Introduction

Kidney cancer is the 13th most common cancer globally. Every year, about 270,000 cases are diagnosed as kidney cancer while 116,000 die from this disease [1]. Clear cell renal cell carcinoma (ccRCC) is a major pathologic form, which accounts for about 90% of kidney malignancies. RCC has a higher incidence in western communities, and in China, about 37.7 men and 16.6 women per 100,000 individuals are diagnosed with RCC every year [2]. In addition to geographic variation, several other epidemiological risk factors have been established in sporadic RCC, including obesity, hypertension, smoking, alcohol intake and so on [3]. However, some people will not develop RCC although they are exposed to these risk factors during their lifetime. Accumulation of studies indicated that both environmental and genetic factors played critical roles in the tumorigenesis of RCC [4].

As we know, dysregulation of cell growth is closely related to cancer development and progression in various types of malignancies. Raf-1 kinase inhibitor protein (RKIP), which is known as one of the phosphatidylethanolamine-binding proteins (PEBP) family [5], has been reported to regulate growth and differentiation in a variety of species [6]. Emerging studies have indicated that RKIP functions as an intracellular regulator via several important signal cascades, including Raf-MEK-MAPK, G protein coupled receptor (GPCR) [7] and NF-κB pathway [8]. Further studies have shown that through these pathways, RKIP works as a...
multifunctional protein in carcinogenesis such as invasion, dysregulation of apoptosis and cell differentiation [9–12]. In 2002, Keller et al. discovered that the expression of RKIP is lower in metastatic prostate cancer cells than that in non-metastatic cells [13]. Subsequently, more and more reports have demonstrated down-regulation of RKIP in various cancers including melanoma [14], breast cancer [15], hepatocellular carcinoma [16,17] and colorectal cancer [18]. In further studies, decreased level of RKIP has been revealed to be related with poor prognosis in prostate cancer, bladder cancer, and colorectal cancer [19–21]. Collectively, these researches suggest that RKIP may act as not only an inhibitor of tumorigenesis, but also a metastasis suppressor. In addition, Moon and his colleagues suggested that RKIP expression was a significant prognostic marker for RCC and its level was tightly associated with progression and metastasis in renal cancer [22]. Recently, a study of 310 RCC cases has suggested RKIP was a significant prognostic marker because of its close correlation with progression and metastasis of RCC. In the same study, researchers also found that reduced RKIP expression was related to higher disease stage, larger tumor size, sarcomatoid subtype and poor overall survival [23].

Considering the critical roles of RKIP played in carcinogenesis, it is possible that single nucleotide polymorphisms (SNPs) located in the functional regions of RKIP may play a role in the development of RCC by influencing the expression RKIP. To test our hypothesis, we selected 5 tagging SNPs (tSNPs) in RKIP and genotyped these tSNPs using SNaPshot method in a case-control study in Chinese population.

Methods

Ethics statement
The study was approved by the Institutional Review Board of the Nanjing Medical University, Nanjing, China. At recruitment, written informed consents were obtained from all participants involved in this study.

Study population
Overall, in this case-control study 859 clear cell renal cell carcinoma cases and 1004 cancer-free controls were enrolled. All subjects are ethnic Han Chinese from different families and have no blood relationship in our study. All these cases were histopathologically confirmed to be incident ccRCC first time without prior history of other cancers, chemotherapy or radiotherapy, and they were consecutively recruited without restriction of age and sex from May 2004 at The First Affiliated Hospital of Nanjing Medical University, Nanjing, China. We classified the disease according to World Health Organization (WHO) criteria and staged it according to the American Joint Committee on Cancer (AJCC) TNM (tumor–node–metastasis) classification. The Fuhrman scale was used to assess tumor nuclear grade. All 1004 controls were enrolled from healthy subjects seeking routine physical examination in the outpatient departments at the same hospital and were frequency matched to the cases on sex and age (±5 years). All the controls were genetically unrelated to the cases and had no individual history of any cancers. Before recruitment, we administered a standard questionnaire containing demographic data and exposure information for every cases and controls by face-to-face interviews. Each of them donated 5 ml venous blood into the EDTA tube after providing a written informed consent. The response rate for both case and control subjects was >85%.

SNP selection
The SNPs were selected by using the genotype data obtained from unrelated Han Chinese in Beijing individuals in the HapMap database (HapMap Data Rel 21a/Phase II, Jan07, on NCBI B35 assembly, dbSNP b125). We reviewed all the SNPs that had a minor allele frequency >5% in Han Chinese in Beijing within a 9.9 kb region spanning the RKIP gene including 2 kb upstream and 2 kb downstream (shown in Table S1). The identification of the tag-SNPs was using the pairwise option of the Haplovew 4.2 software and an r² of 0.8 was selected as a threshold for the analyses. As a result, 5 tag-SNPs (rs17512051, rs1051470, rs2293444, rs904661 and rs2936840) were selected at a resolution of one SNP per 1.90 kb that captured all variant alleles with a mean r² of 0.963. The identification number and relative position of the 5 tag-SNPs as well as the LD plot of the tag-SNPs presented by the Haplovew 4.2 software are shown in Figure 1.

DNA extraction and genotyping
The whole genomic DNA was extracted from the peripheral blood leukocyte by proteinase K digestion and phenol-chloroform extraction (QiAmp DNA Blood Kit). In this study, we genotyped 5 tSNPs using SNapShot assay according to the manufacturer’s instructions (Applied Biosystems). The primers to amplify different fragments containing each SNP were designed using Primer3 software. Briefly, the reaction was performed in 40 μL volume containing 2 μL extracted genomic DNA, 1 μL of 50 mM MgCl2, 1 μL of 10 mM dNTP, 4 μL of 10×Buffer, 1 μL of primer mix and 0.4 μL of 5U Platinum Taq DNA Polymerase in 30 μL of Dnase-free water. The amplification program was performed in

Figure 1. Genetic location and linkage disequilibrium (LD) of the five tag-SNPs. (A) The location of the five tag-SNPs in the RKIP gene. The exons were indicated by black boxes; the 5’ and 3’ untranslated regions were denoted by gray boxes. (B) LD plot among the five tag-SNPs in the RKIP gene (data from HapMap). doi:10.1371/journal.pone.0109285.g001
the start of 95°C for 2 min, followed by 33 cycles of 94°C for 20 sec, 56°C for 30 sec and then 72°C for 40 sec. We performed the Shrimp Alkaline Phosphatase Method to purify the PCR product. In the procedure, 0.3 µL of SAP and 0.2 µL of Exonuclease I were mixed with 2 µL PCR product in 7.5 µL of ddH₂O at 37°C for 100 min followed by 15 min in 75°C. The extension reaction was then carried out in 5 µL volume containing 2.5 µL of Reaction Mix (Applied Biosystems), 1 µL of Probe Mix and 1.5 µL of PCR product and performed in 25 cycles of 96°C, 51°C, 60°C sequentially for 10 s, 5 s and 30 s. And finally, the

| Table 1. Frequency distribution of selected variables between the clear cell renal cell carcinoma cases and control subjects. |
|---------------------------------------------------------------|
| **Variables** | **Cases (n = 859)** | **Controls (n = 1004)** | **P-value** |
| Age (years) (mean ± SD) | 57.0±11.7 | 57.2±12.4 | 0.774 |
| BMI (kg/m2) (mean ± SD) | 24.2±2.9 | 23.8±3.1 | **0.007** |
| Gender | | | |
| Male | 557 | 64.8 | 687 | 68.4 | 0.102 |
| Female | 302 | 35.2 | 317 | 31.6 | |
| Smoking status | | | |
| Never | 530 | 61.7 | 665 | 66.2 | **0.001** |
| Former | 140 | 16.3 | 104 | 10.4 | |
| Current | 189 | 22.0 | 235 | 23.4 | |
| Drinking status | | | |
| Never | 619 | 72.1 | 756 | 75.3 | 0.113 |
| Ever | 240 | 27.9 | 248 | 24.7 | |
| Hypertension | | | |
| No | 522 | 60.8 | 675 | 67.2 | <0.001 |
| Yes | 337 | 39.2 | 251 | 25.0 | |
| Withdraw | 0 | 0 | 78 | 7.8 | |
| Diabetes | | | |
| No | 748 | 87.1 | 870 | 86.7 | <0.001 |
| Yes | 111 | 12.9 | 56 | 5.6 | |
| Withdraw | 0 | 0 | 78 | 7.8 | |
| Clinical stage | | | |
| I | 577 | 67.2 | | |
| II | 170 | 19.8 | | |
| III | 57 | 6.7 | | |
| IV | 55 | 6.4 | | |
| Grade | | | |
| I | 176 | 20.5 | | |
| II | 470 | 54.7 | | |
| III | 171 | 19.9 | | |
| IV | 42 | 4.9 | | |

*Student’s t-test for age and BMI distributions between cases and controls; two-sided χ²-test for others selected variables between cases and controls. doi:10.1371/journal.pone.0109285.t001

| Table 2. The characteristics of the 5 tSNPs in RKIP gene. |
|---------------------------------------------------------|
| **Polymorphism** | **Alleles** | **Location** | **MAF** | **HWE** |
| rs17512051 | T>A | promoter | 0.119 | 0.611 |
| rs1051470 | C>T | 3'UTR | 0.331 | 0.102 |
| rs2293444 | A>C | intron | 0.298 | 0.115 |
| rs904661 | C>T | intron | 0.391 | **0.005** |
| rs2936840 | C>T | intron | 0.190 | 0.062 |

*Goodness-of-fit chi-square test was used to assess Hardy–Weinberg equilibrium (HWE) in controls. doi:10.1371/journal.pone.0109285.t002
extension products were purified by Shrimp Alkaline Phosphatase Method in 0.5 uL of SAP and 2.5 uL of ddH2Oi n3 7uC for 1 h and 75uC for 15 min to inactive SAP. The final purified extension products were analyzed in 96-well PRISM 3730 DNA Sequencer (Applied Biosystems). All our procedure of genotyping was carried out in double-blind control. And in addition, 5% of the samples were selected randomly for confirmation and the results were 100% concordant.

Analysis of RKIP expression

A total of 42 paratumor renal tissues were used to analyze RKIP mRNA levels in vivo. The tissues were taken from the surgically removed samples from the patients and were immediately stored in liquid nitrogen. Total RNA was isolated from about 100 mg frozen tissue with TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to single stranded cDNA using an oligo (dT) primer and Superscript II (Invitrogen) following the manufacturer’s protocol. The RKIP mRNA level was measured by quantitative real-time reverse transcription (RT)-PCR on the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. The primers used for RKIP were 5′- CAATGACATCAGTGG-CACAGTC (forward) and 5′- CACAAGTCATCC-CACTCGGCCTG-3′ (reverse); and for GAPDH were 5′- GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′- TGGTGAAGACGCCAGTGGA-3′ (reverse). The reaction mixture contained 0.1 M of each primer, 26SYBR Green PCR Master Mix (TaKaRa, Berkeley, CA, USA), and 1 mL of cDNA (1:10 dilution). The amplification was performed under the following conditions: 95uC for 30 s, and 40 cycles of 95uC for 15 s and 60uC for 30 s. Each reaction was done in triplicate.

Statistical analysis

We used the Pearson’s chi-square test (for categorical variables) and the student’s t-test (for continuous variables) to compare the differences in variables such as gender, smoking status, drinking status, diabetes and hypertension in logistic regression model.

| Polymorphisms | Cases, n (%) | Controls, n (%) | P* | Adjusted OR (95% CI)b |
|---------------|-------------|----------------|----|----------------------|
| Rs17512051    |             |                |     |                      |
| TT            | 669 (80.2)  | 752 (76.2)     | 0.081 | 1.00 (reference) |
| TA            | 156 (18.7)  | 217 (22.0)     | 0.80 (0.63–1.02) |
| AA            | 9 (1.1)     | 18 (1.8)       | 0.52 (0.23–1.18) |
| TA/AA         | 165 (19.8)  | 235 (23.8)     | 0.039 | 0.78 (0.62–0.99) |
| T allele      | 1494        | 1721           | 1.00 (reference) |
| A allele      | 174         | 253            | 0.026 | 0.79 (0.65–0.97) |
| Rs1051470     |             |                |     |                      |
| CC            | 410 (51.3)  | 501 (51.6)     | 0.084 | 1.00 (reference) |
| CT            | 316 (39.5)  | 407 (41.9)     | 0.98 (0.80–1.20) |
| TT            | 74 (9.2)    | 63 (6.5)       | 1.42 (0.98–2.06) |
| CT+TT         | 390 (48.75) | 470 (48.41)    | 0.885 | 1.04 (0.86–1.27) |
| CC+CT         | 726 (90.8)  | 908 (93.5)     | 1.00 (reference) |
| TT            | 74 (9.2)    | 63 (6.5)       | 0.044 | 1.45 (1.01–2.09) |
| C allele      | 1136        | 1409           | 1.00 (reference) |
| T allele      | 464         | 533            | 0.306 | 1.08 (0.93–1.25) |
| Rs2293444     |             |                |     |                      |
| AA            | 219 (26.9)  | 262 (27.3)     | 0.132 | 1.00 (reference) |
| AC            | 398 (48.8)  | 502 (52.3)     | 0.98 (0.78–1.24) |
| CC            | 198 (24.3)  | 196 (20.4)     | 1.28 (0.97–1.69) |
| AC+CC         | 596 (73.1)  | 698 (72.7)     | 0.843 | 1.06 (0.85–1.32) |
| A allele      | 836         | 1026           | 0.201 | 1.00 (reference) |
| C allele      | 794         | 894            | 1.09 (0.96–1.24) |
| Rs2996840     |             |                |     |                      |
| CC            | 699 (84.4)  | 832 (84.7)     | 0.836 | 1.00 (reference) |
| CT            | 117 (14.1)  | 139 (14.2)     | 0.96 (0.73–1.27) |
| TT            | 12 (1.5)    | 11 (1.1)       | 1.12 (0.48–2.60) |
| CT+TT         | 129 (15.6)  | 150 (15.3)     | 0.858 | 0.97 (0.75–1.26) |
| C allele      | 1515        | 1803           | 0.731 | 1.00 (reference) |
| T allele      | 141         | 161            | 1.04 (0.82–1.32) |

*Two-sided χ2-test for the distributions of either genotype or allele frequencies between the cases and controls.

*genotype-specific ORs were adjusted for age, gender, BMI, smoking status, drinking status, diabetes and hypertension in logistic regression model.

Table 3. Association between the 4 tSNPs in RKIP and risk of clear cell renal cell carcinoma.
The cases and controls. A goodness-of-fit chi-square test was used to assess Hardy-Weinberg equilibrium (HWE) for each 5 tSNPs. Computing odds ratios (ORs) and 95% confidence intervals (CIs) were conducted to estimate the associations between polymorphisms and risk of ccRCC with adjusted data. Differences in RKIP mRNA levels from renal tissues carrying different genotypes were evaluated by Student’s t test or one-way ANOVA test. All data were analyzed with the software SAS 9.1.3 (SAS Institute, Cary, NC, USA) with two-sided test, and the adjusted P value of less than 0.05 was considered to be statistically significant.

Result

Characteristics of the Study Cases and Controls

The frequency distributions of the selected characteristics of 859 cases and 1004 controls are shown in Table 1. No significant differences regarding to age ($P = 0.774$), gender ($P = 0.102$) and drinking status ($P = 0.113$) were observed among the patients and controls. However, there were more hypertension patients, smokers and diabetics among the cases than among the controls (all $P < 0.001$), and the BMI level in cases was statistically higher than that in controls ($P = 0.007$). In our study, 67.2% of the patients were clinical stage I, and 19.8%, 6.6% and 6.4% were found to be stage II, III, IV, respectively. The percentage of nuclear grades from I to IV was 20.5%, 54.7%, 19.9%, and 4.9%, respectively.

The Association between the RKIP Polymorphisms and Risk of Clear Cell Renal Cell Carcinoma

The characteristics of the selected tSNPs are presented in Table 2. The allele frequencies of rs904661 in control group did not conform to HWE, and therefore was excluded from further analysis. The detailed genotype and allele distributions of the remaining 4 tSNPs in the patients and controls are presented in Table 3. No significant difference in genotype and allele distributions of rs2293444 and rs2936840 were observed between the cases and controls ($P = 0.132$ and 0.836, respectively). We found that the rs17512051 in the 5’9 UTR region of RKIP was significantly associated with ccRCC risk. Compared with individuals with homozygote TT genotype, those subjects carrying the variant genotypes (TA/AA) had a significant decreased ccRCC risk (OR = 0.78, 95%CI = 0.62–0.99). Significant association was also observed in allele comparison (OR = 0.79, 95%CI = 0.65–0.97). Another SNP (rs1051470), located in the 3’9 UTR region of RKIP was also found to be associated with ccRCC risk, although the association was less robust. For this SNP, significant association was only observed in the dominant model (TT vs. CC+CT, OR = 1.45, 95%CI = 1.01–2.09). No significant difference in allele frequency distribution was observed between cases and controls ($P = 0.306$).

Stratification analyses of RKIP rs17512051 and risk of RCC

We then further evaluated the effect of RKIP rs17512051 on RCC occurrence stratified by age, gender, BMI, smoking status, drinking status, hypertension and diabetes. As shown in Table 4,
the association between RKIP rs17512051 and RCC risk appeared stronger in subgroups of males (P = 0.016, OR = 0.70, 95% CI = 1.15–3.21), non-smokers (P = 0.041, OR = 0.75, 95% CI = 0.57–0.99) and individuals without history of diabetes (P = 0.021, OR = 0.75, 95% CI = 0.59–0.96). Since the significance of RKIP rs1051470 was less robust, and the number of individuals with rs1051470TT was small (74 and 63 in cases and controls, respectively), which would be further reduced in the stratified analysis, and could cause unstable associations. Therefore, we will not discuss the stratified analysis of RKIP rs1051470 in the study, but the results are presented in Table S2.

### The influence of polymorphisms on RKIP expression

Since the two RKIP rs17512051 and rs1051470 are located in the functional region of RKIP, we then examined whether they had impact on RKIP expression using real-time quantitative RT-PCR. As shown in Figure 2, the preliminary data showed that the renal tissues with RKIP rs17512051 TA/AA genotypes had higher level of RKIP expression, compared with tissues carrying the TT genotype (P = 0.020). However, no significant difference in RKIP expression was observed among renal tissue with different RKIP rs1051470 genotypes (P = 0.726).

### Association between the RKIP rs17512051 polymorphism and the clinicopathological characteristics of ccRCC patients

As expression of RKIP was reported to be involved in the progression of RCC, we then investigated whether the RKIP rs17512051 polymorphism has association with the clinicopathological parameters of ccRCC patients. However, as presented in Table 5, the difference in the distribution of rs17512051 genotype among different stage and grade of ccRCC was not significant (P = 0.134 and 0.440, respectively).

### Discussion

In the present study, we investigated the associations between 4 tSNPs in RKIP and RCC susceptibility in a Chinese population. Our study suggested that the RKIP rs17512051 was associated with a decreased risk of ccRCC. The association study results of rs17512051 were subsequently confirmed by a preliminary functional analysis of the variant, in which we observed that the RKIP mRNA level was increased in individuals who carried the rs17512051A allele. Besides, we also found rs105147 in the 3’UTR region of RKIP was associated with increased RCC risk.

| Category     | TT (n, %) | TA/AA (n, %) | P*     | Adjusted OR (95% CI) |
|--------------|-----------|-------------|--------|----------------------|
| Clinical Stage |           |             |        |                      |
| I            | 460 (68.8)| 101 (61.2)  | 0.134  | 1.00 (reference)     |
| II           | 124 (18.5)| 40 (24.2)   | 1.44 (0.94–2.20)|
| III          | 45 (6.7)  | 9 (5.5)     | 0.91 (0.43–1.95)|
| IV           | 40 (6.0)  | 15 (9.1)    | 1.54 (0.81–2.93)|
| Tumor grade  |           |             |        |                      |
| I            | 144 (21.5)| 28 (17.0)   | 0.440  | 1.00 (reference)     |
| II           | 357 (53.4)| 96 (58.2)   | 1.37 (0.86–2.19)|
| III          | 133 (19.9)| 35 (21.2)   | 1.32 (0.76–2.31)|
| IV           | 35 (5.2)  | 6 (3.6)     | 0.77 (0.29–2.05)|

*Two-sided χ2-test.

1 ORs were adjusted for age, sex, BMI, smoking, drinking status, diabetes and hypertension in logistic regression model. CI: confidence interval; OR: odds ratio.

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however, the significant was less robust, and it seems this polymorphism does not have impact on the expression of RKIP.

As a tumor suppressor, Raf-1 kinase inhibitor protein (RKIP) functions as a multi-aspect factor to inhibit various cellular processes including cell differentiation, cell cycle, cell migration and apoptosis [24]. Collective evidence from researches indicates that the decreased expression level of RKIP can trigger initiation of cancer and even metastasis [14–18]. And in RCC, down-regulation of RKIP was reported to be involved in the renal carcinogenesis and was also a significant prognostic marker for RCC patients [22]. However, there is lack of study on the association between genetic variants in RKIP and susceptibility of RCC, and to our best knowledge, this is the first report to evaluate the above-mentioned associations.

Considering the polymorphism of rs17512051 is located in the promoter of RKIP, it is predicted that the RKIP rs17512051 variant may lead to abnormal expression of RKIP. In addition, according to the UCSC website, dbSNP website in NCBI and the software of TFSEARCH, we deduce that the change of rs17512051T allele to rs17512051A may create a new transcription factor binding site of Cap. Alternatively, when the allele site of SNP rs17512051 is “A”, a new transcription factor of Cap can bind to the promoter region to increase the transcription and expression of RKIP which may induce a higher RKIP expression in the subjects carrying the rs17512051A allele. Therefore, it is biologically plausible that RKIP rs17512051 has an effect on the risk of ccRCC.

Furthermore, through stratification analyses, we observed that the association between RKIP rs17512051 and reduced ccRCC risk was more prominent in males, suggesting that the interaction of gender and genetic variants may contribute to the occurrence of ccRCC together. We also found that the decreased risk was more pronounced in subjects without history of diabetes although this is established factors for ccRCC occurrence, indicating that the effect of the polymorphism might be overwhelmed by effect of plasma glucose on ccRCC. Interestingly, the association was stronger in non-smokers and non-drinkers, suggesting that environmental factors might be more predominant than genetic cause of this polymorphism. Abrupt change of RKIP was reported to contribute to the progression of RCC; however we did not find any significant association between RKIP rs17512051 and ccRCC stage and grade. We speculate that the influence of rs17512051 on RKIP expression is relatively subtle that may slowly affect the development of ccRCC, but not power enough to influence the disease progression.

Since our case-control study was hospital based, we couldn’t rule out a possibility of selection bias of these subjects who might be associated with a particular genotype. In our study, except the rs904661, which had been excluded, the other 4 tSNPs all conformed to HWE. The genotype distributions of the 4 tSNPs in the controls were similar to that in the HapMap database of Han Chinese population. Therefore, the selection bias in terms of genotype distributions could be unsubstantial.

Conclusion

In summary, our result suggests that the potentially functional RKIP rs17512051 polymorphism may affect ccRCC susceptibility through altering the endogenous RKIP expression level. More detailed investigations and further studies on genetic functions are required in the future.

Supporting Information

Table S1 All the single nucleotide polymorphism that were reviewed in the presented study.

(DOC)

Table S2 Stratification analyses between the RKIP rs1051470 polymorphism and risk of clear cell renal cell carcinoma.

(DOC)

Author Contributions

Conceived and designed the experiments: CY PS QC. Performed the experiments: QC JW MZ PL JQ. Analyzed the data: SZ LZ MW CQ MZ. Contributed reagents/materials/analysis tools: XJ ZZ JL MG WZ. Wrote the paper: QC JW MZ.

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