisms were amplified by polymerase chain reaction with the following primers: Sense 5'-TAAACTTGGGAGAACATGGT-3' and antisense

Table 2. Association of IL-4 and IL-4R polymorphisms with the risk of advanced RCC

Polymorphism	Patients	Controls	*P*	Odd Ratio	95% CI
Rs2243250					
CC	6 (5.36%)	8 (5.33%)	0.04	Reference	
CT	31 (27.68%)	64 (42.67%)		1.55	0.49-4.85
TT	75 (66.96%)	78 (52.00%)		0.78	0.26-2.35
C allele	43 (19.20%)	80 (26.67%)	<0.05	Reference	
T allele	181 (80.80%)	220 (73.33%)		0.65	0.43-0.99
Rs1805010					
AA	43 (38.39%)	44 (29.33%)	0.04	Reference	
AG	51 (45.54%)	62 (41.33%)		1.19	0.68-2.08
GG	18 (16.07%)	44 (29.34%)		2.39	1.20-4.77
A allele	137 (61.16%)	150 (50%)	0.01	Reference	
G allele	87 (38.84%)	150 (50%)		1.57	1.11-2.24

Table 3. Association of IL-4 and IL-4R polymorphisms with clinical baseline of advanced RCC patients (N=112, N)

Factors	Rs2243250					Rs1805010				
	CC	CT	TT	X^2	*P*	AA	AG	GG	X^2	*P*
Clinical stage										
III	5	20	53	0.95	0.62	30	33	15	2.18	0.34
IV	1	11	22			13	18	3		
Distant metastasis										
Yes	1	7	13	0.41	0.81	7	13	1	3.75	0.15
No	5	24	62			36	38	17		
Prior therapy										
INF-α	3	12	34	1.50	0.83	17	26	6	3.22	0.52
IL-2	1	8	12			7	9	5		
INF-α+IL-2	2	11	29			19	16	7		

5'-TGGGGAAAGATAGAGTAATA-3' for IL-4 gene fragment [13], Sense 5'-GGCAGGTGTGAGGAGCATCC-3' and antisense 5'-GCCTCCGTTGTTCTCAGGTA-3' for IL-4R gene fragment [18]. The PCR amplification was performed in a 25 μl reaction volume containing 10 × PCR buffer 2.5 μl, dNTP mix (2.5 mM) 0.5 μl, each Primer (10 μM) 1 μl, genomic DNA 1 μl, Taq DNA polymerase (5 U/μl, supplied by Tian Gen) 0.4 μl and sterile deionized water 18.6 μl. After initial denaturation at 94°C for 5 min, the mixture was performed to 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final elongation at 72°C for 10 min [19]. The PCR product was cleaved using Ava II (IL-4, rs2243250) and Rsa I (IL-4R, rs1805010) restriction enzymes (supplied by Thermo) at 37°C for 2 h, then analyzed by electrophoresis on 3% agarose gels. Genotypes for rs2243250 and rs1805010 polymorphisms were identified by two investigators independently. Three genotypes resulting from digestion with Ava II were CC (177, 18 bp), CT (195, 177, and 18 bp) and TT (195 bp). Three genotypes yield from digestion with Rsa I were GG (273 bp), AG (273, 254, and 19 bp) and AA (254, 19 bp).

Clinical measures

The objective response rate (ORR), disease control rate (DCR) and KPS improvement rate was used to examine the immunotherapeutic effects of advanced RCC patients. The incidence of adverse events, including flu-like symptoms, aleukocytosis, thrombocytopenia, hypopiesia, and emesia, was used to investigate the safety of INF-α and IL-2. Furthermore, the prognosis of advanced RCC patients was estimated by progression free survival (PFS) and overall survival (OS).

Statistical analysis

Hardy Weinberg equilibrium was performed using Haploview 4.1 to identify the genotype distributions of these two polymorphisms in IL-4 and IL-4R genes [20]. The following statistical analysis was performed in SPSS 18.0 software. The associations of IL-4 and IL-4R gene polymorphisms with the risk, clinical baseline, immunotherapeutic effects and adverse events of advanced RCC patients were evaluated by X^2 test. The Kaplan-Meier method and the log-rank test for analysis of survival were used to examine the prognostic significance of the respective IL-4 and IL-4R genotypes for PFS and OS [16]. Cox forward stepwise regression model was used in multivariate analysis to determine the independent risk factors of prog-

Table 4. Association of IL-4 and IL-4R polymorphisms with immunotherapeutic effects and adverse events for advanced RCC patients after standardized immunotherapy (N=112, %)

Factors	Rs2243250					Rs1805010				
	CC	CT	TT	X^2	*P*	AA	AG	GG	X^2	*P*
ORR	0.00	6.45	9.33	0.80	0.67	6.98	9.80	5.56	0.43	0.81
DCR	33.33	48.39	62.67	3.29	0.19	58.14	60.78	44.44	1.48	0.48
KPS improvement	16.67	19.35	24.00	0.39	0.82	25.58	15.69	33.33	2.82	0.24
Adverse events	16.67	6.45	8.00	0.71	0.70	11.63	3.92	11.11	2.15	0.34

Table 5. Association of IL-4 and IL-4R polymorphisms with mPFS and mOS for advanced RCC patients (N=112, month)

Polymorphism	mPFS	mOS
Rs2243250		
CC	3.50	5.00
CT	5.00	12.00
TT	8.00	16.00
Log rank X^2	15.01	11.95
P	<0.01	<0.01
Rs1805010		
AA	6.00	18.00
AG	7.00	15.00
GG	3.50	8.50
Log rank X^2	9.03	12.45
P	0.01	<0.01

nosis [21]. A power analysis was performed using the Genetic Power Calculator. All statistical tests were two-tailed, *P* value was adjusted with age and gender, and $P<0.05$ was the threshold level for statistical significance.

Results

Association of SNPs with the risk, clinical baseline, and immunotherapeutic effects of advanced RCC

The genotype frequencies of the IL-4 and IL-4R gene polymorphisms rs2243250 and rs1805010 were in Hardy-Weinberg equilibrium. The distribution of genotype ($P=0.04$) and allele ($P<0.05$) of rs2243250 were significant differences when comparing patients and controls (**Table 2**), and C-allele carriers seemed to decrease the risk of advanced RCC. Furthermore, significant differences were also found in the genotype ($P=0.04$) and allele ($P=0.01$) distribution of rs1805010 when comparing patients and controls (**Table 2**), while G-allele carriers seemed to decrease the risk of advanced RCC. However, no significant difference was demonstrated in the clinical baselines, such as clinical stage, distant metastasis, and prior therapeutic drugs among the patients with different genotype of rs2243250 and rs1805010 ($P>0.05$, **Table 3**). Moreover, after standardized immunotherapy for 112 advanced RCC patients, the immunotherapeutic effects (ORR, DCR, and KPS improvement rate) and the incidence of adverse events were also no significant difference among the patients with different genotypes of rs2243250 and rs1805010 ($P>0.05$, **Table 4**). This study had the power of 0.752 overall.

Association of SNPs with progression free survival and overall survival

In the present study, Kaplan-Meier method and log-rank test were used to investigate the influences of IL-4 and IL-4R gene polymorphisms rs2243250 and rs1805010 on prognosis of advanced RCC patients. Median PFS (mPFS) was 8.00 months in advanced RCC patients carried TT genotype of rs2243250, which was 1.6 and 2.3 fold higher than the patients carried CT and CC genotypes (Log rank $X^2=15.01$, $P<0.01$, **Table 5**). For median OS (mOS), the advanced RCC patients with TT genotype was 16.00 months, which was 1.3 and 3.2 fold higher than CT and TT carriers (Log rank $X^2=11.95$, $P<0.01$, **Table 5**). Moreover, the mPFS was 7.00 months in patients carried AG genotype of rs1805010, which was 1.2 and 2.0 fold higher than the patients carried AA and GG genotypes (Log rank $X^2=9.03$, $P=0.01$, **Table 5**). But, patients carried AA genotype had 18.00 months' mOS, which was 1.2 and 2.1 fold higher than AG and GG carriers (Log rank $X^2=12.45$, $P<0.01$, **Table 5**). Thus, advanced RCC patients carried TT genotype of rs2243250 and AA genotype of rs1805010 showed significantly prolonged survivals and better prognosis under the treatment of immunotherapy. When com-

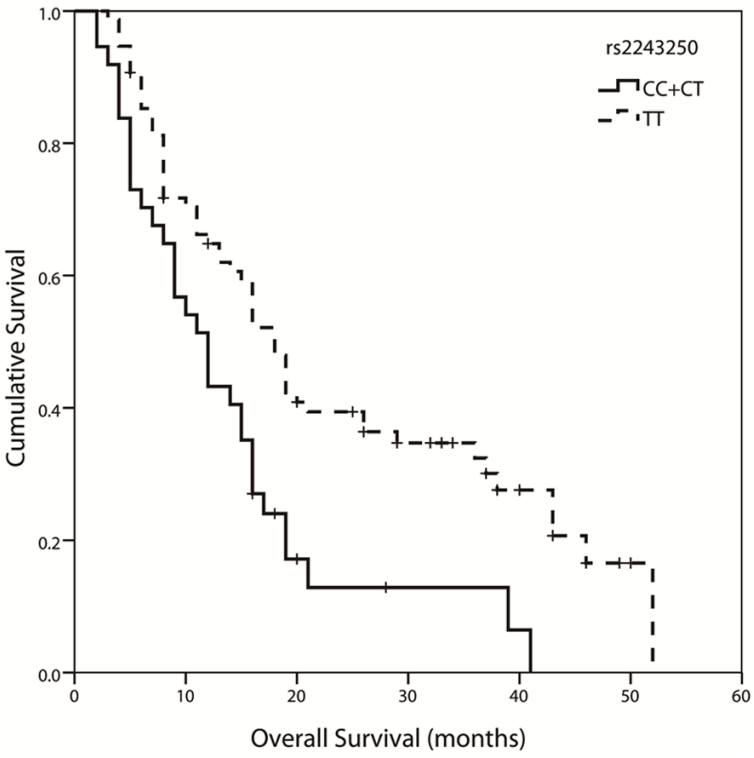

Figure 1. Kaplan-Meier plot of overall survival in relation to the genotype of IL4 gene polymorphism rs2243250. The mOS in advanced RCC patients with TT genotype was 18.00 months, and CC+CT was 12.00 months. Log-rank $P < 0.01$.

pared with rs2243250 C allele carriers of advanced RCC patients, the TT homozygote carriers had increased 1.5 folds' mOS (18.00 vs. 12.00 months, Log-rank $P<0.01$, **Figure 1**). Meanwhile, AA homozygote carriers had 1.7 fold higher mOS than rs1805010 C allele carriers (20.00 vs. 12.00 months, Log-rank $P=0.03$, **Figure 2**).

Multivariate analysis of the effects of clinical characteristics on survival

To adjust for confounding factors, a multivariate analysis of the effects of clinical characteristics (including gender, age, smoke, drink, KPS, distant metastasis, clinical stage, rs2243250, rs1805010, and adverse event) on survival was performed using Cox forward stepwise regression model. Setting the threshold of 0.05, only two variations such as rs1805010 and adverse events entered the final model. Thus, IL-4R gene polymorphism rs1805010 (AA vs. AG+GG) and adverse events of immunotherapy (with vs. without) had prognosis value with a risk ratio of 1.86 ($P=0.01$, 95% CI: 1.16-2.98) and 1.65 ($P=0.01$, 95% CI: 1.02-2.70), respectively (**Table 6**).

Discussion

In the present study, we focused on two RCC-risk polymorphisms in IL-4 and IL-4R gene and systematically investigated its significance in risk, immunotherapeutic effects and prognosis of advanced RCC. No obvious association was found between these SNPs and clinical baselines including tumor stage, metastasis status. Meanwhile, the ORR, DCR, KPS improvement, and adverse events of medications of INF-α and IL-2 treat for RCC was not associated with IL-4 and IL-4R gene polymorphisms. However, we observed that these gene polymorphisms were significantly associated with the disease risk, PFS and OS of RCC patients in Chinese Han population under the treatment of immunotherapy. In addition, T allele of rs2243250 and A allele of rs1805010 carried RCC patients had longer PFS and OS compared with other allele carriers. The polymorphisms in IL-4 and IL-4R gene may contribute to serve as useful genetic marker for evaluating the prognosis of RCC.

The findings in our study are almost novel in Chinese Han population, although previous study has demonstrated that IL-4 gene polymorphisms were associated with the risk of RCC [13]. For clinical characteristics, IL-4 gene rs2243250 CC allele revealed to decrease the disease risk in RCC patients with localized stage and well differentiated carcinoma [13]. It is consistent with our result of the association in RCC risk, but we do not find any association between IL-4 or IL-4R gene and such clinical baselines of RCC patients. The inconsistency might be due to the different selection of the reference population.

Some genes could alter the susceptibility to diseases; meanwhile they may also affect clini-

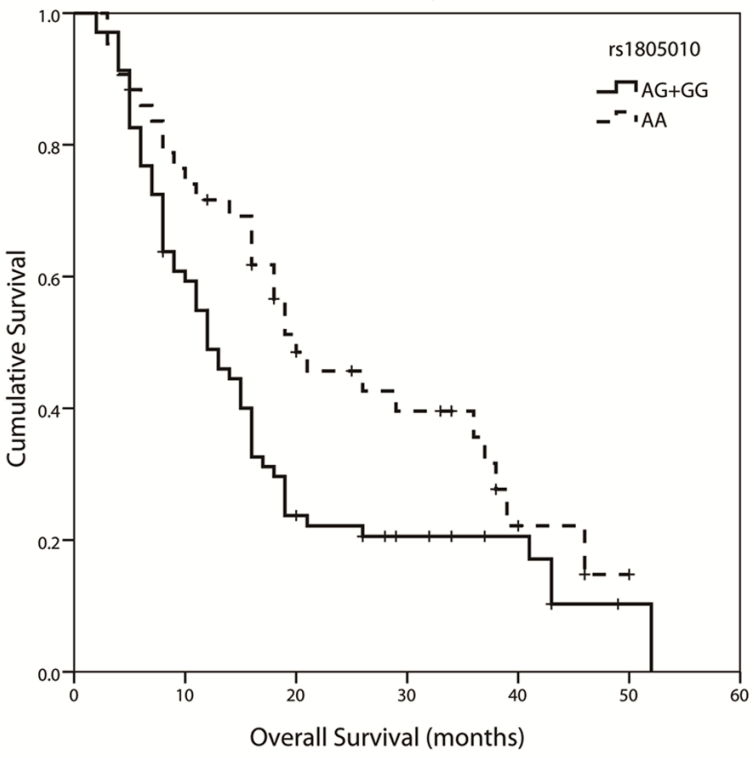

Figure 2. Kaplan-Meier plot of overall survival in relation to the genotype of IL4R gene polymorphism rs1805010. The mOS in advanced RCC patients with AA genotype was 20.00 months, and AG+GG was 12.00 months. Log-rank *P*=0.03.

Table 6. Multivariate analysis of the effects of clinical characteristics on survival

Factors	SE	*P*	Risk Ratio	95% CI
Gender	0.28	0.79	0.93	0.54-1.60
Age	0.01	0.67	0.99	0.97-1.02
Smoke	0.28	0.83	0.94	0.55-1.62
Drink	0.34	0.80	0.92	0.47-1.80
KPS	0.01	0.53	1.01	0.99-1.03
Distant metastasis	0.42	0.69	0.84	0.37-1.94
Clinical stage	0.42	0.51	1.32	0.58-3.01
Rs2243250[a]	0.36	0.56	0.81	0.40-1.94
Rs1805010[b]	0.24	0.01	1.86	1.16-2.98
Adverse events	0.25	0.04	1.65	1.02-2.70

[a]TT vs. CC+CT; [b]AA vs. AG+GG.

cal effects of medications [22-24]. In our results, these two RCC risk gene IL-4 and IL-4R are also affect the long-term follow-up results although they do not associate with short-term effects of immunotherapy such as ORR and DCR. It is almost consistent with a study in Japan which suggested that heterozygote of rs1805010 in IL-4R gene carriers had increasing OS than others [15]. Furthermore, a previous study reported haplotypes in IL-4 gene consisted of rs2243250 and rs2070874 were associated with prognosis of RCC in Australian, and mOS was 3.5 fold increased in patients with homozygote for IL-4 haplotype 4 compared with heterozygote of haplotypes 1 and 4 [16]. Taking together the results of these association analyses, T allele of rs2243250 and A allele of rs1805010 would be protective factors in the transformations of RCC. However, T allele of rs2243250 in IL-4 gene might play a "negative" role in decreasing the risk of RCC [13, 25]. We infer that IL-4 and IL-4R genetic polymorphisms may participate in the carcinogenesis as well as the death of tumor cells through different mechanisms in the whole biological process of RCC. In fact, the interested conclusion was mainly supported by an association analysis in Japanese population [15].

IL-4 plays a central role to regulate the differentiation of antigen-simulated naïve T cells, and then causes these T cells to produce cytokines, such as IL-10 and IL-14, or on the other hand, suppresses $CD4^+$ T cells to secrete IFN-γ [16]. Hence, IL-4 has been seen as the focus of controversy in recent years due to the indeterminate biologic functions on cancers. However, variants in IL-4 and IL-4R genes, such as rs2243250 and rs1805010, may effect on IL-4 signal transduction by change promoter activity of IL-4 gene and conformation of IL-4R protein directly or indirectly [26, 27]. Early studies suggested that IL-4 could help other cytokines or drugs to remove and suppress growth of tumor cells [28, 29]. Conversely, recent studies revealed that IL-4 preferred to inhibit the activity of antitumor from body selves or intervention treatment [30, 31]. In addition, although IL-4 exhibited a growth-inhibitory effect in vitro of RCC [32, 33], the clinical use of IL-4 as an administrated agent failed to inhibit the progression of RCC and other cancers [34, 35].

Previously, Rosenwasser et al. [36] described that T allele of rs2243250 in IL-4 gene was associated with the increased expression of IL-4 in vitro. But IL-4 could not suppress the progression of RCC [34], even exhibit a possible "positive" activity of tumor progression. Taking together, it is probable to explain why T allele of rs2243250 in IL-4 gene was associated with the increased risk of RCC and other cancers. However, this T allele is also related to the subsequent better prognosis of RCC, exhibiting an inhibiting function to tumor progression through other mechanisms which have not been clarified. For the polymorphisms in IL-4R gene, a similar phenomenon was founded. Although A allele of rs1805010 increased the risks of RCC, it is associated with the longer survival of RCC patients.

In summary, we presented an association of IL-4 and IL-4R gene polymorphisms with prognosis of RCC under the treatment of immunotherapy. T allele of rs2243250 in IL-4 and A allele of rs1805010 in IL-4R gene exhibited a potential effect for prolonging the survival of RCC patients. We believe that these results will contribute to understand the role of variants in IL-4 and IL-4R gene for carcinogenesis and immunotherapy, even could consider the possibility of these variants to be biomarkers of RCC. Furthermore, it is also encourage us to next focus on the different mechanisms of IL-4 in carcinogenesis and progression.

Disclosure of conflict of interest

None.

Authors' contribution

Author XF, and LW designed the study. XF wrote the protocol and the first draft of the manuscript. Author XF and ZZ finished the biological experiments. Author XF and YJ undertook the statistical analysis. Author XF, SY, and LW collected clinical samples and data. Author ZZ managed the literature searches and analyses. All authors contributed to and have approved the final manuscript.

Abbreviations

RCC, Renal cell carcinoma; SNP, Single nucleotide polymorphism; IL-4, Interleukin-4; IL-4R, Interleukin-4 receptor; ORR, Objective response rate; DCR, Disease control rate; PFS, Progression free survival; OS, Overall survival; OR, Odd ratio; RR, Risk ratio; CI, Confidence interval.

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