Genetic Variants in Caveolin-1 and RhoA/ROCK1 Are Associated with Clear Cell Renal Cell Carcinoma Risk in a Chinese Population

Ruizhe Zhao*, Kang Liu*, Zhengkai Huang*, Jun Wang, Yongsheng Pan, Yuan Huang, Xiaoheng Deng, Jinliang Liu, Chao Qin, Gong Cheng, Lixin Hua*, Jie Li*, Changjun Yin

State Key Laboratory of Reproductive Medicine, Department of Urology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China

These authors contributed equally to this work.

* drlxhua@126.com(LH); lijie203076@163.com(JL)

Abstract

Background

The RhoA/ROCK pathway and Caveolin-1 (Cav-1) participate in the process of tumorigenesis in numerous types of cancer. Up-regulation of RhoA/ROCK and Cav-1 expression is considered to be associated with the development and progression of clear cell renal cell carcinoma (ccRCC). We investigated the association between genetic variations of RhoA/ROCK and Cav-1 and the risk of ccRCC in the Chinese population.

Methods

Between May 2004 and March 2014, a total of 1,248 clear cell renal cell carcinoma cases and 1,440 cancer-free controls were enrolled in this hospital-based case-control study. Nine SNPs in RhoA/ROCK and Cav-1 were genotyped using the TaqMan assay.

Result

We found two SNPs (Cav-1 rs1049334 and ROCK1 rs35996865) were significantly associated with the increasing risk of ccRCC ($P = 0.002$ and $P < 0.001$ respectively). The analysis of combined risk alleles revealed that patients with 2–4 risk alleles showed a more remarkable growth of ccRCC risk than the patients with 0–1 risk alleles (OR = 1.66, 95%CI = 1.31–2.11, $P < 0.001$). Younger subjects ($P = 0.001$, OR = 1.83, 95% CI = 1.30–2.57), higher weight subjects ($P = 0.001$, OR = 1.76, 95% CI = 1.25–2.47), female subjects ($P = 0.007$, OR = 1.75, 95% CI = 1.17–2.62), nonsmokers ($P < 0.001$, OR = 1.67, 95% CI = 1.26–2.23), drinkers ($P = 0.025$, OR = 1.75, 95% CI = 1.07–2.85), subjects with hypertension ($P = 0.025$, OR = 1.75, 95% CI = 1.07–2.85) and diabetes ($P = 0.026$, OR = 4.31, 95% CI = 1.19–15.62) showed a stronger association between the combined risk alleles and the risk of ccRCC by using the stratification analysis. Furthermore, we observed higher Cav-1 mRNA levels in the presence of the rs1049334 A allele in normal renal tissues.
Conclusion
Our results indicate that the two SNPs (Cav-1 rs1049334 and ROCK1 rs35996865) and genotypes with a combination of 2–4 risk alleles were associated with the risk of ccRCC. The functional SNP rs1049334 may affect the risk of ccRCC by altering the expression of Cav-1 and the relevance between the risk effects and the functional impact of this polymorphism needs further validation.

Introduction
Renal cell carcinoma accounts for the majority, which is more than 80%, of the malignancy of renal and the clear cell type is the most common subtype. It is reported that clear cell renal cell carcinoma (ccRCC) has become the seventh most common cancer type for its steady increase annually, accounting for 270,000 newly diagnosed cases and estimated 116,000 cancer deaths [1]. In China, ccRCC is also a big public health issue because of high incidence and limited medical infrastructure and awareness.

As with most human cancers, the genesis of ccRCC is very complex, and the molecular mechanisms underlying disease occurrence are still largely unknown. It is reported that western people show higher morbidity compared with Chinese population, which may be a result of geographic, lifestyle and genetic variations. Studies associated with the risk factors of ccRCC demonstrated that smoking, drinking, HBP and high BMI might contribute to the tumorgenesis. However, only a small number of people who share the same risk factors suffer from this disease, indicating that genetic variation may be an assignable reason of the origin of tumor.

RhoA is a small GTP-binding protein that acts as a molecular switch that plays important roles in a diversity of cellular processes including motility, mitosis, proliferation and apoptosis. RhoA has a distinct set of effector kinases, including the ROCK, CITRON, and PRK1, all of which regulate cellular processes that contribute to tumorigenesis, invasion, and metastasis [2]. The RhoA/ROCK pathway participates in the process of angiogenesis in numerous types of cancer, by controlling the permeability, migration, proliferation, proliferation and morphogenesis of tumor cells [3]. Cav-1 is secreted as a biologically active molecule of caveolae that promotes cell survival and angiogenesis within the tumor microenvironment, and is overexpressed in the metastatic and primary sites of several tumors. Previous studies demonstrated that Cav-1 functioned as a positive effector of RhoA activation through the phosphorylation of cav-1 by Src kinases under certain circumstances [4]. The association between RhoA/ROCK/Cav-1 and the genesis of tumor have raised increasing concerns.

To our knowledge, there is no report examining the association of single nucleotide polymorphisms (SNPs) in ROCK1/RhoA and Cav-1 and ccRCC risk. Considering that ROCK1/RhoA and Cav-1 may play an important role in initiating the cancer, in the present study, we performed a hospital-based case–control study and selected and genotyped 9 tagging SNPs (tSNP) located in the functional regions of these genes to investigate the association of genetic polymorphisms of ROCK1/RhoA and Cav-1 with ccRCC risk in Chinese population.

Materials and Methods
Ethics statement
The present study was approved by the Institutional Review Board of the Nanjing Medical University, Nanjing, China and each participant involved in this study gave a written informed consent prior to inclusion in the study.
Study Population

In this study, 1248 patients with clear cell renal cell carcinoma and 1440 cancer-free age-matched controls were consecutively recruited from May 2004 to March 2014 at the First Affiliated Hospital of Nanjing Medical University. Patients with blood relationship or from the same region or the same families were excluded from this study in advance. All the diagnosis of clear cell renal cell carcinoma was established by pathological examination of samples resected by surgery and none of the patients had history of other cancers or relative therapy before. Each patient’s ccRCC classification and staging were according to the TNM staging system by American Joint Committee on Cancer (AJCC). The Furman scale was used to assess nuclear grade of ccRCC. The 1440 controls were people who visited for regular health examination in the outpatient departments and declared free of any cancers. They were recruited frequency matched to the cases on age (±5 years) and gender. A written informed consent was given to each patient and all of them approved to donate 5ml venous blood, restoring in a condition of -20°C anticoagulated by EDTA.

SNP Selection

According to the CHB (i.e. Han Chinese in Beijing, China) data from HapMap (http://hapmap.ncbi.nlm.nih.gov/) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and Haploview software 4.2 (http://www.broad-institute.org/haplovew), tag-SNPs in ROCK1/RhoA and Cav-1 via a pair-wise tag-SNP algorithm was selected based on correlation coefficient (r2) linkage disequilibrium. The screening criteria were (i) minor allele frequency > 5% in the Chinese population and (ii) r2 threshold > 0.8. Minor allele frequency (MAF) of all of these genes is more than 5% in the Han Chinese population. Finally, 3 SNPs in Cav-1 (rs1049314, 1049337, rs1049334), 3 SNPs in ROCK1 (rs8089974, rs35996865, rs11874761) and 3 SNPs in RhoA (rs2269736, rs2410, rs2625955) were included in this study.

DNA extraction and Genotyping

Genomic DNA of each individual was isolated and purified from 500 ml EDTA-anticoagulated peripheral blood samples using a DNA extraction kit (Tiangen Biotech, Beijing, China) following the manufacturer’s instructions. Genotyping of the selected SNPs was conducted using the TaqMan SNP Genotyping Assay, which were performed on the 384-well LightCycler 480 Real Time PCR System (Roche, Penzberg, Germany). PCR was performed in a mixture containing 1.5μL SNP Genotyping Assay Mix, 1.5μL TaqMan Universal Master Mix, 1μL Dnase-free water and 1μL genomic DNA. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The LightCycler480 Software (Version 1.5.0) was used to automatically collect and analyze the data and to generate the genotype calls. The quality control was performed in each plate by four negative controls to ensure the genotyping accuracy. Regenotyping was conducted in approximately 10% of the samples and all the results were 100% concordant.

Analysis of Cav-1 and ROCK1 expressions

To further assess the correlations between the Cav-1 and ROCK1 mRNA expressions and the polymorphisms of rs1049334 and rs35996865 in vivo, a total of 64 paratumor renal tissues adjacent to tumour containing 100% normal cells were obtained from patients. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The 1.5-μg total RNA was reverse transcribed in a final volume of 20 μl using random primers under standard conditions using the PrimeScript RT Master Mix (Invitrogen).
The quantitative real-time reverse transcription (RT-PCR) was conducted to measure the mRNA level of Cav-1 and ROCK1 on the Roche LightCycler 480 Real Time PCR System. The primers used for Cav-1 were 5'-CGCCATTCTCTTTTCCTGC-3'(forward) and 5'–AGACG GTGTGAGCTAGA- TG-3'(reverse) and for ROCK1 were 5'- AAGAGAGTAGTTAGAG CAGTT- GGG-3'(forward) and 5'—TTTCTCTATTGTGACAGAACCT-3'(reverse) for β-actin were 5'-ACTGGAACGGTGAAGGTGAC-3' (forward) and 5'-AGAGAA- GTGGGGTG GCCTTTT-3' (reverse). β-actin was used as an internal quantitative control, and each reaction was performed in triplicate. The qRT-PCR reaction included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

Statistical Analysis
SPSS 16.0 (IL, CA, USA) was used for the statistical analysis. To assess the Hardy-Weinberg equilibrium of the genotype distribution, we performed a goodness-of-fit χ² test. The differences in frequency distributions of epidemiological factors like lifestyles and personal health condition between ccRCC cases and controls were tested by using the t test for continuous variables and the χ²-test for categorical variables, respectively. Odds ratios (ORs) and the 95% confidence intervals (CIs) for the association between the polymorphisms and risk of ccRCC was analyzed by unconditional logistic regression, adjusting for age, smoking and drinking status, family history and other factors. Additionally, the false discovery rate (FDR) was used to adjust the P value for multiple comparisons, based on the Benjamini–Hochberg method. The associations were considered statistically significant when Bonferroni FDR-adjusted P values were <0.05. We used the Student’s t test or one-way analysis of variance (ANOVA) to explore associations between the two polymorphisms (rs35996865 and rs1049334) and the mRNA levels of ROCK1 and Cav-1. All statistical analyses were two-sided and P <0.05 was considered statistically significant.

Results
Characteristics and clinical features of all subjects
As is shown in Table 1, age (P = 0.602), gender (P = 0.068) and drinking status (P = 0.618) were matched between cases and controls. Nevertheless, more smokers (P <0.001), higher BMI level (P = 0.002), diabetics (P < 0.001) and hypertension patients (P <0.001) were observed in the ccRCC group compared with the controls. The prevalence of clinical stage I, II, III and IV were 821(65.8%), 243(19.5%), 90(7.2%) and 94(7.5%) in the 1248 ccRCC patients, and those of nuclear grade from I to IV were 240 (19.2%), 645 (51.7%), 277 (22.2%) and 86 (6.9%), respectively.

Association between renal cell carcinoma risk and genetic polymorphisms of Cav-1 and ROCK1/RhoA
A few genetic characteristics of the 9 selected tSNPs were presented in Table 2. The tSNP of rs1049337 in Cav-1 was excluded from further analysis because of the allele frequencies in control group not conforming to HWE (P <0.001). Genotype and allele distributions of the remaining 8 tSNPs in the patients and controls were detailed in Table 3. No significant differences in genotype and allele distributions of tSNPs in RhoA were observed between the cases and controls (rs2269736 P = 0.155, rs2410 P = 0.388, rs2625955 P = 0.595). We found two polymorphisms were significantly associated with ccRCC: rs1049334 in Cav-1 and rs35996865 in ROCK1 (P = 0.002 and P <0.001 respectively).
### Table 1. Distribution of selected characteristics among the ccRCC cases and control subjects.

| Characteristic           | Cases (n = 1248) | Controls (n = 1440) | P*  |
|--------------------------|------------------|---------------------|-----|
| Age (years) (Mean ± SD)  | 56.8±12.3        | 56.6±11.7           | 0.602 |
| BMI (kg/m²) (Mean ± SD)  | 24.1±2.9         | 23.8±3.2            | 0.002 |
| Gender                   |                  |                     |     |
| Male                     | 792              | 63.5                | 962 | 66.8 | 0.068 |
| Female                   | 456              | 36.5                | 478 | 33.2 |
| Smoking status           |                  |                     |     |
| Never                    | 808              | 64.8                | 962 | 66.8 | <0.001 |
| Former                   | 180              | 14.4                | 81  | 5.6  |
| Current                  | 260              | 20.8                | 397 | 27.6 |
| Drinking status          |                  |                     |     |
| Never                    | 908              | 72.8                | 1060| 73.6 | 0.618 |
| Ever                     | 340              | 27.2                | 380 | 26.4 |
| Hypertension             |                  |                     |     |
| No                       | 765              | 61.3                | 1071| 74.4 | <0.001 |
| Yes                      | 483              | 38.7                | 369 | 25.6 |
| Diabetes                 |                  |                     |     |
| No                       | 1087             | 87.1                | 1365| 94.8 | <0.001 |
| Yes                      | 161              | 12.9                | 75  | 5.2  |
| Clinical stage           |                  |                     |     |
| I                        | 821              | 65.8                |     |
| II                       | 243              | 19.5                |     |
| III                      | 90               | 7.2                 |     |
| IV                       | 94               | 7.5                 |     |
| Grade                    |                  |                     |     |
| I                        | 240              | 19.2                |     |
| II                       | 645              | 51.7                |     |
| III                      | 277              | 22.2                |     |
| IV                       | 86               | 6.9                 |     |

* T-test for age and BMI distributions between the cases and controls; two-sided χ² test for others selected variables between the cases and controls.

doi:10.1371/journal.pone.0128771.t001

### Table 2. The characteristics of the 9 tSNPs in Caveolin-1 and RhoA/ROCK1.

| Polymorphism | Alleles | Location     | MAF  | HWE* |
|--------------|---------|--------------|------|------|
| rs2410       | C>A     | 3'-UTR       | 0.467| 0.494|
| rs11874761   | G>A     | 5'-UTR       | 0.085| 0.165|
| rs2625955    | A>C     | 5'-neargene  | 0.427| 0.979|
| rs35996865   | T>G     | 5'-neargene  | 0.085| 0.052|
| rs1049334    | G>A     | 3'-UTR       | 0.159| 0.057|
| rs1049337    | T>C     | 3'-UTR       | 0.387| 0.000|
| rs2269736    | G>A     | 5'-UTR       | 0.317| 0.718|
| rs8089974    | T>G     | 5'-neargene  | 0.083| 0.181|
| rs1049314    | C>T     | 3'-UTR       | 0.169| 0.832|

* χ² test was used to assess Hardy–Weinberg equilibrium (HWE) in controls.

doi:10.1371/journal.pone.0128771.t002
Table 3. The basic information of the genotyped polymorphisms in nine SNPs in the RhoA/ROCK1 and Cav-1 associated with the ccRCC risk.

| Polymorphisms | cases (n = 1248) | controls (n = 1440) | P  | FDR  | Adjusted OR (95% CI) |
|---------------|-----------------|-------------------|----|------|---------------------|
|               | n               | %                 | n  | %    |                     |
| rs2410        |                 |                   |    |      |                     |
| TT            | 350             | 28.0              | 436 | 30.3 | 0.388               |
| GG            | 261             | 20.9              | 303 | 21.0 | 1.08 (0.87–1.36)    |
| GT            | 637             | 51.0              | 701 | 48.7 | 1.19 (0.99–1.43)    |
| GT+GG         | 898             | 72.0              | 1004 | 69.7 | 0.218               |
| T allele      | 1337            | 53.6              | 1573 | 54.6 | 0.442               |
| G allele      | 1159            | 46.4              | 1307 | 45.4 | 1.05 (0.94–1.18)    |
| rs11874761    |                 |                   |    |      |                     |
| GG            | 1020            | 81.7              | 1158 | 80.4 | 0.668               |
| AA            | 9               | 0.7               | 10  | 0.7  | 0.87 (0.34–2.24)    |
| AG            | 219             | 17.5              | 272 | 18.9 | 0.92 (0.75–1.13)    |
| AG+AA         | 228             | 18.3              | 282 | 19.6 | 0.402               |
| G allele      | 2259            | 90.5              | 2588 | 89.9 | 0.436               |
| A allele      | 237             | 9.5               | 292 | 10.1 | 0.90 (0.80–1.17)    |
| rs2625955     |                 |                   |    |      |                     |
| AA            | 480             | 38.5              | 581 | 40.3 | 0.595               |
| CC            | 175             | 14.0              | 192 | 13.3 | 1.06 (0.83–1.36)    |
| CA            | 593             | 47.5              | 667 | 46.3 | 1.05 (0.88–1.24)    |
| CA+CC         | 768             | 61.5              | 859 | 59.7 | 0.323               |
| A allele      | 1553            | 62.2              | 1829 | 63.5 | 0.336               |
| C allele      | 943             | 37.8              | 1051 | 36.5 | 1.02 (0.87–1.21)    |
| rs35996865    |                 |                   |    |      |                     |
| TT            | 955             | 78.9              | 1157 | 80.3 | <0.001             |
| GG            | 36              | 2.9               | 8   | 0.6  | 5.41 (2.47–11.83)   |
| GT            | 257             | 18.2              | 275 | 19.1 | 1.17 (0.96–1.43)    |
| GT+GG         | 293             | 21.1              | 283 | 19.7 | 0.016               |
| T allele      | 2167            | 88.0              | 2589 | 89.9 | <0.001             |
| G allele      | 329             | 12.0              | 291 | 10.1 | 1.39 (1.17–1.65)    |
| rs1049334     |                 |                   |    |      |                     |
| GG            | 765             | 61.3              | 968 | 66.5 | 0.002               |
| AA            | 77              | 6.2               | 60  | 5.1  | 1.68 (1.17–2.41)    |
| AG            | 406             | 32.5              | 412 | 28.3 | 1.30 (1.09–1.54)    |
| AG+AA         | 483             | 38.7              | 472 | 33.5 | 0.001               |
| G allele      | 1936            | 77.6              | 2348 | 80.7 | <0.001             |
| A allele      | 560             | 22.4              | 532 | 19.3 | 1.32 (1.15–1.51)    |
| rs1049337     |                 |                   |    |      |                     |
| TT            | 394             | 31.6              | 453 | 31.5 | 0.502               |
| CC            | 273             | 21.9              | 341 | 23.7 | 0.89 (0.72–1.11)    |
| CT            | 581             | 46.6              | 646 | 44.9 | 1.04 (0.87–1.25)    |
| CT+CC         | 854             | 68.4              | 987 | 68.5 | 0.967               |
| T allele      | 1369            | 54.8              | 1552 | 53.9 | 0.493               |
| C allele      | 1127            | 45.2              | 1328 | 46.1 | 0.95 (0.84–1.22)    |
| rs2269736     |                 |                   |    |      |                     |
| GG            | 436             | 34.9              | 549 | 38.1 | 0.155               |
| AA            | 182             | 14.6              | 216 | 15.0 | 0.98 (0.77–1.25)    |
| AG            | 630             | 50.5              | 675 | 46.9 | 1.18 (0.99–1.41)    |

(Continued)
We found rs1049334 (G>A), which is located in Cav-1, was significantly associated with risk of ccRCC. Subjects with GA and AA genotypes had a significant increased ccRCC risk compared with those with GG (AA vs GG: adjusted OR = 1.68, 95% CI = 1.17–2.41; GA vs GG: adjusted OR = 1.30, 95% CI = 1.09–1.54). When combined the subjects with GA and AA genotypes, an increased risk of ccRCC was also observed in the combined group (adjusted OR = 1.33, 95% CI = 1.14–1.59). Alleles comparison showed similarly ccRCC risk rising (P < 0.001, OR = 1.39, 95%CI = 1.17–1.65). Another SNP (rs35996865) located in ROCK1 was also found to be associated with ccRCC risk. For this SNP, significant association was observed in the subjects with GG genotype and dominant model (GG vs TT, OR = 5.41, 95%CI = 2.47–11.83); GG+ GT vs TT, OR = 1.30, 95%CI = 1.09–1.54). A similar result was observed when compared G alleles with T alleles (P < 0.001, OR = 1.39, 95%CI = 1.17–1.65).

The analysis of combined polymorphisms and ccRCC risk

The two polymorphisms (rs1049334 and rs35996865) were combined based on the number of the risk alleles to explore the genetic power on the risk of ccRCC. As is shown in the Table 4, with the increasing of the number of the risk alleles, elevation of the ccRCC risk was observed and the difference was statistically significant. What’s more, when we divided the patients with two groups, patients with 2–4 risk alleles showed a more remarkable growth of ccRCC risk than the patients with 0–1 risk alleles (OR = 1.66, 95%CI = 1.31–2.11, P < 0.001) comparing with other group combinations. After that, stratification analysis indicated that the increased

---

**Table 3. (Continued)**

| Polymorphisms | cases(n = 1248) | controls(n = 1440) | Pα | FDRβ | Adjusted OR (95% CI)c |
|---------------|----------------|-------------------|-----|------|-----------------------|
|               | n   | %   | n   | %   |          |                      |
| AG+AA         | 812 | 65.1| 891 | 61.9| 0.092    | 1.13(0.96–1.33)     |
| G allele      | 1502| 60.2| 1773| 61.6| 0.3      | 1.15(0.94–1.37)     |
| A allele      | 994 | 39.8| 1107| 38.4|          |                      |
| **rs8089974** |     |     |     |      |          |                      |
| TT            | 1020| 81.7| 1160| 80.6| 0.663    | 1.00 (reference)    |
| GG            | 10  | 0.8 | 10  | 0.7  |          | 0.95(0.38–2.37)     |
| GT            | 218 | 17.5| 270 | 18.8|          | 0.93(0.76–1.14)     |
| GT+GG         | 228 | 18.3| 280 | 19.4| 0.459    | 0.93(0.76–1.14)     |
| T allele      | 2258| 90.5| 2590| 89.9| 0.52     | 0.90(0.71–1.09)     |
| G allele      | 238 | 9.5 | 290 | 10.1|          |                      |
| **rs1049314** |     |     |     |      |          |                      |
| CC            | 1235| 99.0| 1424| 98.9| 0.999    | 1.00 (reference)    |
| TT            | 0   | 0.0 | 0   | 0.0  |          |                      |
| TC            | 13  | 1.0 | 16  | 1.1  | 0.91(0.42–1.96)   |
| TC+TT         | 13  | 1.0 | 16  | 1.1  | 0.91(0.42–1.96)   |
| C allele      | 2483| 99.5| 2864| 99.4| 0.999    | 0.90(0.42–1.93)     |
| T allele      | 13  | 0.5 | 16  | 0.6  |          |                      |

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

b: Bonferroni FDR

c: Adjusted for age, BMI, gender, smoking status, drinking status and history of hypertension and diabetes in logistic regression model; 95% CI: 95% confidence interval

doi:10.1371/journal.pone.0128771.t003
risk was more pronounced among younger subjects ($P = 0.001$, OR = 1.83, 95% CI = 1.30–2.57), higher weight subjects ($P = 0.001$, OR = 1.76, 95% CI = 1.25–2.47), female subjects ($P = 0.007$, OR = 1.75, 95% CI = 1.17–2.62), nonsmokers ($P < 0.001$, OR = 1.67, 95% CI = 1.26–2.23), drinkers ($P = 0.025$, OR = 1.75, 95% CI = 1.07–2.85), subjects with hypertension ($P = 0.025$, OR = 1.75, 95% CI = 1.07–2.85) and diabetes ($P = 0.026$, OR = 4.31, 95% CI = 1.19–15.62) (Table 5). However, in patients with localized (stage I and II) or advanced stage (stage III and IV) and moderately (grade I and II) or poorly differentiated (grade III and IV) nuclear grade, no significant difference was observed (S1 Table).

Associations between Cav-1 rs1049334 and ROCK1 rs35996865 and the expression levels of corresponding mRNA

In our study, 64 normal tumor-adjacent tissue samples were obtained to assess the associations between these two polymorphisms and the expression of Cav-1 and ROCK1 (Fig 1). According to the results, subjects with rs1049334 AA or AG genotypes had higher expression levels of

### Table 4. Analysis between combined risk alleles and ccRCC Susceptibility.

| Number of risk alleles | cases(n = 1248) | controls(n = 1440) | $P^*$ | Adjusted OR (95% CI)$^\Delta$ |
|------------------------|----------------|-------------------|-------|-------------------------------|
|                        | n   | %   | n   | %   |                             |       |
| 0                      | 584 | 46.8| 776 | 53.9| 1.00(reference)              |       |
| 1                      | 474 | 38.0| 520 | 36.1| 0.005 1.28(1.08–1.51)        |       |
| 2                      | 159 | 12.7| 131 | 9.1 | <0.001 1.69(1.30–2.20)       |       |
| 3                      | 27  | 2.2 | 11  | 0.8 | 0.001 3.41(1.66–7.02)        |       |
| 4                      | 4   | 0.3 | 2   | 0.1 | 0.201 3.07(0.55–17.17)       |       |

*Two-sided $\chi^2$ test for either genotype distributions or allele frequencies between the cases and controls.

$\Delta$Adjusted for age, gender, body mass index, smoking status, drinking status, hypertension and diabetes in logistic regression model; 95% CI: 95% confidence interval.

doi:10.1371/journal.pone.0128771.t004

### Table 5. Association between Cav-1 and ROCK1 polymorphism and clinicopathologic characteristics of ccRCC.

| Risk allele | Clinical stage | Grade | | |
|-------------|----------------|-------|-----------------|-----------|
|             | 0–1            | 2–4   | $P^*$           | Adjusted OR(95% CI)$^\Delta$ |
| n           | %   | n   | %   |                        |           |
| I + II      | 896 | 84.2| 168 | 15.8| 0.221 1.00(reference)   |           |
| III + IV    | 162 | 88.0| 22  | 12.0| 0.74(0.45–1.26)        |           |
| I + II      | 745 | 84.2| 140 | 15.8| 0.386 1.00(reference)   |           |
| III + IV    | 313 | 86.2| 50  | 13.8| 0.93(0.64–1.35)        |           |

*Two-sided $\chi^2$ test for number of alleles in cases and controls.

$\Delta$Adjusted for age, gender, body mass index, smoking status, drinking status, hypertension and diabetes in logistic regression model; 95% CI: 95% confidence interval.

doi:10.1371/journal.pone.0128771.t005
Cav-1 compared with those with GG genotypes ($P = 0.003$). However, the ROCK1 expression level in patients carrying three types of rs35996865 genotypes was similar ($P = 0.713$), suggesting that the influence of rs3599686 polymorphism on gene expression is weak.

**Stratification analyses between Cav-1 rs1049334 and risk of ccRCC**

A stratification analysis was then evaluated by the age, gender, BMI, smoking status, drinking status, history of hypertension and diabetes (S2 Table). As a result, we found that the increased risk of ccRCC was more remarkable between older subjects ($P = 0.005$, OR = 1.44, 95% CI = 1.14–1.81), higher weight subjects ($P = 0.015$, OR = 1.37, 95% CI = 1.08–1.74), male subjects ($P = 0.021$, OR = 1.37, 95% CI = 1.11–1.69), nonsmokers ($P = 0.002$, OR = 1.38, 95% CI = 1.13–1.68), nondrinkers ($P = 0.006$, OR = 1.38, 95% CI = 1.14–1.66), subjects without hypertension ($P = 0.006$, OR = 1.34, 95% CI = 1.10–1.64) and without diabetes ($P = 0.001$, 95% CI = 1.14–1.81).
OR = 1.35, 95% CI = 1.14–1.60). Besides, no significant associations between Cav1 rs1049334 polymorphism and clinical stage or tumor grade of ccRCC patients were observed (Table 6).

### Discussion

**Caveolin-1** (Cav1) is a principal functional constituent of caveolae, which are invaginated plasma membrane microdomains and function as a regulator of signal transduction events and cytoskeletal dynamics [5,6]. Glenney first reported that Caveolin-1 was a novel substrate for the src kinase oncogene in virally transformed fibroblasts by binding to several key proteins [7]. Cav-1 has been proved to modulate multiple cancer-associated processes including cellular transformation, tumor growth, cell migration and metastasis, cell death and survival, multidrug resistance and angiogenesis in a number of signaling pathways [8,9]. Collective evidence from researches indicated that the elevated level of Cav-1 was involved in some unfavorable clinical characteristics like larger size, higher grade and stage, resistance to conventional therapies and poor prognosis of various types of malignancy in several organs including colon, liver, stomach, prostate, breast, lung, brain and kidney [10–20]. Although the role of Cav-1 plays in cancer is controversial [12], it is widely confirmed that overexpression of Cav-1 in renal cell carcinoma is associated with poor disease-free survival and metastasis [21–23]. Study in genetic variants revealed a significant association between Cav-1 polymorphisms and ccRCC susceptibility [24].

**RhoA**, which is a predominant member of the well-known Ras superfamily of small guanosine triphosphatases (GTPases), is a small G protein that can exhibit intrinsic GTPases activities that can function as a molecular switch in cellular processes including cytoskeletal dynamics, migration, vesicle trafficking, cell proliferation, apoptosis and transcription [25–27]. A number of studies have shown that RhoA had abilities to control cancer metastasis and progression [28–30] and the expression of RhoA was up-regulated in several common malignancies including gastric, pancreatic and breast cancer [31–33]. Rho-associated coiled-coil forming kinase, which is often referred to as ROCK, is a major effector in the Rho signaling way. ROCK1 is one of the ROCK family and plays a critical role in mediating the effects of RhoA, activated by binding to the Active (GTP-loaded) Rho [34,35]. The Rho/Rho-kinase pathway plays an

### Table 6. The association of Cav1 rs1049334 polymorphism and clinicopathologic characteristics of ccRCC patients.

|                | GG            | AG/AA         | P*               | Adjusted OR (95% CI) △ |
|----------------|---------------|---------------|------------------|------------------------|
|                | n  | %  | n  | %  |               |                         |
| Clinical Stage |    |    |    |    |               |                         |
| I              | 498| 49.7|323| 49.8|0.764|1.00(reference) |
| II             | 386| 38.5|259| 40.0|0.93(0.68–1.27) |
| III            | 57 | 5.7 | 33 | 5.1 |0.84(0.52–1.36) |
| IV             | 61 | 6.1 | 33 | 5.1 |0.90(0.54–1.52) |
| Grade          |    |    |    |    |               |                         |
| I              | 154| 20.1|86 | 17.8|0.408|1.00(reference) |
| II             | 386| 50.5|259| 53.6|1.27(0.93–1.74) |
| III            | 167| 21.8|110| 22.8|1.31(0.88–1.95) |
| IV             | 58 | 7.6 | 28 | 5.8 |0.95(0.52–1.73) |

*Two-sided χ² test for number of alleles in cases and controls.
△Adjusted for age, BMI, gender, smoking status, drinking status and history of hypertension and diabetes in logistic regression model; 95% CI: 95% confidence interval.

doi:10.1371/journal.pone.0128771.t006
important role in various cellular functions and is involved in several proinvasive pathways including src[36]. Accumulating evidences have shown that the interaction between Caveolin-1 and Rho-GTPases could regulate metastasis by controlling the activation of src in malignancies [37,38]. However, study on the association between genetic variants in Cav-1 with RhoA/ROCK and susceptibility of ccRCC is insufficient.

In our study, we genotyped nine polymorphisms in RhoA/ROCK1 and Cav-1 to explored the association between RhoA/ROCK1 and Cav-1 genetic variants and ccRCC susceptibility in Chinese population. The Cav-1 rs1049334 and the ROCK1 rs35996865 significantly differed between ccRCC patients and control participants, indicating that the risk of ccRCC is increased in participants with the A allele of rs1049334 and G allele of rs35996865. The further analysis of the combining risk alleles showed that the group of patients with 2–4 risk alleles was more susceptible to ccRCC compared with those with 0–1 risk alleles. Environmental and epidemiological factor also had certain effects on the risk of ccRCC according to our stratification analyses in combination alleles and Cav-1 rs1049334. Age, BMI, gender, smoking and drinking status, the history of HBP and diabetes are all related with the ccRCC susceptibility, implying that the interaction of the environment, hereditary background and genetic variants may be a complex system contributed to the occurrence of ccRCC.

Additionally, increasing Cav-1 mRNA level was found in individuals who carried the rs1049334 A allele in the preliminary functional analysis of the variant. Interestingly, stratification analyses of the association between the rs1049334 and the risk of ccRCC revealed a little different in history of hypertension and history of diabetes compared with that of risk alleles. We supposed that the role of RhoA/ROCK1 in influencing the level of plasma glucose and vascular contractility may contribute to this procedure [39–41]. No significant association between the polymorphisms and clinicopathological characteristics of ccRCC was observed, we surmise that the effect of rs1049334 on the expression of Cav-1 is not power enough to influence the disease progression.

Limitations should not be ignored in the present study. First, the sample is not large enough that bias there may exist in the analyzing of very low-penetrance SNPs and reduce the statistical power of combined analysis and stratification. Second, selection bias of subjects associated with a particular genotype could not be eliminated because our case-control study is hospital-based. However in present study, except for rs1049337, the rest 8 tSNPs all conformed to HWE. The selection bias might not be substantial because the distribution of genotypes was all similar to the Hapmap database of Chinese population.

In conclusion, we investigated an association between Cav-1 and RhoA/ROCK1 polymorphisms and susceptibility, clinical characteristics in a large sample population of ccRCC patients. Cav-1 rs1049334 (G>A) and ROCK1 rs35996865 (T>G) were significantly associated with the elevated risk of ccRCC in Chinese population, and the combination of risk alleles indicated a positive influence on the ccRCC risk. Furthermore, functional polymorphism in Cav-1 rs1049334 (G>A) altered Cav-1 expression, which may contribute to the genesis of ccRCC. Further functional investigations are expected to confirm our results.

Supporting Information

S1 Table. Stratification analysis of the variant numbers of genotypes by selected variables in ccRCC patients and controls

S2 Table. Stratification analyses between the Cav1 rs1049334 polymorphisms and risk of clear cell renal cell carcinoma
Author Contributions
Conceived and designed the experiments: RZ ZH LH JL YH. Performed the experiments: RZ ZH KL JW YP. Analyzed the data: RZ CQ XD JLL GC JL CY. Contributed reagents/materials/analysis tools: ZH CQ KL. Wrote the paper: RZ ZH.

References
1. Ljungberg B, Campbell SC, Choi HY, Jacqmin D, Lee JE, Weikert S, et al. The epidemiology of renal cell carcinoma. European urology. 2011; 60(4):615–21. Epub 2011/07/12. doi:10.1016/j.eururo.2011.06.049 PMID: 21741761.

2. Teramoto H, Malek RL, Behbahani B, Castellone MD, Lee NH, Gutkind JS. Identification of H-Ras, RhoA, Rac1 and Cdc42 responsive genes. Oncogene. 2003; 22(17):2689–97. Epub 2003/05/06. doi:10.1038/sj.onc.1206364 PMID: 12730683.

3. Chen W, Mao K, Liu Z, Dinh-Xuan AT. The role of the RhoA/Rho kinase pathway in angiogenesis and its potential value in prostate cancer (Review). Oncology letters. 2014; 8(5):1907–11. Epub 2014/10/08. doi: 10.3892/ol.2014.2471 PMID: 25289078; PubMed Central PMCID: PMC4186560.

4. Peng F, Wu D, Ingram AJ, Zhang B, Gao B, Krepsinsky JC. RhoA activation in mesangial cells by mechanical strain depends on caveolae and caveolin-1 interaction. Journal of the American Society of Nephrology: JASN. 2007; 18(1):189–98. Epub 2006/11/24. doi:10.1681/ASN.2006050498 PMID: 17121865.

5. Cohen AW, Razani B, Schubert W, Williams TM, Wang XB, Iyengar P, et al. Role of caveolin-1 in the modulation of lipolysis and lipid droplet formation. Diabetes. 2004; 53(5):1261–70. Epub 2004/10/08. doi:10.1111/j.1513-7669.2004.00126.x PMID: 15111495.

6. Williams TM, Lisanti MP. The Caveolin genes: from cell biology to medicine. Annals of medicine. 2004; 36(8):584–95. Epub 2005/03/17. PMID: 15768830.

7. Glenney JR Jr. Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. The Journal of biological chemistry. 1989; 264(34):20163–6. Epub 1989/12/05. PMID: 2479645.

8. Burgermeister E, Liscovitch M, Rocken C, Schmid RM, Ebert MP. Caveats of caveolin-1 in cancer progression. Cancer letters. 2008; 268(2):187–201. Epub 2008/05/17. doi:10.1016/j.canlet.2008.03.055 PMID: 18482795.

9. Goetz JG, Lajoie P, Wiseman SM, Nabi IR. Caveolin-1 in tumor progression: the good, the bad and the ugly. Cancer metastasis reviews. 2008; 27(4):715–35. Epub 2008/05/29. doi:10.1007/s10555-008-9160-9 PMID: 18503696.

10. Horiguchi A, Asano T, Asakuma J, Sumitomo M, Hayakawa M. Impact of caveolin-1 expression on clinicopathological parameters in renal cell carcinoma. The Journal of urology. 2004; 172(2):718–22. Epub 2004/07/13. doi: 10.1097/01.ju.0000130943.23317.08 PMID: 15247769.

11. Campbell L, Jasani B, Edwards K, Gumbleton M, Griffiths DF. Combined expression of caveolin-1 and an activated AKT/mTOR pathway predicts reduced disease-free survival in clinically confined renal cell carcinoma. British journal of cancer. 2008; 98(5):931–40. Epub 2008/02/20. doi: 10.1038/sj.bjc.6604243 PMID: 18283322; PubMed Central PMCID: PMC2266860.

12. Campbell L, Gumbleton M, Griffiths DF. Caveolin-1 overexpression predicts poor disease-free survival of patients with clinically confined renal cell carcinoma. British journal of cancer. 2003; 89(10):1909–13. Epub 2003/11/13. doi:10.1038/sj.bjc.6601359 PMID: 14612902; PubMed Central PMCID: PMC2394459.

13. Joo HJ, Oh DK, Kim YS, Lee KB, Kim SJ. Increased expression of caveolin-1 and microvessel density correlates with metastasis and poor prognosis in clear cell renal cell carcinoma. BJU international. 2004; 93(3):291–6. PMID: 14764125.

14. Karam JA, Lotan Y, Roehrborn CG, Ashfaq R, Karakiewicz PI, Shariat SF. Caveolin-1 overexpression is associated with aggressive prostate cancer recurrence. The Prostate. 2007; 67(6):614–22. Epub 2007/02/15. doi: 10.1002/pros.20557 PMID: 17299799.

15. Fong A, Garcia E, Gwynn L, Lisanti MP, Fazzari MJ, Li M. Expression of caveolin-1 and caveolin-2 in urothelial carcinoma of the urinary bladder correlates with tumor grade and squamous differentiation. American journal of clinical pathology. 2003; 120(1):93–100. Epub 2003/07/18. doi: 10.1309/292N-HAYN-WAVR-EJ37 PMID: 12866378.

16. Murakami S, Miyamoto M, Hida Y, Cho Y, Fukunaga A, Oshikiri T, et al. Caveolin-1 overexpression is a favourable prognostic factor for patients with extrahepatic bile duct carcinoma. British journal of cancer. 2003; 88(8):1234–8. Epub 2003/05/10. PMID: 12737162.
17. Shi L, Chen XM, Wang L, Zhang L, Chen Z. Expression of caveolin-1 in mucoepidermoid carcinoma of the salivary glands: correlation with vascular endothelial growth factor, microvessel density, and clinical outcome. Cancer. 2007; 108(1):1523–31. Epub 2007/03/08. doi: 10.1002/cncr.22573 PMID: 17342767.

18. Selga E, Morales C, Noe V, Peinado MA, Ciudad CJ. Role of caveolin 1, E-cadherin, Enolase 2 and PKCalpha on resistance to methotrexate in human HT29 colon cancer cells. BMC medical genomics. 2008; 1:35. Epub 2008/08/13. doi: 10.1186/1755-8794-1-35 PMID: 18694510; PubMed Central PMCID: PMC2527490.

19. Ho CC, Kuo SH, Huang PH, Huang HY, Yang CH, Yang PC. Caveolin 1 expression is significantly associated with drug resistance and poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine-based chemotherapy. Lung Cancer. 2008; 59(1):105–10. Epub 2007/09/14. doi: 10.1016/j.lungcan.2007.07.024 PMID: 17890918.

20. Savage K, Lambros MB, Robertson D, Jones RL, Jones C, Mackay A, et al. Caveolin 1 is overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas: a morphologic, ultrastructural, immunohistochemical, and in situ hybridization analysis. Clinical cancer research: an official journal of the American Association for Cancer Research. 2007; 13(1):90–101. Epub 2007/01/04. doi: 10.1158/1078-0432.CCR-06-1371 PMID: 17200343.

21. Campbell L, Al-Jayyoussi G, Gutteridge R, Gumbleton N, Griffiths R, Gumbleton S, et al. Caveolin 1 in renal cell carcinoma promotes tumour cell invasion, and in co-operation with pERK predicts metastases in patients with clinically confined disease. Journal of translational medicine. 2013; 11:255. Epub 2013/10/15. doi: 10.1186/1475-5786-11-255 PMID: 24119769; PubMed Central PMCID: PMC4015803.

22. Steffens S, Schrader AJ, Blasig H, Vetter G, Eggers H, Tranckenschuh W, et al. Caveolin 1 protein expression in renal cell carcinoma predicts survival. BMC urology. 2011; 11:25. Epub 2011/12/14. doi: 10.1186/1471-2490-11-25 PMID: 22152020; PubMed Central PMCID: Pmc3266190.

23. Waalkes S, Eggers H, Blasig H, Atheskefri K, Kramer MW, Hennenlotter J, et al. Caveolin 1 mRNA is overexpressed in malignant renal tissue and might serve as a novel diagnostic marker for renal cancer. Biomarkers in medicine. 2011; 5(2):219–25. Epub 2011/04/09. doi: 10.2217/bmm.11.12 PMID: 21473727.

24. Chang WS, Tsai CW, Wang SW, Wu HC, JiHX, et al. Association of caveolin-1 genotypes with renal cell carcinoma risk in Taiwan. The Chinese journal of physiology. 2014; 57(4):220–6. Epub 2014/09/24. doi: 10.4077/CPJ.2014.BAC213 PMID: 25246063.

25. Vignaud P, Duringer P, Mackay RE, McKee JL, Blondel C, Boisserie JR, et al. Geology and palaeontology of the Upper Miocene Toros-Menalla hominid locality, Chad. Nature. 2002; 418(6894):152–5. Epub 2002/07/12. doi: 10.1038/nature00880 PMID: 12110881.

26. Hasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nature reviews Molecular cell biology. 2008; 9(9):690–701. Epub 2008/08/23. doi: 10.1038/nrm2476 PMID: 18719708.

27. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annual review of cell and developmental biology. 2005; 21:247–69. Epub 2005/10/11. doi: 10.1146/annurev.cellbio.21.020604.150721 PMID: 16212495.

28. Hodge JC, Bub J, Kaul S, Kajdacsy-Balla A, Lindholm PF. Requirement of RhoA activity for increased nuclear factor kappaB activity and PC-3 human prostate cancer cell invasion. Cancer research. 2003; 63(6):1359–64. Epub 2003/03/22. PMID: 12649199.

29. Cardone RA, Bagord A, Bellizzi A, Busco G, Guerra L, Paradiso A, et al. Protein kinase A gating of a pseudopodial-located RhoA/ROCK/p38/NHE1 signal module regulates invasion in breast cancer cell lines. Molecular biology of the cell. 2005; 16(7):3117–27. Epub 2005/04/22. doi: 10.1091/mc0.E04-10-0945 PMID: 15843433; PubMed Central PMCID: PMC1165397.

30. Xia M, Land H. Tumor suppressor p53 restricts Ras stimulation of RhoA and cancer cell motility. Nature structural & molecular biology. 2007; 14(3):215–23. Epub 2007/02/21. doi: 10.1038/nsmb1208 PMID: 17310253.

31. Dreissigacker U, Mueller MS, Unger M, Siegert P, Genze F, Gierschik P, et al. Oncogenic K-Ras down-regulates Rac1 and RhoA activity and enhances migration and invasion of pancreatic carcinoma cells through activation of p38. Cellular signalling. 2006; 18(8):1156–68. Epub 2005/11/01. doi: 10.1016/j.celsig.2005.09.004 PMID: 16257181.

32. Hirsch DS, Wu WJ. Cdc42: an effector and regulator of ErbB1 as a strategic target in breast cancer therapy. Expert review of anticancer therapy. 2007; 7(2):147–57. Epub 2007/02/10. doi: 10.1586/14737140.7.2.147 PMID: 17288526.

33. Liu N, Bi F, Pan Y, Sun X, Xue Y, Shi Y, et al. Reversal of the malignant phenotype of gastric cancer cells by inhibition of RhoA expression and activity. Clinical cancer research: an official journal of the
34. Jacobs M, Hayakawa K, Swenson L, Bellon S, Fleming M, Taslimi P, et al. The structure of dimeric ROCK I reveals the mechanism for ligand selectivity. The Journal of biological chemistry. 2006; 281 (1):260–8. Epub 2005/10/27. doi: 10.1074/jbc.M508847200 PMID: 16249185.

35. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. The EMBO journal. 1996; 15(9):2208–16. Epub 1996/05/01. PMID: 8641286; PubMed Central PMCID: PMC450144.

36. Rivat C, Le Floch N, Sabbah M, Teyrol I, Redeuilh G, Bruyneel E, et al. Synergistic cooperation between the AP-1 and LEF-1 transcription factors in activation of the matrixisin promoter by the src oncogene: implications in cellular invasion. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2003; 17(12):1721–3. Epub 2003/09/06. doi: 10.1096/fj.03-0132fje PMID: 12958188.

37. Arpaia E, Blaser H, Quintela-Fandino M, Duncan G, Leong HS, Ablack A, et al. The interaction between caveolin-1 and Rho-GTPases promotes metastasis by controlling the expression of alpha5-integrin and the activation of Src, Ras and Erk. Oncogene. 2012; 31(7):884–96. Epub 2011/07/19. doi: 10.1038/onc.2011.288 PMID: 21765460; PubMed Central PMCID: PMC3289793.

38. Thomas S, Overdevest JB, Nitz MD, Williams PD, Owens CR, Sanchez-Carbayo M, et al. Src and caveolin-1 reciprocally regulate metastasis via a common downstream signaling pathway in bladder cancer. Cancer research. 2011; 71(3):832–41. Epub 2010/12/15. doi: 10.1158/0008-5472.CAN-10-0730 PMID: 21148751; PubMed Central PMCID: PMC3106590.

39. Chen W, Sang JY, Liu DJ, Qin J, Huo YM, Xu J, et al. Desensitization of G-protein-coupled receptors induces vascular hyporesponsiveness in response to norepinephrine in the mesenteric arteries of cirrhotic patients and rats. Hepatobiliary & pancreatic diseases international: HBPD INT. 2013; 12(3):295–304. Epub 2013/06/08. PMID: 23742775.

40. Rao MY, Soliman H, Bankar G, Lin G, MacLeod KM. Contribution of Rho kinase to blood pressure elevation and vasoconstrictor responsiveness in type 2 diabetic Goto-Kakizaki rats. Journal of hypertension. 2013; 31(6):1160–9. Epub 2013/04/05. doi: 10.1097/HJH.0b013e328360383a PMID: 23552123.

41. Komers R. Rho kinase inhibition in diabetic nephropathy. Current opinion in nephrology and hypertension. 2011; 20(1):77–83. Epub 2010/11/16. doi: 10.1097/MNH.0b013e32834131f8 PMID: 21076299.