Over-Expression of ZNF677 Inhibits Proliferation, Invasion and Induces Apoptosis in Clear Cell Renal Cell Carcinoma 786-0 Cells

W Liang
Guangxi Medical University First Affiliated Hospital
SH Chen
Guangxi Medical University First Affiliated Hospital
GL Yang
Guangxi Medical University First Affiliated Hospital
JY Feng
Guangxi Medical University First Affiliated Hospital
Q Ling
Guangxi Medical University First Affiliated Hospital
B Wu
Guangxi Medical University First Affiliated Hospital
HB Yan
Guangxi Medical University First Affiliated Hospital
JW Cheng (sss79568685@163.com)
Guangxi Medical University First Affiliated Hospital https://orcid.org/0000-0001-6802-5448

Research

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Abstract

Background: ZNF677 has been reported downregulation in several cancers due to DNA methylation. Recent studies have demonstrated that ZNF677 acts as a tumor suppressor gene in cancer. However, the expression and function of ZNF677 in ccRCC is still unclear. In this study, we aimed to investigate the expression of ZNF677 in ccRCC tissues and the malignant biological behavior of ZNF677 on 786-0 cells.

Methods: Combining with bioinformatics analysis,qRT-PCR and IHC analysis was carried out to detect ZNF677 expression in 60 Clear cell Renal cell carcinoma (ccRCC) tissues and 47 normal tissues and 786-0 cells. In vitro, lentiviral vector containing negative control and ZNF677 vector was constructed and transfected into 786-0 cells, respectively. CCK8 and clone formation assays were used to measure the proliferation of 786-0 cells, and apoptosis was assessed by flow cytometry assay, and transwell assay was carried out to measure the invasion ability of 786-0 cells. Apoptosis markers and EMT markers expression was evaluated by western blot.

Results: ZNF677 is hypermethylated in ccRCC and associated with clinicopathologic features,and functional assays suggested that ZNF677 inhibits tumor cell proliferation, invasion and induce apoptosis. Further prognosis analysis indicated that low expression of ZNF677 was associated with shorter overall survival (OS) and ZNF677 suppressed invasion and EMT of 786-0 cells through activating PI3K/AKT signaling pathway, but the exact mechanism remains unknown.

Conclusions: ZNF677 can be used as an adverse prognostic biomarker and potential therapeutic target for ccRCC.

1. Introduction

Renal cell carcinoma (RCC) is the third prevalent urological malignancy worldwide, ranking sixth and eighth among all cancers in men and women, respectively [1]. The morbidity of RCC is rising by 2-4% each year [1]. The mainly subtype of RCC is Clear cell Renal cell carcinoma (ccRCC), which accounts for about 70-80% of all RCC. The most effective therapeutic strategy is partial or radical nephrectomy for localized ccRCC, depending on the tumor size and location, and the 5-years survival rate is approximately 93% [1]. Unfortunately, some ccRCC patients have already metastasis when they were initial diagnosis, and the 5-years survival rate descended dramatically [2]. Advanced ccRCC is not sensitive to radiotherapy and chemotherapy. At present, targeted therapy has become an important therapeutic method of ccRCC [2]. However, the prognosis of ccRCC is still unsatisfactory due to drug resistance. Therefore,it is imperative to identify novel biomarkers and therapeutic targets that may improve the therapeutic effect of ccRCC.

The occurrence and development of cancer is closely related to the activation of oncogenes and the inactivation of tumor suppressor genes,including DNA methylation, chromosome remodeling, histone modification and noncoding RNA regulation [3]. In recent years, many molecular biomarkers for ccRCC have been explored. For example, ABCG1 and P4HB could serve as independent diagnostic and

prognostic markers for ccRCC [4–5]. CYP2J2 was higher expression in ccRCC and prolonged the survival outcome of ccRCC patients [6]. Transcription factors play an important role in regulating gene expression. Zinc finger proteins(ZNFs) are the largest family of transcription factors in the human genome,which have a “finger-like” domain. The majority of ZNFs belong to the Krupple associated box domain zinc finger protein (KRAB-ZNF) superfamily, which bind to DNA, RNA, and proteins through their specific zinc finger structure,playing an important role in cellular differentiation, proliferation, apoptosis, invasion and metastasis [7–9]. Over the last few decades, lots of literature revealed that ZNFs play crucial roles in the occurrence and development of cancer. For instant, high expression of ZNF143 was correlated with lymph node metastasis in gastric cancer, indicating that ZNF143 plays an important role in the metastase of gastric cancer [10]. ZNF281 could activate the Wnt/β-Catenin Signaling and then enhance the invasion and proliferation of Pancreatic Cancer Cells [11]. ZNF830 acts as an oncogene in lung and gastric cancer patients and is correlated with poor outcome [12].

Zinc finger protein 677 (ZNF677),which comprises C2H2-type zinc fingers (ZFs) and a Krüppel associated box (KRAB) domain, is a member of the KRAB-ZFP family. It is located at the chromosomal region 19q13, a region where frequent loss of heterozygosity in cancer occurs [13]. Sequence analysis has demonstrated that the ZNF677 promoter contains a CpG island, which implies that CpG methylation may become a common mechanism in silencing its expression in cancer. Recent studies have demonstrated that ZNF677 is low expression and functions as a tumor suppressor in thyroid cancer, lung cancer, and gastric cancer as a result of DNA methylation [13–15]. However, there is still no report regarding the expression and function of ZNF677 in ccRCC .

In this study,we revealed that ZNF677 could act as a tumor suppressor in ccRCC. We investigated the expression of ZNF677 in ccRCC and further explored its function in the development and progression of ccRCC. The results indicated that ZNF677 could be considered as an unfavorable prognostic indicator and a potential therapeutic target for ccRCC.

2. Materials And Methods

2.1. Patients and samples

We included 60 ccRCC tissues and 47 normal renal tissues from ccRCC patients at the First Affiliated Hospital of Guangxi Medical University between 2015 to 2021. All histopathological parameters were evaluated according to the 2017 TNM classification of the International Union Against Cancer (UICC). All samples were confirmed by histopathological examination and handled according to ethical and legal standards. Ethical consent was obtained from the Committee for Ethical Review of Research of the First Hospital of Guangxi Medical University. Informed consent was obtained from all enrolled patients.

2.2. Cell culture and plasmid transfection

We obtained renal cancer cell line 786-0 and immortalized human proximal tubular cell line HK-2 from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), which were grown in a humidified CO_2

incubator at 37°C and maintained in RPMI 1640 medium, and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Both the negative control and plasmid DNA encoding human ZNF677 were purchased from Origene (Rockville, MD, USA), and then subcloned into the pcDNA 3.1 plasmid vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was confirmed by western blot.

2.3. RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

RNA was extracted from ccRCC samples and 786-0 cells using TRIzol RNA Isolation Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The first cDNA strand for ZNF677 was synthesized from 1 μg of RNA using the cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT-PCR was carried out using the SYBR® Premix Dimer Eraser Kit (TaKaRa, Dalian, China) on an Applied Biosystems 7,500 Real-Time PCR System. GAPDH was used as an internal control to normalize the expression of ZNF677. The 2 − ΔΔCT method was used to calculate the relative expression of ZNF677. The primers used in this study were as follows: for GAPDH, forward, 5'-AAGGCCGGTTATCAACGT-3'; reverse,5'-GCCAGTCCCTCACTGCTCT-3'; for ZNF677, forward, 5'-ACAAGCAAGGGATTATCACCAAA-3'; reverse, 5-CAGGCTGTCAAACTTAGGCAT-3.

2.4. Western blot analysis

Western blotting was performed as previously described [16]. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Massachusetts, USA). Blots were blocked in 5% skim milk in TBST and then incubated with antibodies against GAPDH (ab16651, 1:150, Abcam, UK), ZNF677(ab155075,1:100,Abcam,UK),Caspase-3(9662,1:500,CST,Danvers,MA,USA),Caspase-9(9508,1:500,CST,Danvers,MA,USA),PARP(9532,1:300,CST,Danvers,MA,USA), bcl-2(ab32124,1:100,abcam,UK),E-cadherin (3195,1:500,CST, Danvers, MA, USA), vimentin (5741s, 1:500,CST, Danvers, MA, USA), and p-AKT (1:600; 2965, CST, Danvers, MA, USA). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature after washing and visualized with a super ECL chromogenic substrate (Applygen, Beijing, China). GAPDH was used as an internal control.

2.5. CCK8 assays

CCK8 assay was performed as previously described [16]. Briefly, 5,000 786-0 cells/well were planted in a 96-well cell culture plate at 37oC for 24 hours, and then placed with serum for another 6 hours. Afterwards, 786-0 cells were transfected with pcDNA, ZNF677, and negative control pcDNA, respectively. On the day of assessing the growth rate of the cells, 100μl of spent medium was replaced with an equal volume of fresh medium including 10% CCK8, then the cells continued to be incubated at 37oC for 3 hours, and the absorbance was finally determined at 450 nm using a micro plate reader.

2.6. Clone formation assay

Clone formation assay was performed as previously described [16]. Briefly,500 cells/well of 786-0 cells was seeded into 12-well plates and incubated under routine conditions for 1-2 weeks. Phosphatebuffered saline was used to wash the plates twice and then fixed the plates with 4% paraformaldehyde for 10 min at room temperature. After staining with 0.1% crystal violet for 30 min, the cells were photographed and the numbers of colonies were counted.

2.7. Flow cytometry assay

Flow cytometry assay was performed as previously described [16]. Briefly, after 48h of infection treatment, cells in each well were collected, and cell apoptosis was detected by annexin using the V-FITC/PI staining method. Each sample was tested in triplicate, and analyses were performed by Kaluza Analysis 2.0 (Beckman Coulter, Inc.) according to the manufacturer's guidelines.

2.8. Transwell invasion assay

Transwell invasion assay was performed as previously described [16]. A 24-well Transwell unit (Corning, New York, USA) with an 8-μm pore size polycarbonate filter was used following the manufacturer's protocol. Briefly, 3×10^5 cells were seeded into the upper chamber of the insert with Matrigel (BD, Franklin Lakes, NJ, USA) in serum-free medium. The lower chamber was filled with medium and PBS. After incubation for 48 hours, cells that invaded through the extracellular matrix (ECM) were stained with methanol and crystal violet and counted in five random fields under a light microscope. The experiments were performed thrice.

2.9. Immunohistochemical analysis

Immunohistochemical analysis was performed as previously described [16]. The Monoclonal anti-ZNF677 (ab155075,1:100,Abcam,UK) was used for immunohistochemistry. For each slide, five random fields were selected for scoring and the mean score of each slide was used in the final analyses. The percentage of positivity of the tumor cells and the staining intensity were then multiplied to generate the immunoreactivity score (IS) for each tumor sample. Unequivocal nuclear and cytoplasm staining patterns for ZNF677 were both considered positive. The staining intensity was determined as follows: no staining, 0; weak staining, 1; intermediate staining, 2; heavy staining, 3. The percentage of stained cells was scored as follows:0-10% positive cells,1; 10-50% positive cells,2; >50% positive cells,3. The immunohistochemistry scoring was performed by two independent pathologists without knowledge of the clinical features of the cases.

2.10. UALCAN Analysis

UALCAN (http://ualcan.path.uab.edu/) is an online, open-access platform that contains TCGA data, including gene expression, protein expression (CPTAC dataset), promoter methylation, miRNA expression, and clinicopathological data. In this study, UALCAN (http://ualcan.path.uab.edu/cgi-bin/ualcan-res.pl) was used to assess the expression, promoter methylation and prognosis of ZNF677 in ccRCC from TCGA database.

2.11. Statistical analysis

All data analyses were performed using SPSS (version 22.0; SPSS Inc., Chicago, USA). The χ^2 test was used to analyze the differences in the RNA expression patterns between the ccRCC and normal renal tissue groups, and the relationship between ZNF677 expression and clinicopathological variables. Survival data were analyzed with Kaplan–Meier curves and the log-rank test. Statistical significance was set at $p < 0.05$.

3. Results

3.1 ZNF677 was lower expression in ccRCC and served as a tumor suppressor gene.

To evaluate the mRNA expression patterns of ZNF677 in ccRCC, we first used the online TCGA database containing 533 ccRCC tissues and 72 normal tissues to examine the mRNA expression of ZNF677 and found that ZNF677 was considerably downregulated in tumor tissues in comparison to normal tissues (Fig. 1,A).The expression of ZNF677 at different ccRCC grades and stages showed in Fig. 1B-D. To validate the results of TCGA,the expression of ZNF677 mRNA and protein in 60 tumor tissues and 47 normal tissues then were examined by qPCR and IHC, respectively.qPCR results showed that ZNF677 mRNA levels were downregulated in ccRCC tissues compare to normal tissues (Fig. 2,A). Furthermore, ZNF677 protein levels were found to be downregulated in cancer tissues by IHC.Moreover, the expression of ZNF677 protein was gradually decreased in the primary tumor and metastatic tissues when compared with the adjacent normal tissues (Fig. 2,B). The staining of the primary tumor tissues was weak, but the staining in the metastatic tissues was absent. qPCR and western blot also showed that ZNF677 was downregulated expression in ccRCC 786-0 cells than immortalized human proximal tubular cell line HK-2 cells (Fig. 2,C).These results indicated that ZNF677 may be a tumor suppressor gene in ccRCC, which is consistent with previous studies in gastric cancer, lung cancer and thyroid cancer [13–15].

3.2. Low expression of ZNF677 was due to hypermethylation and was correlated with clinicopathologic features and poor prognosis.

Since ZNF677 is often expressed in normal tissues but decreased or absent in cancer tissues because of aberrant methylation, we detected the methylation status of ZNF677 in ccRCC. First, the methylation status of ZNF677 in primary ccRCC tissues and normal tissues from TCGA database was analysed. As showed in Fig. 3, ZNF677 methylation was far more common in ccRCC tissues than in normal tissues (Fig. 3,A).We then evaluated *ZNF677* methylation in ccRCC tissues by using MSP assay.The result suggested that *ZNF677* methylation was found in 51/60 (85%) ccRCC tissues, whereas it was only found in 2/47 (4.2%) normal tissues (Fig. 3,D;Table 1), suggesting the frequent methylation of ZNF677 in ccRCC. To further confirm whether ZNF677 expression is regulated by methylation, we treated 786-0 cells with the demethylation drug Aza combined with the HDAC inhibitor trichostatin A (TSA), and the western blot results indicated that the expression of ZNF677 dramatically restored after treatment (Fig. 3,B).

Evidently, we investigated the association of ZNF677 methylation with clinicopathologic features of ccRCC patients. The data showed that ZNF677 methylation was significantly positively associated with pathological T stage, Fuhrman classification, and lymph node metastasis, but not with age, sex, smoking and distant metastasis (Table 2).To further explore the potential prognostic value of ZNF677 in ccRCC, a prognostic analysis was next performed using the TCGA database. Results suggested that patients with higher ZNF677 mRNA expression levels had longer survival time compared to those with low ZNF677 mRNA levels (Fig. 3,C).

Table 1 Methylation status of ZNF677 between normal tissues and ccRCC tissues.

	ZNF677		
	Methylation	Unmethylation	Frequency of methylation
Normal	2	47	4.2%
Tumor	51	9	85%

Table 2 The correlation between ZNF677 methylation and clinicopathological feature in ccRCC

Item	ZNF677 methylation status		X^2	*p* value
	methylated	unmethylated		
Age (years)				
≤55	22	4	0.005	0.941
＞55	29	5		
Sex				
Male	26	5	0.064	0.800
Female	25	4		
Smoking status				
NO	23	6	1.425	0.232
YES	28	3		
Pathological T stage				
pT1	14	6	5.294	0.021
pT2-T4	37	3		
Lymph node metastasis				
N1	19	7	5.116	0.023
NO	32	2		
Distant metastasis				
M0	45	9	1.176	0.278
M1	6	0		
Fuhrman classification				
I-II	21	8	6.974	0.008
III-IV	30	1		

Collectively,like other cancers, these results indicated that the low expression of ZNF677 in ccRCC may be due to hymethylation and ZNF677 may be an adverse prognostic factor in ccRCC.

3.3. ZNF677 inhibited 786-0 cell proliferation and induced apoptosis.

To explore the effect of ZNF677 in ccRCC, we first established cell lines that stably ectopic ZNF677. 786-0 cells were transfected with empty pcDNA3.1 and pcDNA ZNF677. Western blot was used to verify the transfection effect (Fig. 4,A). Colony formation and CCK-8 assays were used to estimate the effect of ZNF677 on cell proliferation. CCK-8 assay showed that cell viability was reduced at 24, 48, and 72 h in ZNF677-expressing cells (Fig. 4,B). A reduction of approximately 70–80% in colony formation was observed in ZNF677-expressing cells compared to control cells (Fig. 4,C). These results demonstrated that ZNF677 inhibited ccRCC 786-0 cell growth.

Flow cytometry analysis was performed to assess the effect of ZNF677 on cell apoptosis. Flow cytometry indicated that the percentage of apoptotic cells was significantly increased after ZNF677-transfection in 786-0 cells (Fig. 4,D).We then examined the expression of apoptotic protein markers by western blot. The result showed that ZNF677 significantly increased the expression of Cleaved-caspase3, Cleaved-caspase9, and Cleaved PARp and decreased BCL-2 expression (Fig. 4,E).

3.4 Ectopic expression of ZNF677 suppressed invasion and EMT of 786-0 cells through the inactivated PI3K/AKT signaling pathway.

Transwell assays were carried out to investigate the effect of ZNF677 on 786-0 cell invasion. The results showed that ectopic expression of ZNF677 remarkably decreased the number of invaded cells (Fig. 5,A). Furthermore, western blot indicated that ectopic expression of ZNF677 resulted in downregulation of vimentin expression and upregulation of E-cadherin expression in 786-0 cells (Fig. 5,B). These results suggested that ZNF677 inhibited ccRCC invasion by reversing Epithelial-mesenchymal transition (EMT). Previous studies have confirmed that PI3K/AKT signaling pathway is crucial in ZNF677 mediating invasion and EMT in thyroid cancer [14, 17]. Thus, we want to know whether AKT signaling was involved in ZNF677 mediating 786-0 cell invasion. Western blot assays revealed that the ectopic expression of ZNF677 decreased the expression of p-AKT, suggesting that this molecular pathway may be involved in the ZNF677-driven EMT (Fig. 5,C).

4. Discussion

Genetic and epigenetic changes can lead to tumorigenesis and development.

Substantial evidences have verified that epigenetic modifications, such as DNA methylation alterations, nucleosome remodeling, and histone modification are involved in the biological process of maintaining normal development and gene expression [18]. DNA methylation alteration is one of the most common events in tumorigenesis and plays an important role in the initiation and development of tumors [19]. It has been recognized that ZNF family proteins are frequently decreased by DNA methylation in various types of human cancer .For example, ZNF471 is one of the most significantly hypermethylated with low mRNA expression in esophageal cancer as compared with normal tissues [20]. It has been confirmed that ZNF671 functions as a tumor suppressor in multiple carcinomas due to DNA methylation [21–22].

Previous studies have demonstrated that DNA methylation alterations participate in tumorigenesis and are significantly correlated with the clinicopathological features of ccRCC [23]. Dysregulated of gene methylation has been shown to increase ccRCC cell proliferation and invasion, and may contribute to the progression and recurrence of ccRCC [24–25]. ZNF677, a member of the ZNFs family, recently has been reported that downregulated in several different types of cancer due to promoter hypermethylation [13–15].The present study demonstrated that,combining with the online data, ZNF677 acted as a tumor suppressor gene with significantly higher methylation in ccRCC tumor tissues compared to normal

tissues. Clinical data showed that ZNF677 methylation was significantly positively associated with clinicopathologic features. In addition, hypermethylation was negatively correlated with mRNA expression, thereby suggesting that ZNF677 silenced may be due to aberrant methylation. Moreover, drug demethylation experiments showed that treated with the demethylation drug Aza combined with the HDAC inhibitor trichostatin A restored the expression of ZNF677 in 786-0 cells. These results indicate that the hypermethylation of ZNF677 regulates its downregulation expression in ccRCC, which is consistent with previous studies in thyroid cancer,lung cancer, and gastric cancer, implying that ZNF677 methylation may be a common event in tumorigenesis [13–15]. Furthermore, we used online data analysis to explore the potential prognostic value of ZNF677 in ccRCC. We found that low expression of ZNF677 predicted poor survival in ccRCC. These data suggested that ZNF677 may be an unfavorable prognostic factor in ccRCC. To further estimate the potential biological function of ZNF677 in ccRCC, we carried out a series of experiments about proliferation, apoptosis, and invasion on 786-O cells. And the results showed that ectopic expression of ZNF677 could significantly inhibit cell proliferation, invasion and induce apoptosis. Therefore, we infer that ZNF677 may serve as a potential therapeutic target to improve the prognosis of ccRCC.

Metastasis is the main cause of death of ccRCC. Epithelial Mesenchymal transition (EMT) is an essential process of tumor metastasis [26]. Recent studies have proposed that EMT is a key event in cancer cell development, invasion, and metastasis. EMT can enhance the mobility and reduce the adhesion of tumor cells,and then promotes tumor metastasis and invasion [27]. The decreased expression of epithelial marker genes and the increased expression of mesenchymal marker genes are the markers of EMT. Therein, the reduced expression of E-cadherin is what marks the threshold of the EMT process [28]. The influence of ZNFs factors on EMT and cancer invasion has been confirmed in various numbers of cancers. ZNF703 has been reported that induced EMT by inhibiting E-cadherin expression and enhanced breast cancer cell invasion and resist to sorafenib [29]. ZNF281 has been proved to play a crucial role in controlling cellular stemness expression and EMT by EMT induction and regulation of EMT-associated gene expression in colorectal cancer [30]. ZNF471 suppressed cervical cancer cell invasion through modulating EMT by negatively regulating Wnt/ β-catenin signaling pathway [31]. In our study, we observed that ZNF677 significantly reduced 786-0 cell invasion. Furthermore, ectopic expression of ZNF677 significantly elevated E-cadherin protein expression and reduced vimentin protein expression in 786-0 cells, indicating that ZNF677 decreased 786-0 cell invasion by reversing EMT. The finding was consistent with other researchers in thyroid cancer, thus supporting the concept that ZNF677 suppressed cancer metastasis and invasion through regulating EMT.

EMT can be triggered by many signaling pathways. Aberrant activation of AKT signaling pathway has been revealed in many cancers that attribute to cancer cell metastasis and EMT [32–34]. The precise molecular mechanisms of ZNF677 involved in tumor invasion and EMT are currently elusive, but activation of the PI3K/AKT signaling pathway has been reported [15, 17]. Siraj AK etal. demonstrated that overexpression of ZNF677 inhibited thyroid cancer cell invasion, decreased E-cadherin expression, and increased N-cadherin expression. Whereas, knockdown PI3K/AKT signaling pathway reduced the expressions of N-cadherin, Twist, and Zeb1, with an accompanying expression of E-cadherin in PTC cell

lines. This result proposed that ZNF677 mediated thyroid cancer cell invasion and EMT through AKT signaling pathway-dependent. Therefore, we sought to determine whether PI3K/AKT signaling pathway was involved in ZNF677 regulated ccRCC cell EMT. Western blot analysis showed that ectopic expression of ZNF677 significantly elevated E-cadherin protein expression and reduced vimentin protein expression in 786-0 cells, accompanied with inhibition of cell invasion. Meanwhile,the expression of p-AKT protein was decreased. Thus, the PI3K/AKT signaling pathway may play a role in ZNF677 promoted 786-0 cell EMT. However, additional studies are still required to explore the exact molecular mechanism underlying the effects of ZNF677 and ccRCC cell EMT.

Some limitations in our study should not be ignored. First, there are no in vivo experiments in our study, all results are based on public data and cellular experiments, and require to further validate by in vivo experiments. Second,because there is only 786-0 cell line in our laboratory, only one cell line was examined in the functional experiment.Third,the exact mechanism of ZNF677 inactivating AKT signaling pathway remains unclear. Given that ZNGF677 is a putative transcription factor, we speculate that it may bind to downstream target genes and then regulate the PI3K/AKT signaling pathway. However, the specific mechanism needs to be further investigated.

In summary, this is the first report to evaluate the expression and function of ZNF677 and ccRCC. Our findings reveal that ZNF677 is hypermethylated in ccRCC, and functional assays suggested that ZNF677 inhibits tumor cell proliferation, invasion and induces apoptosis. Further prognosis analysis indicated that low expression of ZNF677 was associated with shorter overall survival (OS) and ZNF677 suppressed invasion and EMT of 786-0 cells through inactivating PI3K/AKT signaling pathway, but the mechanism remains unknown. Further studies are needed to clarify this issue. Our results confirm that ZNF677 can be used as an adverse prognostic biomarker and a potential therapeutic target for ccRCC.

5Declarations

Ethical Approval

and Consent to participate

Ethical consent was granted from the Committee for Ethical Review of Research of First Hospital of Guangxi Medical University.

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Consent for publication

All authors approved this publication.

Availability of supporting data

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors have declared that no competing interest exists.

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Authors' contributions

W Liang and JW Cheng contributed to the experiment design, manuscript draft, and data analysis. SH Chen and GY Yang collected clinical samples. JY Feng and B Wu created the tables and Figures. Q Ling and HB Yan participated in the experimental procedures. All authors read and approved the final manuscript.

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Authors' information

All Authors' information were listed in the title page.

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Figures

Fig 1

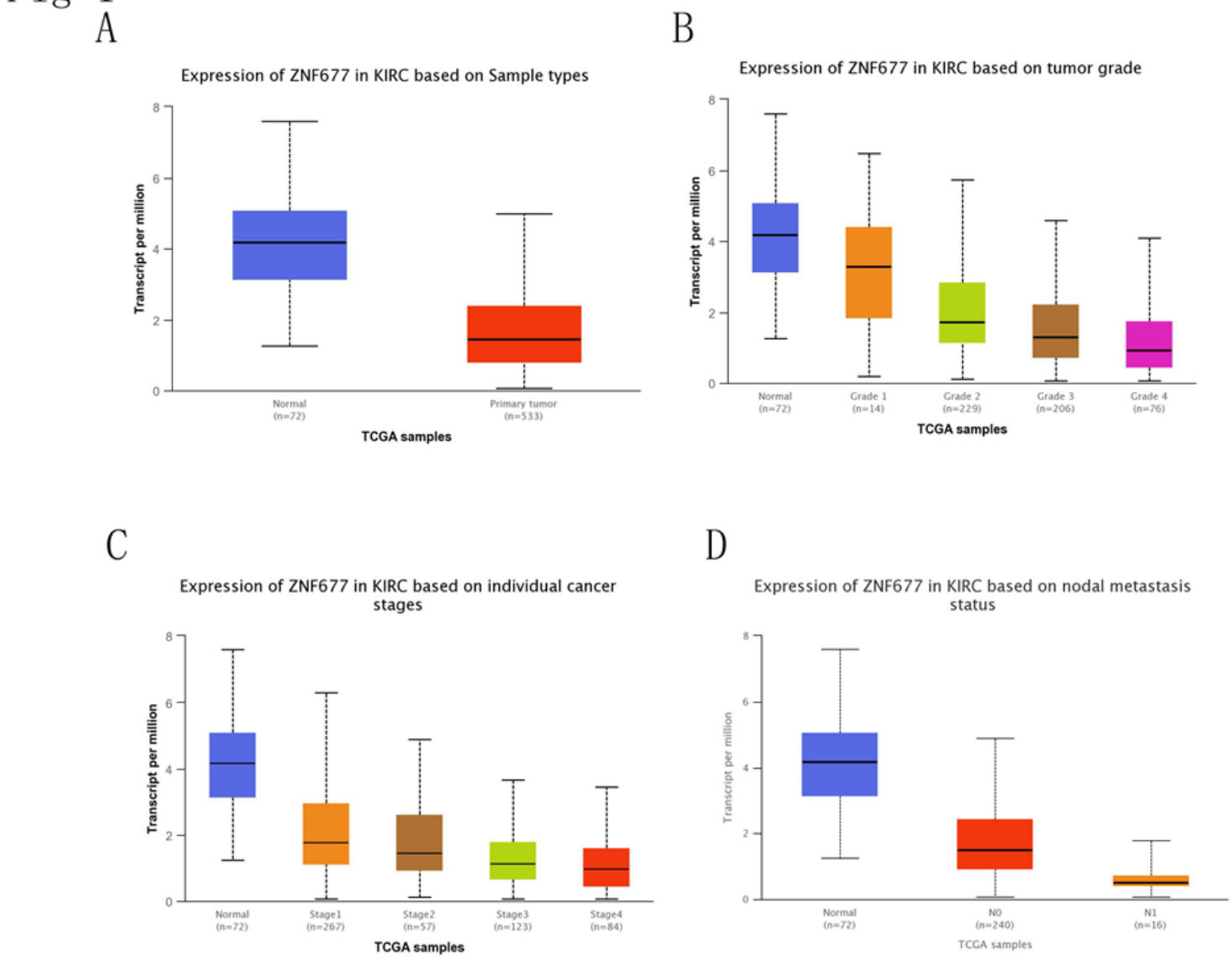

Figure 1

Online database showed that ZNF677 was considerably down-regulated in tumor tissues. (A) Expression of ZNF677 in 533 ccRCC tissues and 72 normal tissues from TCGA database. (B) Different expression of ZNF677 in ccRCC based on tumor grade. (C) Different expression of ZNF677 in ccRCC based on individual cancer stages. (D) Different expression of ZNF677 in ccRCC based on metastasis status.

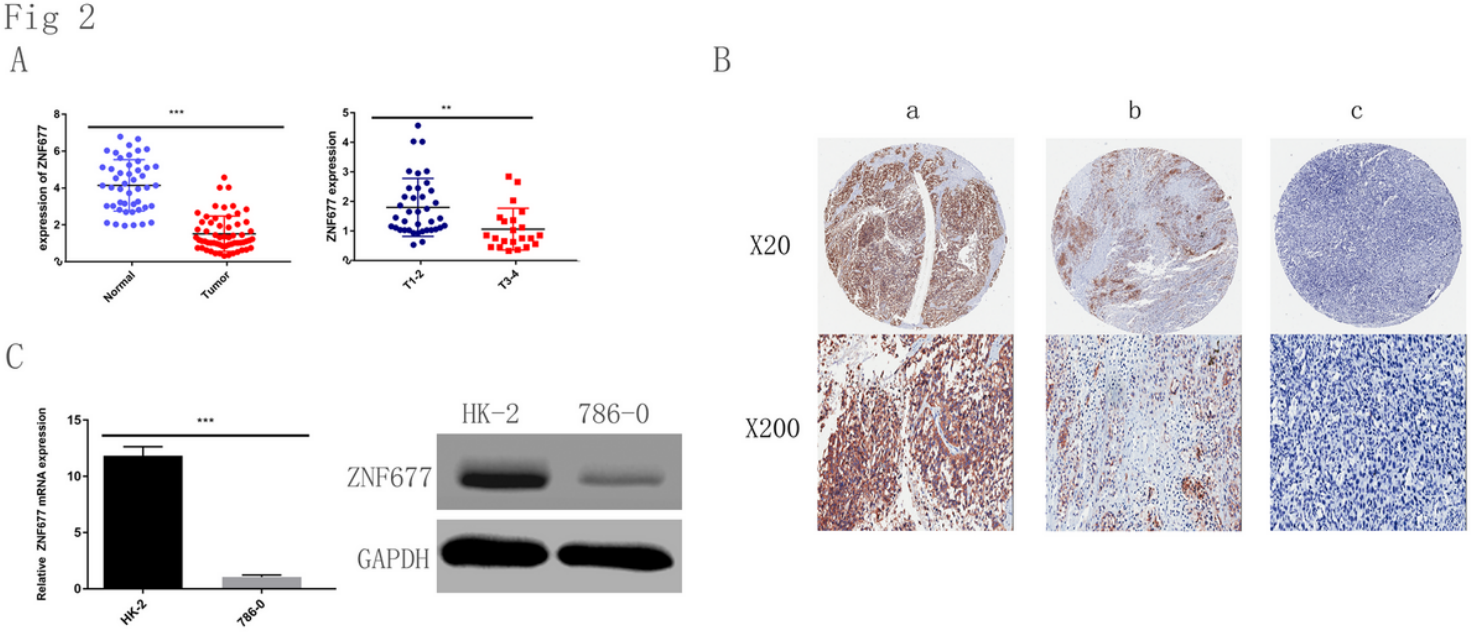

Figure 2

Expression of ZNF677 in ccRCC samples and normal tissues and 786-0 cells. (A) The relative expression levels of ZNF677 mRNA in 60 ccRCC tissues and 47 normal tissues were determined by qRT-PCR. (B) The relative expression levels of ZNF677 protein in 60 ccRCC tissues and 47 normal tissues were measured by IHC. ZNF677 protein levels were found to be down-regulated in cancer tissues in comparison to normal tissues. Moreover, the expression of ZNF677 protein was gradually decreased in the primary tumor and metastatic tissues. a.normal tissue b.not metastasis cancer tissue c.metastasis cancer tissue (C)The relative expression level of ZNF677 mRNA and protein in ccRCC 786-0 cell line was clearly decreased compared with the immortalized human proximal tubular cell line HK-2 cells evaluated by qPCR and western blot.

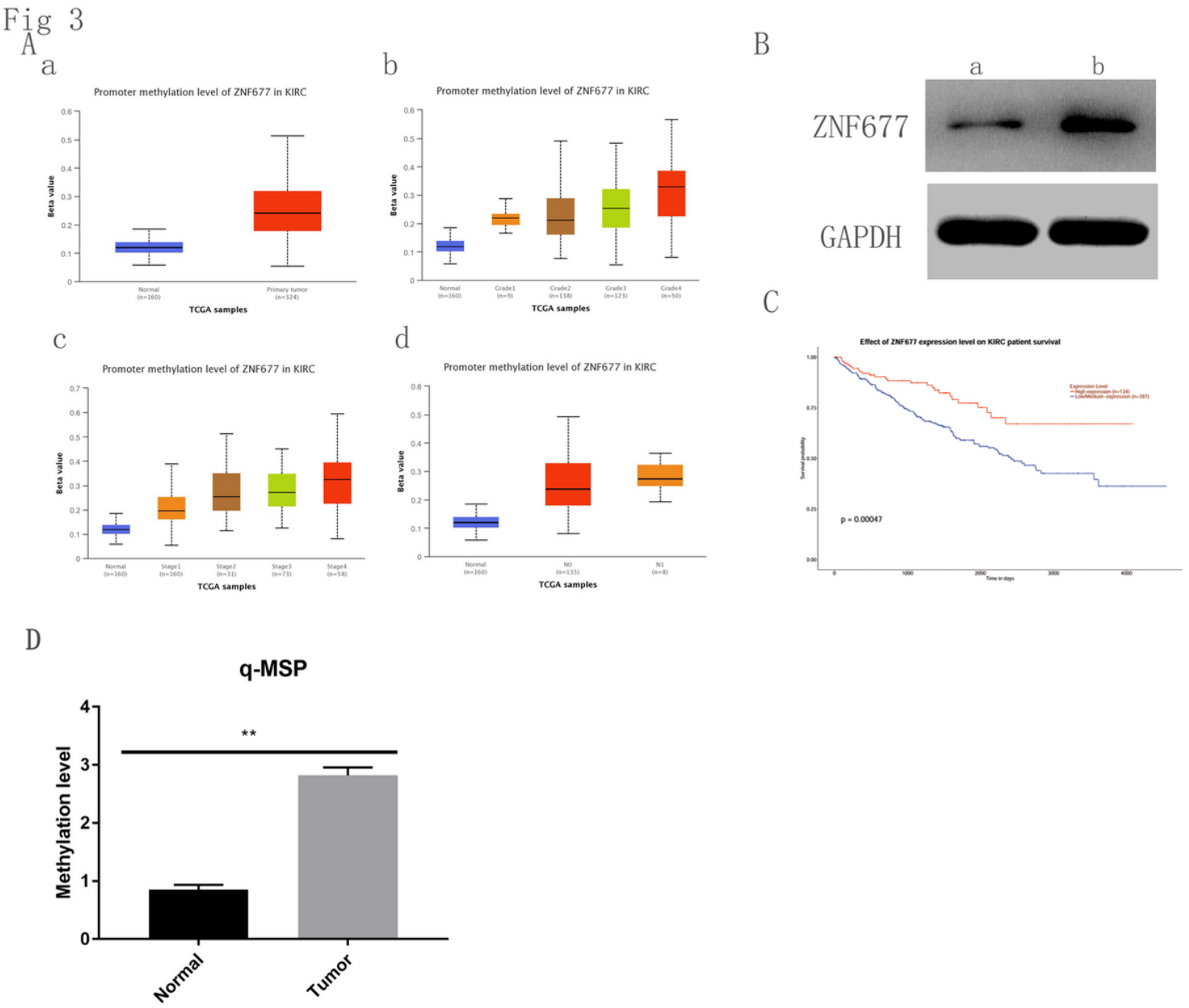

Figure 3

Low expression of ZNF677 was due to hypermethylation. The methylation status of ZNF677 in primary ccRCC tissues and normal tissues from TCGA database.(B) drug demethylation experiment showed that treated with the demethylation drug Aza combined with the HDAC inhibitor trichostatin A could restored the expression of ZNF677 in 786-0 cells, assessed by western blot. (a)drug(-) (b)drug(+). (C)The prognostic effect of ZNF677 expression in ccRCC from TCGA database. Patients with higher ZNF677 mRNA expression levels had longer survival time compared to those with low ZNF677 mRNA levels. (D) Q-MSP results showing ZNF677 methylation levels in ccRCC tissues and normal tissues.

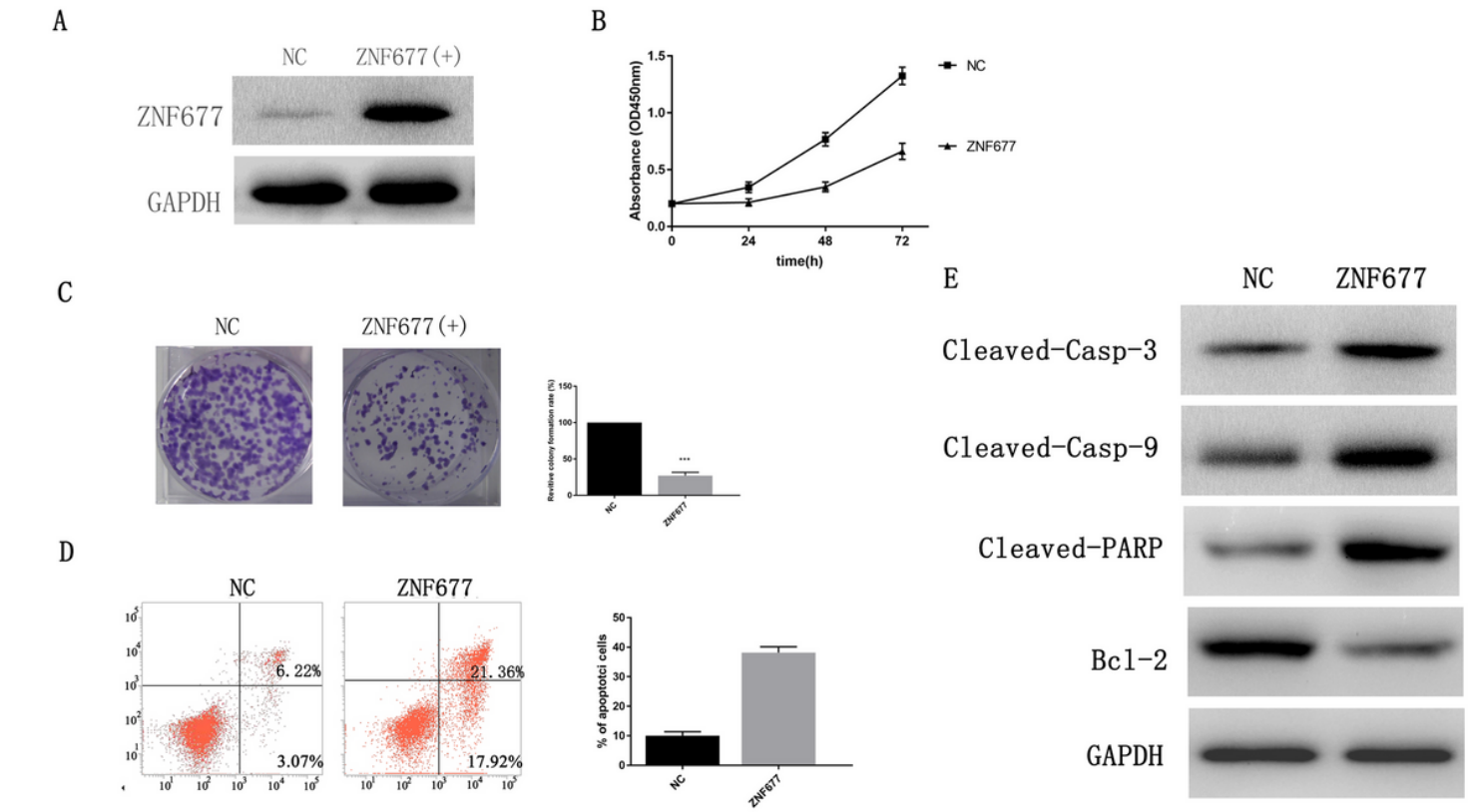

Figure 4

ZNF677 inhibits 786-0 cells proliferation and induces apoptosis. (A) Ectopic expression of ZNF677 inhibited 786-0 cells invasion in vitro evaluated by transwell assay. (B) CCK8 assays showed that ZNF677 suppressed 786-0 cells growth. (C) Clone formation assays showed that over-expression of ZNF677 reduced 786-0 cell colony formation. Colonies larger than 1 mm in diameter were counted. a. negative control group b.ZNF677(+)group. (D)Over-expression of ZNF677 induced 786-0 cells apoptosis. Apoptotic cells of different groups were measured by flow cytometry after 48 hours transduction. The cell populations of Annexin-V+/PI- and Annexin-V+/PI+ were used to assess apoptotic events. a. negative control group. b. ZNF677(+)group. (E) Effect of over-expression of ZNF677 on the expression of apoptosis-related genes, measured by western blot. Cleaved-caspase3, Cleaved-caspase9, Cleaved-PARP were increased in ZNF677(+) group cells than control cells, while apoptosis protein Bcl-2 was decreased.

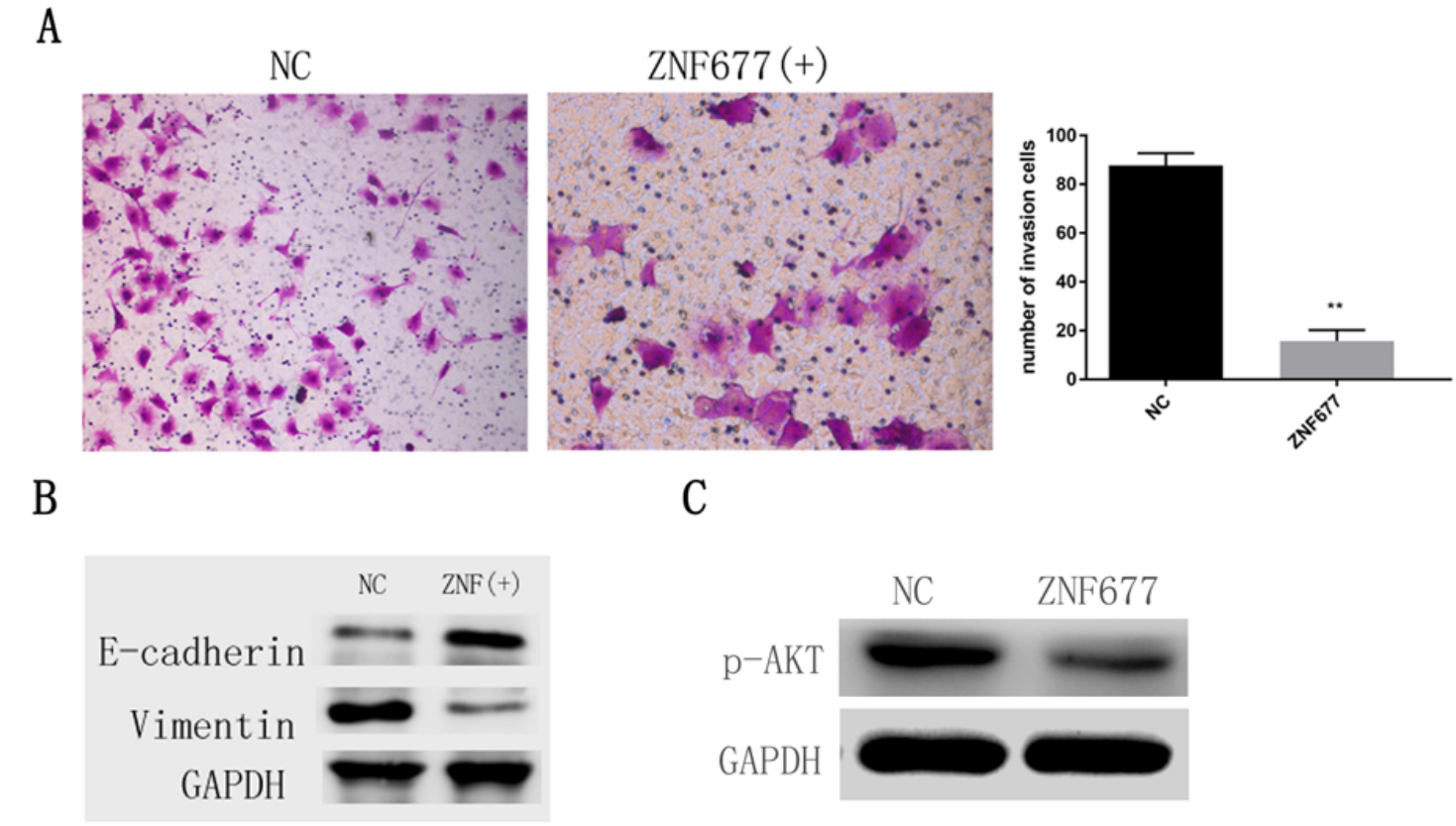

Figure 5

Ectopic expression of ZNF677 suppress invasion and EMT of 786-0 cells through inactivating PI3K/AKT signaling pathway. (A) Ectopic expression of ZNF677 inhibited 786-0 cells invasion in vitro evaluated by transwell assay. (B) Changes in EMT-related markers between control group cells and ZNF677(+)group cells, measured by Western blot analysis. (C) Changes in p-AKT protein expression between control group cells and ZNF677(+)group cells, measured by Western blot analysis