ORIGINAL ARTICLE

A functional epidermal growth factor (EGF) polymorphism, EGF serum levels and renal cell carcinoma risk in a Chinese population

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The epidermal growth factor (*EGF*) gene encodes a growth factor that binds to the EGF receptor (EGFR), which is involved in activating pathways that promote cellular proliferation, survival, migration and differentiation, and lack of control is characteristic of malignant development. Previous studies showed that serum EGF levels may influence the risk of cancer. In this study, we genotyped the *EGF* G61A polymorphism (rs4444903) and measured serum EGF levels using an enzyme immunoassay in a hospital-based case–control study of 345 patients with diagnosed renal cell carcinoma (RCC) and 346 cancer-free controls in a Chinese population. Compared with the *EGF* 61GG genotype, the AA genotype had a significantly increased RCC risk (odds ratio=1.80, 95% confidence interval=1.04–3.12). Besides, the mean serum EGF levels in RCC patients (858.94 ± 391.54 pg ml⁻¹) were significantly lower than those in controls (1281.52 ± 568.42 pg ml⁻¹, $P < 0.001$). In addition, individuals carrying AA genotype had lower serum EGF levels than GA or GG carriers. These results suggested that the *EGF* G61A polymorphism is involved in the etiology of RCC and thus may be a marker for genetic susceptibility to RCC in Chinese populations. Larger studies are warranted to validate our findings.

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Keywords: *EGF* polymorphism; EGF serum levels; genetic susceptibility; RCC

INTRODUCTION

Renal cell carcinoma (RCC) represents 2–3% of all cancers and is the third leading cause of death among genitourinary malignancies, with the highest incidence occurring in the developed countries.¹ It is estimated that ~37.7 men and 16.6 women per 100 000 Chinese individuals are diagnosed with RCC every year.² Accumulative epidemiological studies have suggested that gender, obesity, cigarette smoking and a history of hypertension, along with some other less certain factors such as alcohol consumption, occupational exposures, diet, physical activity and family history of cancer are associated with RCC.^{3,4} Cigarette smoking, for example, doubles the risk for RCC and contributes to as much as one-third of all cases, yet only a fraction of smokers and a low number of nonsmokers develop RCC.⁵ This suggests that interindividual differences including genetic susceptibility in critical genes may have an important role in RCC carcinogenesis.

Epidermal growth factor (EGF) is a member of the EGF superfamily, which also includes transforming growth factor- α , heparin-binding EGF-like growth factor, epiregulin, betacellulin and amphiregulin.⁶ EGF is encoded by a 4.8-kb mRNA transcript from a

gene that is 110 kb in length, contains 24 exons and is located on chromosome 4q25–27.⁶ As a growth factor, EGF can activate DNA synthesis and cellular proliferation and stimulate mitogenesis in epidermal tissue.^{7,8} In 2002, Shahbazi *et al.*⁹ first identified a G>A substitution at position 61 in the 5'-untranslated region of *EGF*, and the presence of the variant 61A allele led to a decreased *EGF* production in peripheral blood mononuclear cells. Since then, numerous studies focused on the association between the *EGF* G61A polymorphism and risk of cancers, including melanoma,^{10,11} gastric cancer,^{12,13} breast cancer¹⁴ and others.

As EGF is important for tumor growth and progression, the dysregulation of *EGF* gene may modulate the progression and spread of cancer. To the best of our knowledge, no reports have been published regarding the role of the *EGF* G61A polymorphism in RCC. Therefore, our present study was designed to investigate the association of *EGF* G61A gene polymorphism with risk of RCC in our ongoing hospital-based case–control study in a Chinese population. Besides, to further assess the potential functional effect of *EGF* G61A in RCC risk, we investigated the association of serum EGF levels with RCC risk.

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MATERIALS AND METHODS

Study subjects

This case-control study consisted 345 RCC cases and 346 cancer-free controls. All subjects were genetically unrelated ethnic Han Chinese. Cases were patients with histopathologically confirmed incident RCC, recruited in an ongoing study starting in July 2006 at The First Affiliated Hospital of Nanjing Medical University, Nanjing, China. The cancer-free control subjects were genetically unrelated to the cases and had no individual history of cancer. The controls were recruited from healthy subjects who were seeking health care in the outpatient departments at the hospital. All controls of appropriate age and sex for frequency matching with the cases were recruited and included if they gave their informed consent. As for the cases, exclusion criteria for the controls were significant mental impairment or blood transfusion in the past months; controls were also excluded if they had symptoms suggestive of RCC, such as hematuria. Before recruitment, a standard questionnaire was administered by trained interviewers to obtain information on demographic data and related factors through face-to-face interviews. After the interview, about 5 ml venous blood sample was collected from each subject. The study was approved by the institutional review board of Nanjing Medical University.

Genotyping

Genomic DNA was isolated from leukocytes of venous blood by proteinase K digestion and phenol/chloroform extraction. The *EGF* G61A polymorphism was determined using the PCR-restriction fragment length polymorphism method, as previously described.⁹ The PCR primers for the *EGF* G61A polymorphism were 5'-TGTCACAAAGGAAAGGAGGT-3' (forward) and 5'-TTACAGAGTTTAACAGCCC-3' (reverse). PCR was performed in a total volume of 20 μ l containing 50 ng genomic DNA, 10 $\times$ Taq buffer, 0.02 mmol l⁻¹ of MgCl₂, 0.05 mmol l⁻¹ of dNTP mix, 10 pmol μ l⁻¹ of each primer and 1 U Taq DNA polymerase. After initial denaturation at 95 °C for 5 min, the reaction was carried out at 95 °C denaturation for 30 s, annealing for 40 s at 55 °C and extension for 45 s at 72 °C for a total of 34 cycles, and a final elongation at 72 °C for 10 min. The 242 bp amplified products were incubated with 3 U of AluI (New England Biolabs(Beijing) Ltd., Beijing, China) restriction enzyme at 37 °C overnight. The restriction fragments were then analyzed by electrophoresis in 3% agarose gel stained with 0.5% ethidium bromide and photographed under UV illumination. AluI digested the 242 bp PCR products containing the 61G allele into 193, 34 and 15 bp fragments, whereas the 61A allele produced 102, 91, 34 and 15 bp fragments. The polymorphism analysis was carried out by two persons independently in a blinded manner. More than 15% of the samples were randomly selected for confirmation, and the results were 100% concordant.

EGF serum levels quantitative measurement

Serum samples were available for 70 RCC cases and 96 controls. EGF levels were measured in serum as opposed to plasma because the major source of EGF in the blood is platelets.¹⁵ The serum was allowed to clot for 30 min at 4 °C before centrifugation at 2000 r.p.m. for 10 min at 4 °C. Serum was isolated and stored at -80 °C before use. We detected EGF concentration by using a sandwich enzyme immunoassay (Quantikine EGF immunoassay kit, R&D Systems Inc, Minneapolis, MN, USA). The EGF concentration was calculated by referring to a standard curve according to the manufacturer's instructions.

Statistical analyses

Differences in the distributions of demographic characteristics, selected variables, and frequencies of genotypes of *EGF* G61A variants between the cases and controls were evaluated using the Student's *t*-test (for continuous variables) or χ^2 -test (for categorical variables). Hardy-Weinberg equilibrium was tested using a goodness-of-fit χ^2 -test. The associations between *EGF* genotypes and risk of RCC were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from unconditional logistic regression analysis with the adjustment for possible confounders. *P* < 0.05 was considered statistically significant, and all statistical tests were two sided. All the statistical analyses were performed with Statistical Analysis System software (9.1.3; SAS Institute, Cary, NC, USA).

RESULTS

Characteristics of the study population

The frequency distributions of selected characteristics of the cases and controls are presented in Table 1. There were no differences between the cases and controls on age, sex, body mass index, smoking and

Table 1 Distribution of selected variables between the RCC cases and controls

Variables	Cases (n=345)		Controls (n=346)		P-value ^a
	n	%	n	%	
Age (years) (mean $\pm$ s.d.)	56.5 $\pm$ 11.8		55.7 $\pm$ 11.4		0.342
BMI (kg m ⁻²) (mean $\pm$ s.d.)	24 $\pm$ 2.9		23.7 $\pm$ 3.3		0.270
Sex					
Male	222	64.3	208	60.1	0.251
Female	123	35.7	138	39.9	
Smoking status					
Never	214	62	225	65	0.413
Ever	131	38	121	35	
Former	36	10.4	20	5.8	
Current	95	27.6	101	29.2	
Pack-years of smoking					
0	214	62	225	65	0.260
0-20	95	27.5	97	28	
> 20	36	10.5	24	7	
Drinking status					
No	240	69.6	231	66.8	0.429
Yes	105	30.4	115	33.2	
Hypertension					
No	214	62	243	70.2	0.023
Yes	131	38	103	29.8	
Diabetes					
No	301	87.2	330	95.4	<0.001
Yes	44	12.8	16	4.6	
Stage					
I	239	69.3			
II	52	15.1			
III	17	4.9			
IV	37	10.7			
Grade					
I	61	17.7			
II	174	50.4			
III	82	23.8			
IV	28	8.1			
Histology					
Clear cell	295	85.5			
Papillary	9	2.6			
Chromophobe	21	6.1			
Unclassified	20	5.8			

Abbreviations: BMI, body mass index; RCC, renal cell carcinoma.

^aStudent's *t*-test for age and BMI distributions between cases and controls; two-sided χ^2 -test for other selected variable distribution between cases and controls.

drinking status (all $P > 0.05$). However, there were more subjects with hypertension (38.0%) and diabetes (12.8%) among the cases than among the controls (29.8 and 4.6%, respectively), and these differences were statistically significant ($P = 0.023$ for hypertension and $P < 0.001$ for diabetes). The majority of patients (85.5%) had the conventional clear cell carcinoma. Papillary carcinoma was presented in 9 (2.6%) patients and 21 (6.1%) had chromophobe carcinoma. In addition, there were 20 (5.8%) unclassified RCC patients. Approximately 69% of patients were in stage I, whereas 15.1, 4.9 and 10.7% of patients were found to have stage II, III and IV diseases, respectively. The nuclear grades from I to IV were 17.7, 50.4, 23.8 and 8.1% respectively.

EGF genotype and RCC risk

The genotype and allele distributions of the *EGF* G61A polymorphism in the cases and controls were shown in Table 2. The observed genotype frequencies for this polymorphism were in Hardy–Weinberg equilibrium in the controls ($\chi^2 = 0.0026$, $P = 0.96$). For the *EGF* G61A polymorphism, the frequencies of the GG, GA and AA genotypes were 46.7, 40.0 and 13.3%, respectively, among the cases, and 52.9, 39.6 and 7.5%, respectively, among the controls ($P = 0.031$). After adjusting for possible confounders (age, sex, pack-years of smoking, drinking status, hypertension and diabetes), subjects carrying GA heterozygote and AA homozygote had an increased risk of RCC (OR = 1.14, 95% CI = 0.82–1.58 for GA versus GG, and OR = 1.80, 95% CI = 1.04–3.12 for AA versus GG) ($P_{\text{trend}} = 0.026$), compared with GG homozygote. In addition, subjects carrying AA homozygote had a 1.82-fold increased risk (95% CI = 1.09–3.06) than those carrying GG/GA genotypes ($P = 0.012$), and the A allele displayed a high prevalence of RCC compared with the G allele ($P = 0.016$). These data indicated that individuals carrying variant A allele may have a higher risk for developing RCC.

In this study, we further evaluated the effect of *EGF* G61A variant on RCC risk stratified by age, body mass index, sex, smoking status, pack-years of smoking and drinking status. As shown in Table 3, we found that the increased risk was more pronounced among older patients (OR = 2.54, 95% CI = 1.23–5.23) and never alcohol drinkers (OR = 2.03, 95% CI = 1.07–3.85).

Table 2 Genotype and allele frequencies of the *EGF* G61A polymorphism among the cases and controls and the associations with risk of RCC

Genotype	Cases (n=345)		Controls (n=346)		P-value ^a	Adjusted OR (95% CI) ^b
	n	%	n	%		
GG	161	46.7	183	52.9	0.031	1 (reference)
GA	138	40	137	39.6		1.14 (0.82–1.58)
AA	46	13.3	26	7.5		1.80 (1.04–3.12)
GG+GA	299	86.7	320	92.5	0.012	1 (reference)
AA	46	13.3	26	7.5		1.82 (1.09–3.06)
G allele	460	66.7	503	72.7	0.016	1 (reference)
A allele	230	33.3	189	27.3		1.33 (1.06–1.68)
P_{trend}					0.026	

Abbreviations: CI, confidence interval; EGF, epidermal growth factor; OR, odds ratio; RCC, renal cell carcinoma.

^aTwo-sided χ^2 -test for either genotype distributions or allele frequencies between the cases and controls.

^bAdjusted for age, sex, pack-years of smoking, drinking status, hypertension and diabetes in logistic regression model.

EGF serum levels and RCC risk

Serum samples were available for 70 RCC cases and 96 controls in this case–control study. The mean serum EGF level was $858.94 \text{ pg ml}^{-1}$ (± 391.54) in cases and $1281.52 \text{ pg ml}^{-1}$ (± 568.42) in controls ($P < 0.001$) (Figure 1), suggesting that serum EGF level may serve as an independent protective marker against RCC.

Then, the associations between serum EGF levels and risk of RCC were further evaluated by unconditional logistic regression analyses. As shown in Table 4, when the median EGF level in controls ($1281.15 \text{ pg ml}^{-1}$) was used as the cutoff value for calculating the ORs, 14.3% (10 of 70) of the cases were above this median level. The serum EGF levels above this median level was associated with a dramatic decreased risk of RCC after adjustment for age, sex, pack-years of smoking, drinking status, hypertension and diabetes (OR = 0.12, 95% CI = 0.05–0.29). In addition, when the control quartiles of EGF levels were used to categorize the variable, a significant dose–response relationship between increased serum EGF levels and declined RCC risk was evident (ORs = 0.57, 0.17 and 0.13 for the second, third and fourth quartiles, respectively) (Table 4). Furthermore, serum EGF levels in subjects carrying AA ($714.64 \text{ pg ml}^{-1}$) genotype was lower than those with GA ($855.97 \text{ pg ml}^{-1}$) or GG ($893.14 \text{ pg ml}^{-1}$) genotypes, but the results were not statistically significant (AA versus GA, $P = 0.233$; AA versus GG, $P = 0.295$).

DISCUSSION

EGF, a potent mitogenic peptide, is a growth factor that activates a signaling transduction cascade responsible for activating genes involved in cell proliferation, differentiation or survival through binding to its receptor (EGFR),¹⁶ having an important role in carcinogenesis.¹⁷ Therefore, genetic variant in the *EGF* gene was hypothesized to have a critical role in tumorigenesis. In this study, we analyzed the association between a functional G61A polymorphism of *EGF* and the risk for developing RCC in a Chinese population. We found that the variant 61AA genotype was associated with an increased risk of RCC, compared with their GG/GA genotypes, and this increased risk was more pronounced among the older people and never drinkers.

Previous studies showed that low-serum EGF level may increase the risk of endometrium carcinoma¹⁸ and breast cancer.¹⁴ In this study, we found that there was a significantly lower serum EGF levels in RCC cases than that in controls, and individuals carrying AA homozygote had a lower serum EGF level than the GA or GG carriers. EGF is a growth factor that activates a signal transduction pathway promoting proliferation, migration and differentiation; nevertheless, some investigators showed that, after EGF binding to its receptor, they are both internalized, sourcing the formation of endosomes and ultimately degradation.^{19,20} The protective effect for the *EGF* overexpression in G allele carriers that we observed in this study may be explained by the increased bioavailability of EGF and the subsequent removal of EGFR from the cell surface due to its degradation. In fact, other studies showed that EGFR expression on cell surface decreases abruptly when a certain concentration of EGF is added to cell lines.^{21,22} Specific EGF ligands promote a long-lasting effect, inhibiting EGFR recycling.²² A low availability of EGFR will decrease the EGF/EGFR pathway activation and eventually protect RCC development. Another recent study reported that, in some cell lines, EGF paradoxically inhibits proliferation in high concentrations and induces loss of adhesion, cell-cycle arrest and apoptosis.²³

In 2002, Shahbazi et al.⁹ first reported that *EGF* 61GG genotype was significantly associated with risk of malignant melanoma compared with the AA genotype (OR = 4.90, 95% CI = 2.30–10.20), which may

Table 3 Stratification analyses between *EGF* G61A genotypes and risk of RCC in cases and controls

Variables	Cases/controls (N)	Genotypes (cases/controls)				P-value ^a	Adjusted OR (95% CI) ^a
		GG+GA		AA			
		n	%	n	%		
Total	345/346	299/320	86.7/92.5	46/26	13.3/7.5	0.023	1.82 (1.09–3.06)
Age (years)							
≤56	173/187	158/173	91.3/92.5	15/14	8.7/7.5	0.585	1.24 (0.57–2.69)
>56	172/159	141/147	82/92.5	31/12	18/7.5	0.012	2.54 (1.23–5.23)
BMI							
≤23	124/142	107/133	86.3/93.7	17/9	13.7/6.3	0.056	2.37(0.98–5.72)
>23	221/204	192/187	86.9/91.7	29/17	13.1/8.3	0.131	1.65(0.86–3.14)
Sex							
Male	222/208	191/190	86/91.4	31/18	14/8.6	0.080	1.76 (0.94–3.31)
Female	123/138	108/130	87.8/94.2	15/8	12.2/5.8	0.078	2.36 (0.91–6.13)
Smoking status							
Never	214/225	182/206	85.1/91.6	32/19	14.9/8.4	0.083	1.73 (0.93–3.19)
Ever	131/121	117/114	89.3/94.2	14/7	10.7/5.8	0.103	2.26 (0.85–6.01)
Pack-years							
0	214/225	182/206	85.1/91.6	32/19	14.9/8.4	0.083	1.73 (0.93–3.19)
0–20	95/97	83/92	87.4/94.9	12/5	12.6/5.1	0.052	3.08 (0.99–9.58)
>20	36/24	34/22	94.4/91.7	2/2	5.6/8.3	0.721	0.66 (0.07–6.28)
Drinking status							
Never	240/231	206/215	85.8/93.1	34/16	14.2/6.9	0.030	2.03 (1.07–3.85)
Ever	105/115	93/105	88.6/91.3	12/10	11.4/8.7	0.291	1.65 (0.65–4.20)

Abbreviations: CI, confidence interval; EGF, epidermal growth factor; OR, odds ratio; RCC, renal cell carcinoma.

^aAdjusted for age, sex, pack-years of smoking, drinking status, hypertension and diabetes in logistic regression model. **Figure 1** Distribution of serum EGF levels in 70 cases and 96 controls. Mean levels of serum EGF were significantly different between cases and controls (* $P < 0.001$).**Table 4** Logistic regression analyses on associations between serum EGF levels and risk of RCC

Serum EGF levels cut off by the control quartile (pg ml ⁻¹)	Cases		Controls		Adjusted OR (95% CI) ^a
	n	%	n	%	
EGF ≤ 790.06	45	64.4	24	25	1 (reference)
790.06 < EGF ≤ 1281.15	15	21.4	24	25	0.57 (0.29–1.13)
1281.15 < EGF ≤ 1569.68	5	7.1	24	25	0.17 (0.06–0.47)
EGF > 1569.68	5	7.1	24	25	0.13 (0.05–0.37)
Serum EGF levels cut off by the control median (pg ml ⁻¹)					
EGF ≤ 1281.15	60	85.7	48	50	1 (reference)
EGF > 1281.15	10	14.3	48	50	0.12 (0.05–0.29)

Abbreviations: CI, confidence interval; EGF, epidermal growth factor; OR, odds ratio; RCC, renal cell carcinoma.

^aAdjusted for age, sex, pack-years of smoking, drinking status, hypertension and diabetes in logistic regression model.

appear contradictory to our present results. The discrepancy could be interpreted to suggest that different mechanisms underlie the development of malignant melanoma and RCC, and *EGF* gene may have different role in different tumorigenesis. It has been suggested that EGF has pleiotropic cell responses that are regulated by signals when

numerous negative regulatory mechanisms act, such as availability of the ligand to the receptor and terminal signal inactivation through receptor internalization and degradation.^{19,20} Moreover, this regulation varies according to the arrangements of ligand–receptor engagement, tyrosine phosphorylation and subsequent receptor dimerization

combinations; as well as, depending on the stage and context of cell type and cell growth, different signaling transduction pathways can be activated.²⁰ As active pathways are not known in different tumors, we can explain that our contradictory results for different regulatory mechanisms of *EGF* may depend on specific tissue or tumor type.

We also analyzed the distribution of *EGF* genotypes among the East Asian population and Caucasian population from the previous studies, and found that, the *EGF* genotypes were significantly different between the East Asian population and Caucasian population. In this study, the *EGF* 61A allele frequency (0.273) in control was close to that reported in the Chinese (0.297, 0.305),^{14,24} Korean (0.284)²⁵ and Japanese populations (0.313, 0.304),^{12,13} but was significantly lower than that in the Caucasians (higher than 0.500).^{10,11,26,27} Therefore, we confirmed that *EGF* genotypes were significantly different between the East Asian and Caucasian populations. The ethnic heterogeneity in this polymorphism suggests the possibility that constitutive *EGF* expression may vary between different ethnic groups. Moreover, this ethnic heterogeneity results in discrepancy between risk association case-control studies with different ethnic groups.

Our present study has some limitations. First, our sample size was small, and we did not have detailed information on environmental factors, such as occupational exposures, diet and physical activity; these may limit the statistical power of our study, especially for gene-environment interaction analyses. Second, our study was a hospital-based study design, because we could not rule out the possibility of selection bias of subjects who may have been associated with a particular genotype. However, the agreement with Hardy-Weinberg equilibrium and similar allele frequency of our controls to those reported in the East Asian populations suggested that the selection bias in terms of genotype distribution would not be substantial.

In conclusion, our study showed that the *EGF* G61A polymorphism was associated with RCC risk. We found that the variant AA genotype was associated with an increased risk of RCC, compared with their wild-type GG homozygote. We also observed that lower serum *EGF* levels were associated with an increased RCC risk. To explore the exact biological mechanism of *EGF* genotype and their interaction with environmental factors, further functional studies and larger well-designed prospective studies are warranted.

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