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*Original Article*

# Association between EPAS1 gene single nucleotide polymorphisms and risk and prognosis of renal clear cell carcinoma

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**Abstract:** Background: This study aimed to determine whether rs11894252 and rs6758592 of EPAS1 gene are associated with susceptibility to renal clear cell carcinoma (RCCC) and analyze whether rs11894252 and rs6758592 can be useful prognostic markers in RCCC patients in the Chinese Han population. Methods: 163 patients diagnosed as RCCC and 192 healthy controls were enrolled. Based on the EPAS-1 gene loci found in GWAS studies, rs11894252 and rs6758592 were selected. The associations between genotypes and survival were analyzed in 163 RCCC patients. Results: Significant differences between cases and controls were observed in smoking ($P$=3.00×10$^{-10}$) and hypertension ($P$=0.0001). When stratified by gender, the decreased risk was observed in males carrying variant allele of rs11894252 compared to those with the wild-type genotype (OR, 0.29; 95% CI, 0.13-0.64). A significantly decreased risk was also shown for people younger than 55 years with at least one variant allele of rs11894252 (OR, 0.40; 95% CI, 0.19-0.82). However, histological grade ($P$=2.83×10$^{-11}$) and clinical stage ($P$=9.45×10$^{-14}$) were significantly associated with survival. Patients carrying variant allele had a significantly decreased risk of dying compared to those with the wild-type genotype (HR=0.06; 95% CI: 0.01-0.58). This result was also significant in a dose-dependent manner (HR=0.18; 95% CI: 0.03-1.00). Conclusion: Rs11894252 combined with age and gender can be used to predict the risk of the RCCC. Rs11894252 may be used as a potential RCCC prognosis marker in Chinese Han population, which should still be studied by further functional research and verified by studies with larger sample size.

**Keywords:** EPAS1 (endothelial PAS protein-1) gene, single nucleotide polymorphisms, renal clear cell carcinoma, risk, prognosis, PCR reaction

## Introduction

Renal cell carcinoma is one of the most common malignant tumors in urinary system, in which clear cell carcinoma is the main histological type [1]. In recent years, the incidence of renal cell carcinoma increased gradually [2]. Given that the etiology is not clear, it is necessary to study the possible risk factor for incidence in renal clear cell carcinoma (RCCC). With the development of molecular biology and genetics, it's possible to have a better understanding of the disease from genetic level. Single nucleotide polymorphisms (SNPs) are the most common forms of genetic variations of human genomic DNA and core information to determine the susceptibility of human diseases [3]. SNPs have been widely used for exploring etiology and therapy [3]. In this study, we investigated the relationships between EPAS1 gene polymorphisms and cancer risk in RCCC, which revealed the role of EPAS1 gene polymorphisms in the carcinogenesis and progression of RCCC.

## Materials and methods

### *Study population and data collection*

This study has been approved by the Ethics Committee of Shanghai Ruijin Hospital, Shanghai Jiaotong University School of Medicine (2008-11-1J). The study was based on 163 newly diagnosed, histologically confirmed RCCC patients at the institute of Urology, Ruijin Hospital, Shanghai (China). All cases of RCCC confirmed by puncture or surgical pathology

**Table 1.** Information of EPAS1 gene single nucleotide polymorphisms

| Gene | SNPs | Location mutation | Base |
|---|---|---|---|
| EPAS-1 | rs11894252 | intron | T>C |
| | rs6758592 | intron | T>C |

were collected from Nov. 2008 to Nov. 2011. There were no restrictions on age, gender, or cancer stage in recruitment. Histological grade of RCCC was classified by Fuhrman grade recommended by WHO in 1997. Grade I and II were combined into well-differentiated, while grade III was moderate-differentiated and grade IV was poorly-differentiated or undifferentiated [4]. 192 healthy control subjects with no history of cancer, no urinary system-related diseases, such as kidney cysts, kidney stones and other urinary system diseases, were selected from the same period at the same hospital. The controls were frequency matched to the cases by age (±5 y). In order to avoid the impact of ethnic difference, all samples selected were cases of Chinese Han population. All study participants provided signed informed consent and epidemiological data, which included medical history like hypertension and diabetes, lifestyle like smoking history and alcohol consumption. In addition, a 5 ml peripheral blood sample was drawn into coded and heparinized tubes. Genomic DNA was extracted from peripheral blood lymphocytes and stored at -80°C until use for subsequent analysis.

*Polymorphism selection and genotyping*

Based on the EPAS-1 gene loci found in GWAS studies by Purdue et al [5] in 2010, rs11894252 and rs6758592 were selected to be analyzed by using PCR reaction which was induced by sequence specific primers (PCR-SSP), agarose gel electrophoresis and gel imaging system (Tanon) [6] (**Table 1**). All the primers are listed in Supplementary Table 1.

*Statistical analysis*

Statistical analyses were performed using STATA software. The Student's t-test was used to analyze continuous variables and the Pearson $\chi^2$ test was used to analyze categorical variables and individual SNPs genotypes of the case and control groups.

The maximum likelihood ratio $\chi^2$ test was applied to assess whether the distribution of the various polymorphic loci in the control population in line with Hardy-Weinberg equilibrium (Hardy-Weinberg equilibrium, HWE). Logistic regression model was used to calculate different genetic models (Dominant, Recessive, Additive) to estimate OR (odds ratios, ORs) and their 95% confidence intervals (confidence intervals, CIs), adjusting for age, sex, smoking, drinking, hypertension and diabetes. All statistical analyses were two sided and $P \leq 0.05$ was considered statistically significant. The single factor affected on RCCC patients' overall survival was analyzed using log-rank tests and Kaplan-Meier curves. Cox's proportional hazards model was used to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs) for the multivariate survival analyses, adjusted for age, sex, clinical stage, histological grade, hypertension, diabetes, smoking and drinking status.

## Results

*Patient characteristics*

As described in **Table 2**, 355 cases were enrolled in this study, including 163 patients and 192 healthy controls. The mean age of the patient group and the control group was 54.67±12.14 and 54.38±10.14 years, showing no significant difference ($P$=0.81). In addition, there were no significant differences between patients and healthy controls with respect to gender ($P$=0.12), drinking status ($P$=0.93) and diabetes ($P$=0.498). However, significant differences between cases and controls were observed in smoking (cases versus controls 35.58% vs.8.33%, $P=3.00\times10^{-10}$) and hypertension (cases versus controls: 47.85% vs. 28.13%; $P$=0.0001).

*Distribution and analysis of EPAS-1 gene polymorphism*

The success rates of rs6758592 and rs11894252 genotyping (Supplementary Figure 1) between case group and control group were 95.8% and 97.5%. In the correlational research of EPAS-1 gene polymorphism with susceptibility to RCCC, the detection results of polymorphic loci rs6758592 and rs11894252 and the minimum allele frequency (MAF) were founded in **Table 3**. Since statistical analysis shows that

**Table 2.** Demographic characteristics and risk factors in cases and controls

| Characteristic | Cases, n (%) | Controls, n (%) | $X^2$ | $P^a$-value |
|---|---|---|---|---|
| Age, mean, years | 54.67±12.14 | 54.38±10.14 | 23.55 | 0.81 |
| Gender | | | | |
| Male | 113 (69.33) | 118 (61.46) | | |
| Female | 50 (30.67) | 74 (38.54) | 45.33 | 0.12 |
| Smoking history | | | | |
| No | 105 (64.42) | 176 (91.67) | | |
| Yes | 58 (35.58) | 16 (8.33) | 19.00 | $3.00*10^{-10}$ |
| Drinking history | | | | |
| No | 143 (87.73) | 169 (88.02) | | |
| Yes | 20(12.27) | 23 (11.98) | 34.22 | 0.93 |
| Hypertension | | | | |
| No | 85 (52.15) | 138 (71.88) | | |
| Yes | 78 (47.85) | 54 (28.13) | 45.66 | 0.0001 |
| Diabetes | | | | |
| No | 146 (89.57) | 176 (91.67) | | |
| Yes | 17 (10.43) | 16 (8.33) | 34.22 | 0.498 |

Notes: [a]The Student's t-test was used to analyze age variable and the Pearson $\chi^2$ test was used to analyze other categorical variables.

**Table 3.** MAF of Epas-1 and test of HWE

| SNPs | Location mutation | Base | Cases | | Controls | |
|---|---|---|---|---|---|---|
| | | | MAF | HWE $P^a$ | MAF | HWE $P^a$ |
| rs6758592 | intron | T>C | 0.37 | 0.001 | 0.39 | $1.09*10^{-7}$ |
| rs11894252 | intron | T>C | 0.44 | 0.89 | 0.47 | 0.80 |

Notes: [a]Pearson $\chi^2$ test was used to analyze MAF of the case and control groups.

the detection results of rs6758592 did not conform to the hardy Weinberg equilibrium (case group HWE $P$=0.001; control group HWE $P=1.09\times10^{-7}$), and further study was only made on the rs11894252 which was not statistically different between cases and controls (case group HWE $P$=0.89; the control group HWE $P$=0.80).

*Analysis of relation between EPAS-1 gene polymorphism and susceptibility to RCCC*

The individual carrying genotype of TT, TC and CC of rs11894252 was 51, 79 and 32 respectively in the case group, while which was 52, 90 and 42 respectively in the control group. The results showed that there was no significant association between genotypes and RCCC risk under the three genetic models. (OR, 0.93; 95% CI, 0.56-1.56, $P$=0.79 dominant model; OR, 0.97; 95% CI, 1.69-0.55, $P$=0.91 recessive model; OR, 0.96; 95% CI 0.69-1.34, $P$=0.81 additive model).

A further analysis was made on the relationship between the rs11894252 of EPAS-1 gene and the risk of RCCC when stratified by demographic characteristics and risk factors. The result showed that the risk of RCCC in males carrying at least one variant rs11894252 allele (TC/CC genotype) decreased significantly compared to those with the wild-type genotype (TT genotype) (OR, 0.29; 95% CI, 0.13-0.64, $P$=0.002), whereas the risk of RCCC in females was opposite (OR, 3.18; 95% CI, 1.39-7.29, $P$=0.006). Moreover, a significantly decreased risk was also shown for people under the age of 55 years with at least one variant allele of rs11894252 (OR, 0.40; 95% CI: 0.19-0.82, $P$=0.01). There was no relationship between the rs11894252 and the risk of RCCC when stratified by smoking, drinking, hypertension and diabetes in the study (**Table 4**).

*General demography, risk factors and prognosis of RCCC patients*

After checking the information of 163 RCCC patients, all data could be used for statistical analysis. The median survival time (MST) was 28.41 months (2.30-45.60). Gender, race, smoking, drinking, hypertension and diabetes were not associated with clinical outcomes. However, histological grade ($P=2.83\times10^{-11}$) and clinical stage ($P=9.45\times10^{-14}$) were significantly associated with survival (**Table 5** and **Figure 1**).

*Analysis of EPAS-1 gene polymorphism and prognosis of RCCC*

The success ratios of rs11894252 and rs6758592 genotyping were 99.4% and 96.9% (case group). EPAS-1: rs11894252 was significantly associated with the prognosis of RCCC. The survival individuals carrying the rs11894252 genotype TT, TC and CC were 46, 74 and 31 while the number of dead individuals was 5, 5 and 1, respectively.

**Table 4.** Analysis of rs11894252 and susceptibility to RCCC

| Characteristic | Genotype (TT/TC/CC) | | OR (95% CI) | $P^a$-value | OR (95% CI) | $P^a$-value | OR (95% CI) | $P^a$-value |
|---|---|---|---|---|---|---|---|---|
| | Cases | Controls | *Dominant model* | | *Recessive model* | | *Additive model* | |
| All samples | 51/79/32 | 52/90/42 | 0.93 (0.56-1.56) | 0.79 | 0.97 (0.55-1.69) | 0.91 | 0.96 (0.69-1.34) | 0.81 |
| Gender | | | | | | | | |
| Male | 37/54/21 | 14/69/31 | 0.29 (0.13-0.64) | 0.002 | 0.69 (0.34-1.44) | 0.32 | 0.54 (0.33-0.87) | 0.01 |
| Female | 14/25/11 | 38/21/11 | 3.18 (1.39-7.29) | 0.006 | 1.60 (0.59-4.34) | 0.35 | 1.88 (1.09-3.23) | 0.02 |
| Age | | | | | | | | |
| ≤55 | 33/37/14 | 27/54/20 | 0.40 (0.19-0.82) | 0.01 | 0.86 (0.38-1.98) | 0.73 | 0.64 (0.40-1.02) | 0.06 |
| >55 | 18/42/18 | 25/36/22 | 2.58 (1.04-6.39) | 0.04 | 0.87 (0.39-1.94) | 0.74 | 1.31 (0.79-2.17) | 0.30 |
| Smoking history | | | | | | | | |
| No | 32/50/23 | 47/82/39 | 0.98 (0.55-1.73) | 0.94 | 0.98 (0.54-1.80) | 0.96 | 0.99 (0.69-1.41) | 0.94 |
| Yes | 19/29/9 | 5/8/3 | 0.25 (0.03-2.26) | 0.22 | 0.53 (0.11-2.60) | 0.43 | 0.48 (0.16-1.43) | 0.19 |
| Drinking history | | | | | | | | |
| No | 44/69/29 | 46/79/36 | 1.09 (0.63-1.89) | 0.76 | 1.04 (0.58-1.87) | 0.89 | 1.05 (0.74-1.49) | 0.78 |
| Yes | 7/10/3 | 6/11/6 | 0.17 (0.02-1.61) | 0.12 | 0.32 (0.03-4.21) | 0.39 | 0.32 (0.07-1.46) | 0.14 |
| Hypertension | | | | | | | | |
| No | 28/37/20 | 35/68/29 | 0.74 (0.38-1.14) | 0.38 | 1.16 (0.58-2.32) | 0.67 | 0.94 (0.62-1.42) | 0.77 |
| Yes | 23/42/12 | 17/22/13 | 1.56 (0.66-3.70) | 0.31 | 0.65 (0.24-1.70) | 0.38 | 1.04 (0.59-1.83) | 0.88 |
| Diabetes | | | | | | | | |
| No | 44/71/30 | 46/83/39 | 0.93 (0.54-1.60) | 0.78 | 1.00 (0.56-1.78) | 0.99 | 0.97 (0.69-1.37) | 0.87 |
| Yes | 7/8/2 | 6/7/3 | 0.98 (0.16-6.02) | 0.98 | 0.75 (0.05-10.80) | 0.83 | 0.92 (0.25-3.37) | 0.90 |

Notes: [a]Gender, age, smoking, drinking, hypertension and diabetes were adjustment factors when using Logistic regression model to analyze.

Patients carrying variant allele had a significantly decreased risk of dying compared to those with the wild-type genotype (HR=0.06, 95% CI: 0.01-0.58, $P$=0.01). This result was also significant in a dose-dependent manner (HR=0.18; 95% CI: 0.03-1.00, $P_{trend}$=0.05) (**Table 6**).

Since half of the patients did not die during the follow-up period, the Kaplan-Meier survival analysis showed that there was no significant relationship between the rs11894252 and the survival time of the patients (Log rank $P$=0.25) (**Figure 2**). Carrying the rs6758592 mutation allele C had a protective effect on the prognosis of RCCC, but it did not reach statistical significance.

## Discussion

Endothelial PAS protein-1 (EPAS-1, also known as hypoxia-inducible factor-2alpha (HIF-2alpha)) is a protein that in humans is encoded by the EPAS1 gene which is located on the short (P) arm of chromosome 2 between positions 21 and 16 [7]. This protein is one part of a larger protein complex called HIF, which plays a critical role in the body's ability to adapt to changing oxygen levels [8-10].

HIF controls several important genes just like vascular endothelial growth factor (VEGF), erythropoietin (EPO), endothelin-1 (ET-1) and nitric oxide synthase (NOS) etc. which were involved in the formation of new blood vessels, the production of red blood cells and vascular contraction, etc. [11]. It is the major regulatory factor called vascular endothelial growth factor (VEGF), which controls vascular growth. HIF-2α is constantly produced in the body. When adequate oxygen is available, other proteins target HIF-2α to be degraded so it does not build up. However, when oxygen levels are lower than normal, HIF-2α is degraded at a slower rate. Consequently, more HIF is available to stimulate the formation of new blood vessels. These activities help maximize the amount of oxygen that can be delivered to the body's organs and tissues. Therefore, HIFs plays an important role in the process of organism growth, development, physiology and pathology [12, 13].

One of the important steps in tumor growth is that the cancer cells meet the tolerance capacity through regulating energy metabolism on cell hypoxia microenvironment. The other step is that the supply system of rapidly generating new blood vessels provides conditions for

**Table 5.** Selected characteristics of RCCC patient population (N=163)

| Characteristic | Alive, n (%) | Dead, n (%) | *P*-value |
|---|---|---|---|
| MST (months) | 28.41 (2.30-45.60) | | |
| Mean age (years) | 54.54±12.41 | 56.45±7.57 | 0.61 |
| Gender | | | |
| Male | 105 (69.08) | 8 (72.73) | |
| Female | 47 (30.92) | 3 (27.27) | 0.80 |
| Histological grade | | | |
| Well-differentiated | 118 (77.63) | 4 (36.36) | |
| Moderate-differentiated | 32 (21.05) | 2 (18.18) | |
| Poorly-differentiated | 2 (1.32) | 5 (45.45) | $2.83*10^{-11}$ |
| Clinical stage | | | |
| I | 56 (36.84) | 1 (9.09) | |
| II | 78 (51.32) | 0 | |
| III | 16 (10.53) | 5 (45.45) | |
| IV | 2 (1.32) | 5 (45.45) | $9.45*10^{-14}$ |
| Smoking history | | | |
| No | 100 (65.79) | 5 (45.45) | |
| Yes | 52 (34.21) | 6 (54.55) | 0.17 |
| Drinking history | | | |
| No | 133 (87.50) | 10(90.91) | |
| Yes | 19 (12.50) | 1 (9.09) | 0.74 |
| Hypertension | | | |
| No | 79 (51.97) | 6 (54.55) | |
| Yes | 73 (48.03) | 5 (45.45) | 0.87 |
| Diabetes | | | |
| No | 135 (88.82) | 11 (100.00) | |
| Yes | 17 (11.18) | 0 | 0.24 |

tumor proliferation, invasion and metastasis. Many studies have also shown that VEGF promotes tumor growth, invasion and metastasis by stimulating angiogenesis, which is also closely related to the prognosis [14, 15]. Renal cell carcinoma is a highly vascularized solid tumor. Therefore, angiogenesis is a key step in the growth of renal cell carcinoma.

EPAS-1 can stimulate the formation of tumor angiogenesis through promoting vascular endothelial growth factor (VEGF) and other angiogenic factors. Recently, some studies have also found that EPAS-1 is abnormally expressed in renal cell carcinoma and the extent expression of its increasing is related to the degree of damage in advanced tumor stage [16, 17], which plays an important role in the occurrence and development of renal cell carcinoma.

It was reported that the rs11894252 and rs6758592 of EPAS-1 gene were associated with the susceptibility to renal cell carcinoma by Purdue et al [5] in the GWAS study in 2010. Then we studied the two sites in depth to make it clear whether they were related to the incidence of RCCC in the Chinese Han population, which was the first report in Chinese Han population. We found that the detection results of rs6758592 did not conform to the hardy Weinberg equilibrium by statistical analysis and there was no significant association between genotypes and RCCC risk under the three genetic models.

In addition, like any other malignant tumor, the pathogenesis of RCCC involves multi-factor and multi-step process and may be associated with hereditary and environmental factors. Smoking, drinking, obesity, hypertension and diabetes may be the risk factors of RCCC and our results showed the occurrence of RCCC was related to smoking and hypertension, but not related to drinking and diabetes. To control confounding factors, stratified analysis for age, gender, smoking, drinking, hypertension and diabetes was made to analyze the relations between genotype and susceptibility of RCCC.

The result showed that the risk of RCCC in males or people less than 55 years carrying at least one variant rs11894252 allele (TC/CC genotype) decreased significantly compared to those with the wild-type genotype (TT genotype), whereas the risk of RCCC in females or people over age 55 did not decrease. The result might be influenced by selection bias because the case-control was based on a hospital, but the selection bias could be minimized by propensity age and gender matching as well as adjustment in the later statistical analysis.

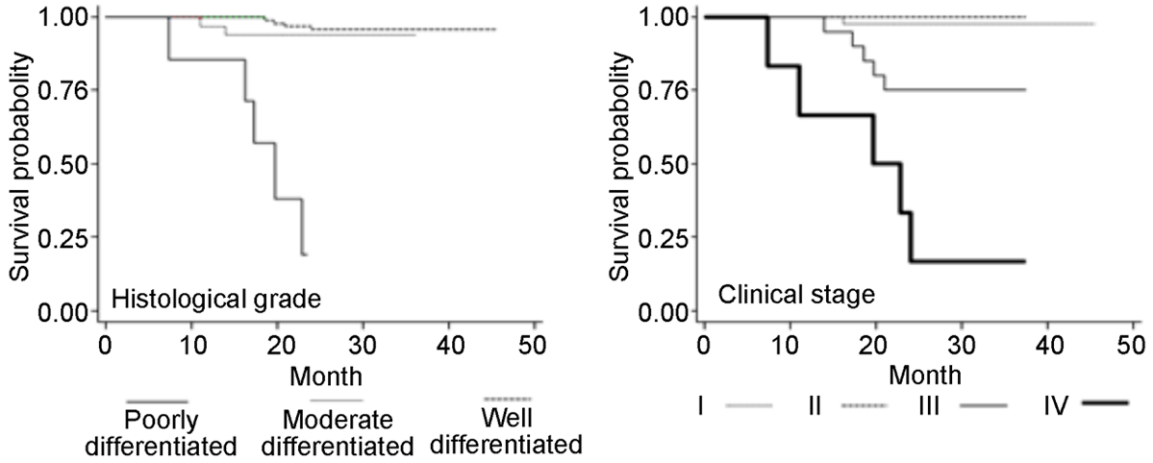


**Figure 1.** Kaplan Meier survival curves for RCCC patients according to histological grade and clinical stage.

**Table 6.** Analysis of EPAS-1 gene polymorphism and prognosis of RCCC

| SNP | Genotype (TT/TC/CC) | | Model[a] | HR (95% CI) | *P*-value[b] |
|---|---|---|---|---|---|
| | Alive, n | Dead, n | | | |
| rs6758592 | 50/86/11 | 4/6/1 | dom | 0.37 (0.05-2.53) | 0.31 |
| | | | rec | 0.12 (0.004-3.94) | 0.24 |
| | | | add | 0.36 (0.08-1.74) | 0.21 |
| rs11894252 | 46/74/31 | 5/5/1 | dom | 0.06 (0.01-0.58) | 0.01 |
| | | | rec | 1.08 (0.08-15.06) | 0.95 |
| | | | add | 0.18 (0.03-1.00) | 0.05 |

Notes: [a]: Genetic model of inheritance: dom, dominant model; rec, recessive model; add, additive model. [b]: Adjusted for age, gender, clinical stage, histological grade, hypertension, diabetes, smoking and drinking status.

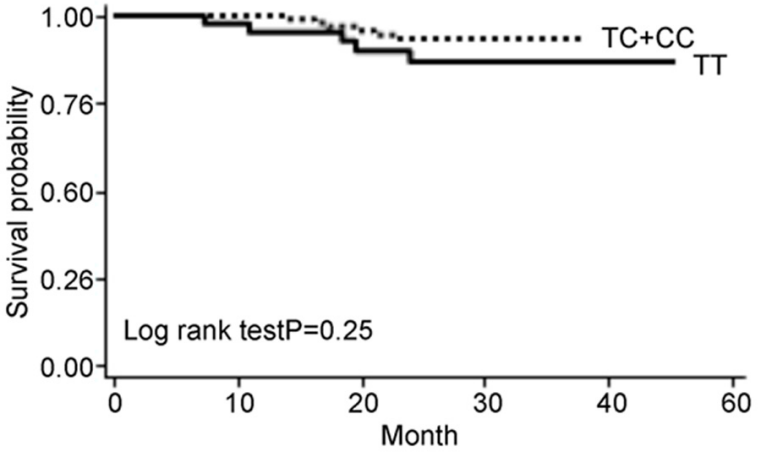


**Figure 2.** Kaplan Meier survival curves for RCCC patients according to EPAS-1: rs11894252.

Univariate survival analysis showed that histological grade and clinical stage were prognostic factors of RCCC, which reaffirmed that the early detection and diagnosis of RCCC has an important role for improving prognosis. However, there were no significant correlation between the prognosis and indicators of gender, smoking, drinking, hypertension and diabetes in our study.

Furthermore, EPAS-1: rs11894252 was associated with not only susceptibility but also prognosis with different gender and age of RCCC in our research, which indicated the occurrence and development of RCCC was a continuous process. RCCC is one of the most well vascularized solid tumors. The growth and metastasis of RCCC is accompanied with tumor angiogenesis. The increase of tumor angiogenesis infers poor prognosis.

It has been demonstrated that VEGF is known as one of the strongest factors which can promote the generation of blood vessels and it can promote cell proliferation and division, migration and then massive angiogenesis formation according to the basic research [18]. EPAS-1 plays a key role in signal transduction pathway to regulate the expression of VEGF, which

can enhance the transcriptional activity of VEGF and increase the stability of VEGF mRNA under hypoxia conditions [19]. Consistent with this, from our research, it is found that the mutant rs11894252 may change the function of EPAS-1, which may weaken the expression of VEGF and then improve the prognosis of RCCC although its specific mechanism needs further study. In addition, we did not find a correlation between rs6758592 and the prognosis of patients with RCCC.

We suggest that rs11894252 may be a potential prognostic marker for RCCC since it was associated with prognosis and survival of RCCC patients and it can provide a reference for the clinical treatment strategies and individual treatment programs. However, the site needs to be re-observed in different populations and its mechanism of action is still needed. If we integrate rs11894252 into clinical pathological indicators to establish a predictive model of prognostic to analysis, it will be helpful for enhance the evaluation of prognosis of patients with RCCC.

## Conclusions

In conclusion, we believe that rs11894252 combined with age and gender can be used to predict the risk of the RCCC in Chinese Han population. Furthermore rs11894252 may be used as a potential RCCC prognosis marker in Chinese Han population, which should still be studied by further functional research and verified by large-sample-size studies.

## Disclosure of conflict of interest

None.

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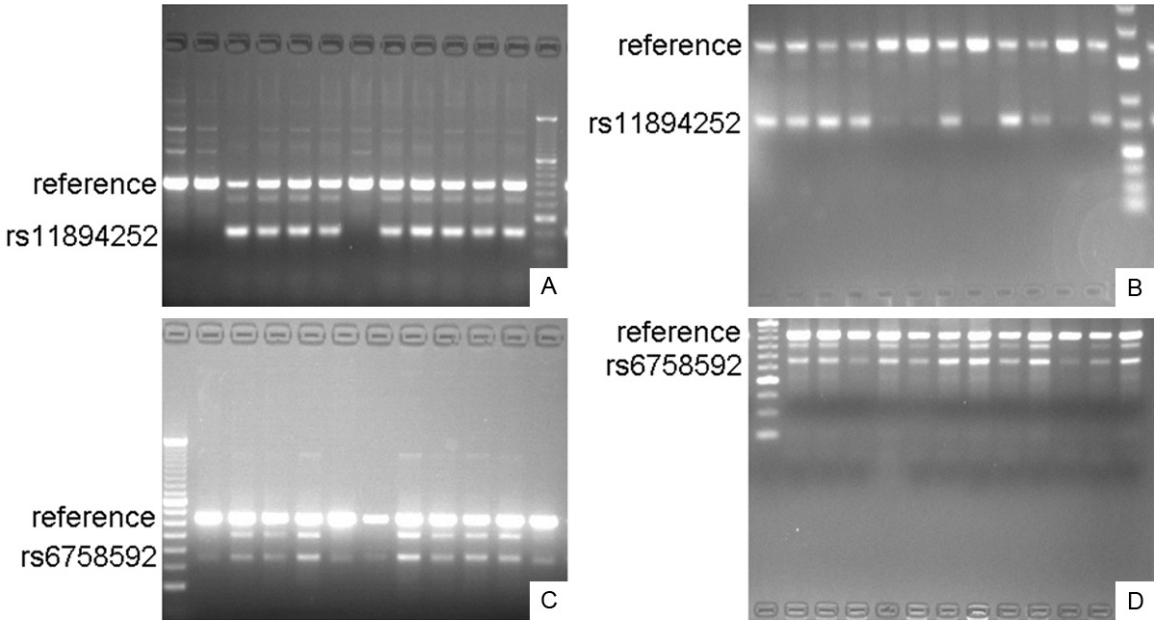
**Supplementary Table 1.** The primers for PCR-SSP

| SNPs | Alleles | Forward primers (5'-3') | Reverse primers (5'-3') | Forward primers for reference (5'-3') |
|---|---|---|---|---|
| rs11894252 | C | AATACACACATTTCACTC | ACTGGAGCCCAAGGTGGT | CCTGATGCTGGCCTGTGA |
| | T | AATACACACATTTCACTT | | |
| rs6758592 | C | ATTCCAAGGAGCCATACTTC | CTCCGCTTAGTTATTTCC | ACTGGGCTGTCAGTTCCT |
| | T | ATTCCAAGGAGCCATACTTT | | |




**Supplementary Figure 1.** PCR-SSP of rs11894252 and rs6758592. A: rs11894252 of EPAS-1 gene (C allele); B: rs11894252 of EPAS-1 gene (T allele); C: rs6758592 of EPAS-1 gene (C allele); D: rs6758592 of EPAS-1 gene (T allele).