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CYBA as a Potential Biomarker for Renal Cell Carcinoma: Evidence from an Integrated Genetic Analysis

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Abstract. *Background/Aim: Oxidative stress plays an important role in various pathogenic processes, and disruption in the coordinated production of NADPH oxidase (NOX)-derived reactive oxygen species has been associated with carcinogenesis. However, little is known about whether genetic variants in NOX can contribute to the development of renal cell carcinoma (RCC). Patients and Methods: This study aimed to bridge this knowledge gap by analysing the association of 10 single-nucleotide polymorphisms in the phagocyte NOX genes, CYBA and CYBB, with RCC risk and tumour characteristics in 630 RCC patients and controls. Differential gene expression and patient prognosis analyses were performed using gene expression data obtained from public databases. Results: Multivariate analysis and multiple testing corrections revealed the A allele of rs7195830 in CYBA to be a significant risk allele for RCC, compared to the G allele [odds ratio (OR)=1.70, 95% confidence interval (CI)=1.27-2.26, p<0.001]. A pooled analysis of 17 renal cancer gene expression datasets revealed a higher CYBA expression in RCC than in normal tissues. Moreover, high CYBA expression was associated with advanced tumour characteristics and worse patient prognosis. Conclusion: CYBA might play an oncogenic role in RCC and serve as a predictive indicator of patient prognosis.*

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Renal cell carcinoma (RCC) is the most common type of renal cancer in adults and accounts for approximately 90% of all cases (1). In 2020, a total of 431,288 new cases of renal cancer, with 179,368 renal cancer-associated deaths were recorded worldwide (2). The global incidence of RCC has increased by 2-3% annually over the past two decades (3), making it a growing public health concern. The incidence of RCC is higher in developed countries and is more common in men than in

Table I. *Association of phagocyte NADPH oxidase gene polymorphisms with risk, grade, and stage of renal cell carcinoma.*

Gene	SNP ID	Chromosome	Position	MAF	HWE	Allele	Risk		Grade		Stage	
							p-Value	*q*	*p*-Value	*q*	*p*-Value	*q*
CYBA	rs899729	16	88635372	0.127	0.482	C>A	0.676	0.734	0.121	0.560	0.410	0.690
CYBA	rs7195830	16	88643304	0.263	0.480	G>A	0.001	0.024	0.365	0.690	0.780	0.734
CYBA	rs12933505	16	88646359	0.124	0.858	G>A	0.745	0.734	0.133	0.560	0.254	0.690
CYBA	rs4673	16	88646828	0.076	1.000	G>A	0.392	0.690	0.269	0.690	0.478	0.690
CYBA	rs117176859	16	88647264	0.040	0.618	G>A	0.777	0.734	0.011	0.087	0.141	0.560
CYBA	rs16966671	16	88651867	0.200	0.391	G>C	0.010	0.087	0.926	0.736	0.721	0.734
CYBA	rs11649119	16	88653666	0.180	0.430	A>G	0.404	0.690	0.552	0.690	0.502	0.690
CYBB	rs35158198	X	37777783	0.076	–	C>T	0.554	0.690	0.579	0.690	0.237	0.690
CYBB	rs5917471	X	37793265	0.103	–	C>T	0.908	0.736	0.483	0.690	0.875	0.736
CYBB	rs139892460	X	37797313	0.041	–	C>T	0.440	0.690	0.800	0.734	0.892	0.736

SNP, Single nucleotide polymorphism; MAF, minor alleles frequency; HWE, Hardy-Weinberg equilibrium. –, not calculated for the SNPs on the X chromosome.

women. Risk factors for the disease include smoking, obesity, hypertension, and a history of renal disease (4). Early detection is crucial for improving the outcomes and increasing the likelihood of successful treatment. Therefore, further research is required to understand the underlying causes of RCC and to develop new and effective treatments.

The phagocyte NADPH oxidases (NOX) are multi-protein complexes that generate reactive oxygen species (ROS) in phagocytes, such as neutrophils and macrophages. NOX are activated by various stimuli, including cytokines and growth factors, leading to the transfer of electrons from NADPH to oxygen and resulting in the generation of ROS (5). These ROS, in turn, act as stimuli and serve as signals in various cellular processes, including immune responses, inflammation, and tissue injury (6). The catalytic subunits of the phagocyte NOX are composed of the $p22^{phox}$ (encoded by *CYBA*) and $gp91^{phox}$ (encoded by *CYBB*) proteins, and mutations in these genes are associated with chronic granulomatous disease, which is an inherited disorder characterized by recurrent infections and abnormal inflammation (7). Besides the phagocyte NOX, *CYBA* is shared by several other NOX in a variety of nonphagocytic cells with different cellular functions (8). Dysregulation of the NOX activation, which leads to elevated ROS levels, has been linked to various pathophysiologies, such as cardiovascular diseases, diabetes, neurodegenerative diseases, and cancer (9). The ROS produced by NOX has been recently linked with the proliferation of human colon cancer cells and silencing NOX1 using short hairpin RNA hindered the cell cycle progression in the G_1/S phase due to reduced cyclin D1 expression (10).

Single-nucleotide polymorphisms (SNPs) can serve as suitable markers to predict the predisposition to various diseases (11), including cancer, and represent a pathway towards personalized medicine. To date, limited studies have elucidated the role of genetic variations in *NOX* genes and their link with RCC. Consequently, this present case-control study, involving 630 participants, aimed to investigate the relationship between phagocyte *NOX* gene polymorphisms and the risk of RCC, as well as tumour characteristics. Furthermore, we examined the correlation between *NOX* gene expression levels and the clinical and pathological features in RCC patients.

Patients and Methods

Study population. A total of 312 patients with RCC, along with 318 sex- and age-matched, unrelated, healthy participants without any history of cancer, were recruited from three hospitals across Taiwan: Taipei Medical University Hospital, Taipei Municipal Wan Fang Hospital, and National Taiwan University Hospital. The details of the recruitment criteria, participation, and data collection have been described previously (12, 13). The distributions of body mass index and smoking behaviour did not differ between the controls and patients (14). The alcohol intake was lower, and the prevalence of diabetes and hypertension was higher among the patients than among the controls ($p<0.001$). A total of 68 (24.8%) and 55 (18.6%) patients were diagnosed with the stage III-IV and grade III-IV (a more aggressive form of RCC), respectively. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee of National Taiwan University Hospital (9100201527). Written informed consent was received from all the participants.

SNP selection and genotyping. Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations and stored at -80˚C until further analysis. The SNPs in phagocyte NOX genes, *CYBA* and *CYBB*, were selected using the Han Chinese data in the 1000 Genomes Project and the Haploview software with a pairwise linkage disequilibrium $r^2>0.8$ and minor allele frequency >0.05 (15, 16). Finally, 10 haplotype-tagging SNPs were determined and genotyped using the Affymetrix Axiom Genotyping arrays at the National Centre for Genome Medicine, Taiwan (17). The overall genotyping rate was between 98.0 and 99.8%.

Table II. *Association between CYBA rs7195830 and the risk of renal cell carcinoma.*

Genotype	Controls, *n* (%)	Patients, *n* (%)	OR (95% CI)	*p*-Value	*q*	OR (95% CI)[a]	*p*-Value[a]
GG	194 (61.4)	152 (49.8)	1.00			1.00	
GA	108 (34.2)	121 (39.7)	1.43 (1.02-2.00)	0.037		1.59 (1.10-2.30)	0.015
AA	14 (4.4)	32 (10.5)	2.92 (1.50-5.66)	0.002		3.27 (1.55-6.93)	0.002
Trend			1.57 (1.21-2.02)	0.001	0.024	1.70 (1.27-2.26)	<0.001

OR, Odds ratio; CI, confidence interval. [a]ORs were adjusted for sex, age, body mass index, smoking status, alcohol intake, and histories of diabetes and hypertension.

Table III. *Regulatory annotation of CYBA rs7195830.*

Chromosome	Position	SNP ID	Reference allele	Alternate allele	ASN frequency	Variant type	Enhancer histone marks	DNAse	Proteins bound	eQTL hits	Motifs changed
16	88642676	rs931138	C	T	0.77		IPSC, BLD, SKIN	BLD, MUS, LIV	POL2	1 hit	ATF3, ATF4, ELF1, Maf
16	88642935	rs9925947	A	G	0.77		IPSC, BLD, SKIN	IPSC, BLD, LIV, BLD, SKIN	ZNF263	1 hit	THAP1
16	88643304	rs7195830	A	G	0.77	3'-UTR	IPSC, BLD,	LIV	POL2	6 hits	Hoxb8
16	88643420	rs1049254	A	G	0.77	Missense	SKIN			1 hit	BCL, BHLHE40, E2F, ELF1, ETF, Egr-1, Ets, HEY1, Myc, NRSF, Pou2f2, TATA, YY1, Znf143, p300
16	88644425	rs3180279	C	G	0.75	Intronic	BLD	LNG		3 hits	HNF1
16	88644474	rs3794622	T	C	0.75	Intronic	BLD	BLD			AIRE, HNF4
16	88644480	rs3794623	G	T	0.75	Intronic	BLD	BLD			AIRE, HNF4
16	88645911	rs12709102	T	C	0.22	Intronic	ESDR, BLD, GI	BLD		7 hits	AP-1, Elf3, Elf5, GATA, LXR, Mef2, Nanog, p53
16	88647093	rs4782393	A	G, T	0.23	Intronic	ESDR, IPSC, BLD, SKIN, GI, MUS	IPSC		1 hit	

Bioinformatic analyses. The functional prediction for the risk-associated SNP rs7195830 was performed with HaploReg (18), and the correlation between the rs7195830 and *CYBA* expression was assessed using the Genotype-Tissue Expression (GTEx) database (19).

Statistical analyses. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS; IBM, Armonk, NY, USA). The clinical characteristics were presented as the proportion (%) of participants, and the differences between the healthy controls and patients with RCC were analysed using Chi-squared (χ^2) tests. The association between the SNPs and risk, grade, and stage of RCC was estimated by examining odds ratios (ORs) and 95% confidence intervals (CIs) which were determined using logistic regression analysis. The expression levels of *CYBA* were compared between the renal cancer and adjacent normal tissues using the standardized mean difference (SMD) and 95% CI using a random effects model with Review Manager (Cochrane, Oxford, UK). The correlations between the *CYBA* mRNA expression and tumour grade, stage, and survival of RCC were assessed using Spearman's rank correlation tests and Kaplan-Meier survival curves from The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma (TCGA-KIRC) dataset. Correction for multiple testing was performed using the false discovery rate (*q*-value) (20). *p*- and *q*-values <0.05 were considered statistically significant.

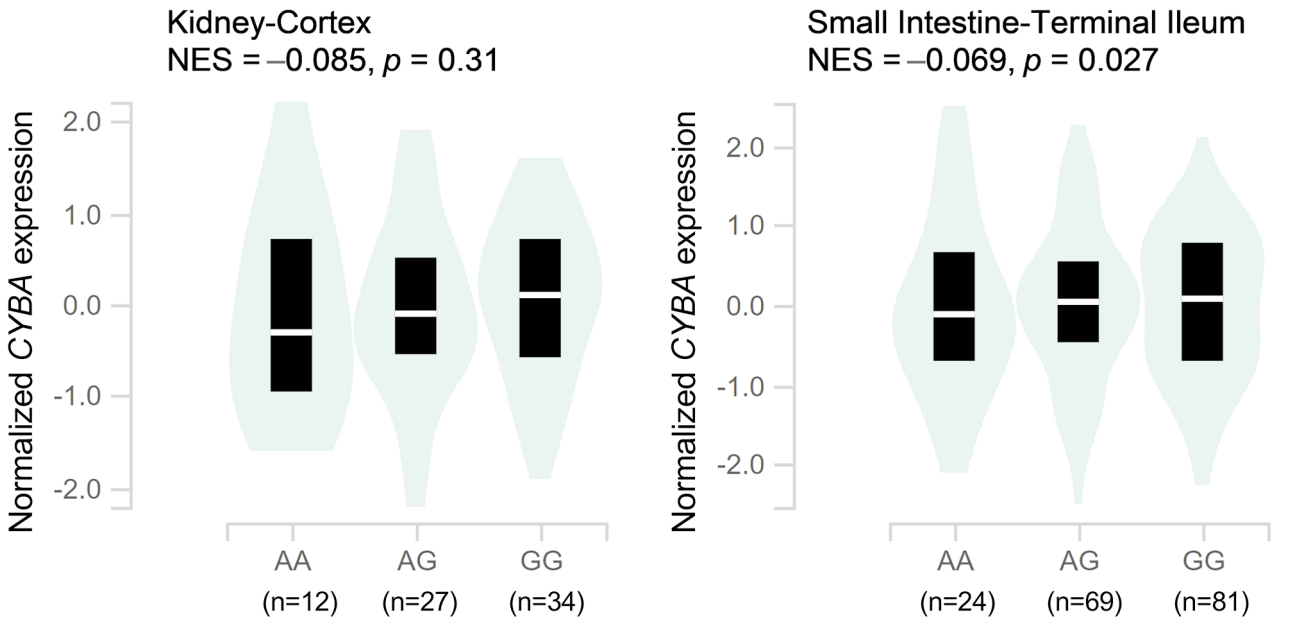

Figure 1. *Correlation of rs7195830 genotypes with CYBA expression in the renal cortex and small intestine–terminal ileum tissues based on the Genotype-Tissue Expression dataset. The values in brackets indicate the number of patients in each subgroup. NES, Normalized effect size.*

Results

Logistic regression was used to investigate the association of genetic variants in phagocyte NOX genes with the risk, grade, and stage of RCC (Table I). After adjusting for multiple testing (q<0.05), only *CYBA* rs7195830 showed a significant association with RCC risk, but none were found to be associated with tumour grade and stage. Specifically, individuals with the A allele at *CYBA* rs7195830 were at a significantly higher risk of developing RCC than those with the G allele (OR=1.57, 95% CI=1.21-2.02, p=0.001, q=0.024, Table II). Moreover, this association remained statistically significant when examined using the multivariate logistic regression analysis that was adjusted for sex, age, body mass index, smoking status, alcohol intake, and histories of diabetes and hypertension (adjusted OR=1.70, 95% CI=1.27-2.26, p<0.001, Table II).

The results of the HaploReg analysis indicated that rs7195830 and its linked SNPs reside in an open chromatin region with enhancer histone marks, DNase I hypersensitivity, RNA polymerase II binding, and expression quantitative trait loci signals, and are likely to affect transcription factor binding and *CYBA* expression (Table III). The three-dimensional organization of human genomes, such as topologically associating domains (TADs), play a crucial role in gene regulation by constraining interactions between promoters and cis-regulatory elements. Chromatin conformation capture Hi-C data from the 3D genome browser (21) revealed that both rs7195830 and *CYBA* are situated within the same TAD across various tissues and cell lines. The GTEx database was further used to evaluate the correlation of rs7195830 with *CYBA* expression. The results demonstrated that individuals harbouring the risk allele of rs7195830 A exhibited a higher *CYBA* expression in the small intestine-terminal ileum tissues compared to those harbouring the G allele [normalized effect size (NES)=–0.069, p=0.027, Figure 1]. Moreover, a similar Genotype-gene expression relationship was observed in the renal cortex tissues, although this relationship was not statistically significant (NES=–0.085, p=0.31, Figure 1).

To investigate the potential role of *CYBA* in renal cancer, the TCGA, Gene Expression Omnibus, and ArrayExpress databases were used to evaluate the effects of *CYBA* expression on tumour characteristics and patient prognosis. A total of 1418 renal cancer and 400 adjacent normal tissues from 17 independent datasets were included in the pooled analysis. The results revealed that *CYBA* expression was higher in the renal cancer tissues than in the noncancerous tissues (SMD=0.61, 95% CI=0.16-1.05, p=0.008, Figure 2). Analysis using the TCGA-KIRC dataset showed that high *CYBA* expression was positively correlated with an advanced tumour stage and grade (p<0.001, Figure 3). Moreover, the Kaplan-Meier survival analysis demonstrated that patients showing a high *CYBA* expression (separated by the median) exhibited shorter progression-free, overall, and RCC-specific survivals (p<0.001, Figure 3). However, *CYBA* expression was not associated with cancer progression and patient survival in the TCGA kidney renal papillary cell carcinoma and kidney chromophobe datasets, possibly as a result of smaller sample sizes (data not shown).

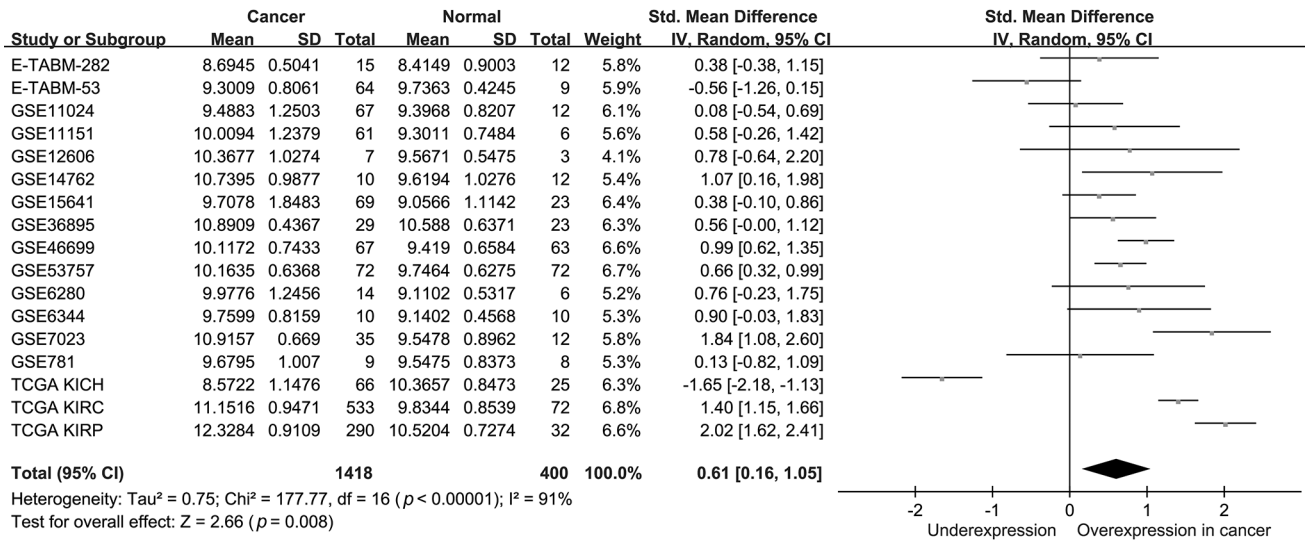

Figure 2. *Forest plot illustrating the differential CYBA expression between renal cancer and normal tissues. SD, Standard deviation; IV, inverse variance; CI, confidence interval; Std, standardized; TCGA, The Cancer Genome Atlas; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; df, degrees of freedom.*

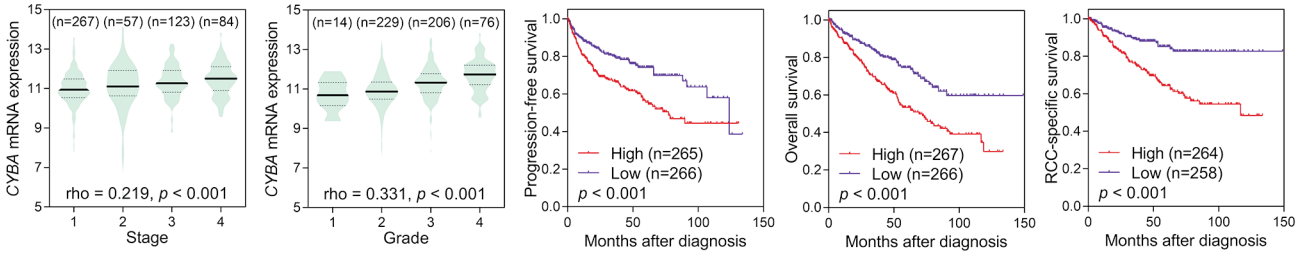

Figure 3. *CYBA expression is correlated with the aggressiveness of renal cancer and predicts worse clinical outcomes. CYBA expression is elevated during stage and grade progression, and a high CYBA expression is associated with worse progression-free, overall, and disease-specific survivals in The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma dataset. rho, Spearman's rank correlation coefficient. The values in brackets indicate the number of patients in each subgroup.*

Discussion

Despite numerous studies demonstrating the involvement of NOX-derived oxidative stress in cancer, our understanding of the relationship between NOX gene expression levels and polymorphisms with RCC remains limited. In this study, we found that *CYBA* rs7195830 was independently associated with the risk of RCC after adjustment for the known factors and correction for multiple testing. Furthermore, the expression of *CYBA* was up-regulated in the renal cancer tissues compared to the normal tissues, and high *CYBA* expression levels were found to be significantly associated with poorer survival in clear cell RCC.

The *CYBA* gene, located on chromosome 16, encodes a critical $p22^{phox}$ subunit of NOX. The NOX family comprises seven members, NOX1–5 and dual oxidases (DUOX1–2), and many NOX enzymes require association with CYBA to promote transmembrane electron transfer and to generate superoxide and hydrogen peroxide (22). NOX-derived ROS have been implicated in carcinogenesis. NOX1 is implicated in colon cancer, wherein its ability to produce ROS may promote tumour cell proliferation and metastasis (10, 23). The knockdown of *NOX1* using short hairpin RNA in HT-29 human colon cancer cells inhibits mitogen-activated protein kinase (MAPK) signalling, impairs cyclin D1 expression, and blocks cell cycle progression in the G_1/S phase (10). In myeloid leukemic cells, high levels of NOX2 hinder the destruction of malignant cells by triggering ROS-induced apoptosis of adjacent antileukemic lymphocytes (24). Moreover, the expression of *NOX2* in cancer stem cells has been linked to leukemogenesis by promoting the maintenance of leukemic stem cell self-renewal and proliferation (25). In Epstein-Barr virus-infected gastric cancer cells, *NOX2* expression increases by down-regulating miRNA34a to promote cell survival (26). Furthermore, NOX3 mediates the insulin-

induced angiogenic response through p42/44 MAPK signalling in hepatocellular carcinoma cells (27). NOX4 is overexpressed in several forms of cancer, including RCC (28), glioma (29), and melanoma (30). In RCC, NOX4 can increase hypoxia-induced interleukin (IL)-6 and IL-8 secretion, resulting in cell invasion (28). CYBA protein levels and NOX-derived ROS production are elevated in von Hippel-Lindau (VHL)-deficient RCC cells, and the reintroduction of VHL into these cells leads to reduced *CYBA* expression and ROS production (31). Furthermore, gene silencing of *CYBA* results in the inhibition of AKT phosphorylation, and the use of specific NOX inhibitors decreases RCC cell growth and reduces tumour formation *in vivo* (31). Several studies have also reported significant associations between genetic variants in *CYBA* and the risks of cervical cancer (32), colorectal cancer (33), and end-stage renal disease (34), further supporting the significance of *CYBA* in cancer. Consistent with this, our data revealed that *CYBA* mRNA expression in the RCC tissue specimens is significantly higher than in the noncancerous tissue specimens. Furthermore, a high *CYBA* expression was correlated with a poorer survival rate, indicating that *CYBA* may have an oncogenic role in RCC. However, the risk allele rs7195830 A exhibited only a marginal increase in *CYBA* expression in the GTEx renal cortex tissues. Therefore, further investigations are required to identify additional molecular mechanisms that may alter *CYBA* expression in RCC.

Conclusion

Although the exact role of *CYBA* in RCC is not well understood, more in-depth research may uncover therapeutic targets within NOX pathways. The current study establishes a link between *CYBA* and RCC; however, this study has certain limitations, which include being limited to samples from Taiwanese individuals, which may impact its generalizability to other populations. Other crucial NOX-related genes that may have contributed to the development of RCC were not evaluated in the current study. Additionally, despite correcting for multiple testing with *q*-values, false positives remain a possibility. Furthermore, our sample size is relatively small compared to other genetic association studies. Lastly, our understanding of the biological mechanisms by which rs7195830 affects *CYBA* function and RCC progression remains incomplete. Although additional functional experiments are necessary, this study indicates that *CYBA* rs7195830 confers an increased risk of RCC, and targeting *CYBA* could be a promising therapeutic approach for treating RCC.

Conflicts of Interest

The Authors declare that they have no potential conflicts of interest in regard to this study.

Authors' Contributions

CFC performed data collection and analysis. SPH and CYH contributed to project development and funding acquisition. YMH and PLC performed data collection. CHL and JHG performed data analysis. BYB contributed to project development, data analysis, and funding acquisition. All Authors prepared and agreed to the published version of the manuscript.

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