

KIFC1 accelerates the proliferation, migration and invasion of papillary thyroid cancer cells via MAPK signaling

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Research

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Abstract

Kinesin family member C1 (KIFC1) acts as a kind of minus end-directed motorized protein and is considered as an oncogene of some cancer types. However, no studies have fully elucidated its biological activity and molecular mechanisms in papillary thyroid cancer (PTC). The study focused on reporting the overexpression of KIFC1 in cell lines and tissues of PTC. Moreover, clinicopathological features analysis showed that KIFC overexpression is significantly correlated with extrathyroidal invasion and lymph node metastasis. Knockdown of KIFC1 significantly reduced cell growth, migration and invasion in PTC cells, and concomitant increased levels of differentiation markers, such as Tg and Nis. Knockdown of KIFC1 markedly increased the expression level of epithelial cell marker (E-cadherin), and decreased the expression levels of epithelial-mesenchymal transition (EMT) related transcriptional factor N-cadherin, Snail and ZEB1. Further study revealed that knockdown of KIFC1 downregulated stemness markers ALDH2 and SOX2, and inhibited the MAPK signaling cascades and downstream signaling, including p-ERK, ERK, p-JNK, JNK, MMP2, and MMP9, which can affect the expression of the EMT associated factors. Taken together, we reported that KIFC1 might promoted the proliferation, migration and invasion of PTC cells and offer a candidate molecular target for therapeutic intervention.

Introduction

Thyroid cancer (TC) is a common endocrine system related malignancy. A rapid increase in the incidence of TC in the last decades, especially due to over-diagnosis [1, 2]. Papillary thyroid cancer (PTC), a representative histopathological type of TC, which accounts for the largest proportion of cases with TC. [3]. Although recent improvements in treating PTC, for example surgery, radioiodine and endocrine therapy. PTC cells still showed occasional metastasis, loss of differentiation, anaplastic thyroid and high aggressiveness [4, 5]. Hence, the elucidation of mechanism and novel targets of signaling involved in PTC oncogenesis are still required.

The kinesin family member C1 (KIFC1) acts as a kind of minus end-directed motorized protein, which participates many physiological processes, including centrosome clustering, microtubule transportation and axle formations during mitosis [6]. Besides, KIFC1 is thought to be a potential oncogenic biomarker and gain of function mutations in different types of cancers, such as ovarian adenocarcinoma, breast cancer and lung cancer, which represents a therapeutic target due to its pathogenic role [7, 8, 9]. Studies have revealed that overexpression of KIFC1 driving docetaxel resistance in breast cancer, which is associate with a poor prognosis of patients with brain metastasis in advanced breast cancer [10].

The epithelial-mesenchymal transition (EMT) could remarkably affect the carcinoma metastasis, by which obtain mesenchymal characteristics and enhance migration and invasion abilities [11, 12]. The mitogen-activated protein kinase (MAPK) assists in controlling different cellular processes [13]. Previous researches showed that activation of the MAPK signaling drives the process of EMT in PTC progression [14, 15]. The study aims to investigate the role of KIFC1 in PTC. We also explored the mechanisms of KIFC1 in the regulation of PTC proliferation and metastasis through inhibiting EMT via targeting MAPK

signaling. Our findings offer new insight into KIFC1 function and suggest KIFC1 could be a potential target for PTC therapies.

Materials And Methods

Expression analysis in TCGA datasets and clinical specimens

The mRNA-seq data of PTC patients were provided by The Cancer Genome Atlas (TCGA; <http://www.cancer.gov/about/nci/>), and expression profile (GSE3678) from Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) database. 46 cases of PTC had been pathological diagnosis at the Harbin Medical University Cancer Hospital from October 2018 to September 2019. Paraffin embedded tissue samples and adjacent non-cancerous tissues were frozen after collection, followed by being stored in liquid nitrogen until use. This study has obtained the approval from the Ethics Committee of the Harbin Medical University Cancer Hospital.

Cell lines and transfection

Human PTC cell lines (PTC-1, NPA87, KTC-3, BCPAP) and primary thyroid follicular epithelial cell line of normal human (Nthy-ori 3 – 1) were provided by the Institute of BeNa Culture Collection (Beijing, China). These cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS) in a humid incubator at 37° C with 5% CO².

We obtained the short hairpin RNA (shRNA) target KIFC1 or negative control (NC) from GeneChem (Shanghai, China). The shRNA sequences: shKIFC1-1: 5'- GCAACATCCGTGTATTCTGCC – 3'; shKIFC1-2: 5'- CCAGGGCTATCAAATAAAGAA-3'. Retrovirus particles were produced in NPA87 and KTC-3 by transient transfection plasmids harboring KIFC1 shRNA-1, shKIFC1- shRNA-2. The cells were transfected with shRNAs by virtue of Lipofectamine 2000 transfection reagent (Invitrogen, USA) based on the protocol of manufacturers. qRT-PCR and Western blot analysis measured the silencing efficiency exhibited by KIFC1. The cells were gathered and divide into three groups for subsequent experiments as follows: KIFC1 shRNA-1 group, KIFC1 shRNA-2 group and NC shRNA group.

CCK-8 assay

The proliferation capacity of cells was evaluated with the cell-counting kit 8 (CCK8). PTC cell line NPA87 and KTC-3 were plated in 96-well black bottom well plates at 1.5×10^3 for 24 h. CCK-8 reagent (Dojindo, Kumamoto, Japan) was added incubation for 2 hours at 37°C. A microplate reader ((SpectraMax M2, Molecular Devices, CA, USA) assisted in evaluating the optical density (OD) at the 450 nm absorbance.

Colony formation assay

We first seeded PTC cells into 6-well plates (500 cells/each well), and then incubated them at different time for colony formation after transfection. Staining solution with 1% formaldehyde, 0.05% crystal violet,

and 10% methanol buffered with PBS were used for staining the fixed colonies. Then, getting images by Nikon microscope (Tokyo, Japan) and performing it in triplicate.

Cell migration and invasion assay

Putting the PTC cells into Boyden's chambers (24-well insert; with pore size of 8 μ m; BD Biosciences, San Jose, CA, USA) were used in the migration and invasion assay. Matrigel (BD biosciences) assisted in coating membranes in the invasion assay. We plated cells in the top chamber of the medium with serum-free, and filled the lower chamber with 10% FBS as the chemoattractant. After 48 hours of incubation, 4% paraformaldehyde (PFA) and crystal violet were used to fix and stain cells, respectively.

Wound-healing assays

Here, we seeded PTC cells were in 6-well plates and cultivated them until confluent. A sterile 100 ml pipette tip facilitated scratch the monolayer for creating artificial wounds, and a culture media was used to wash cells. A microscope (Nikon, Japan) was applied for capturing wound areas' images at 0 h and 24 h.

Luciferase Reporter Assays

The luciferase reporter assay was performed as previously described [16]. The activity in Nis and Tg promoter region was assessed by transfected with pNis 2.8 [17] and pGI-Tg [18] plasmids in PTC cell lines. In brief, 4×10^4 cells were seeded in 24-well plates. After 72 hours, the 300 ng reporter plasmid plus 30 ng pRL (Renilla luciferase) were used to transfect cells by virtue of Lipofectamine 2000 (Invitrogen, USA). Dual-Glo Luciferase Assay (Promega) measured the luminescence following the transfection.

RNA isolation and Real-time PCR

The real time-PCR technique assessed the mRNA expression. Briefly, total RNA was isolated using RNeasy Mini kit (Invitrogen, Carlsbad, CA, USA) following the instruction of manufacturers, and a reverse transcription kit (Applied Biosystems, CA, USA) assisted in synthesizing cDNA. The reactions were performed by A Super Real PreMix Plus (SYBR Green) Kit (TaKaRa, Biotechnology Co. Dalian, China) and real-time PCR analysis (Applied Biosystems, CA, USA). The primers were shown below:

KIFC1 Fwd: 5'-ACTACAGTGCCACAGACA-3' and Rev: 5'-CCTGATGTGCCAGACTTC-3';

ALDH2 Fwd: 5'- ATGGCAAGCCCTATGTCATCT - 3' and Rev: 5'- CCGTGGTACTTATCAGCCCA - 3;

SOX2 Fwd: 5'- GCCGAGTGGAACTTTTGTCTG-3' and Rev: 5'- GGCAGCGTGTACTTATCCTTCT-3;

MMP2 Fwd: 5'-TCGGAATGGGACAGACCTACT-3' and Rev: 5'- TCAAAGGGGTACATTGCTCC - 3';

MMP9 Fwd: 5'-AGACCTGGGCAGATTCCAAAC-3' and Rev: 5'- CGGCAAGTCTTCCGAGTAGT-3';

GAPDH Fwd: 5'-ACAACCTTTGGTATCGTGGAAGGA-3' and Rev: 5'-GCCATCACGCCAGTTTC-3

GAPDH was used as the positive control. The $2^{-\Delta\Delta Ct}$ method was adopted to calculate the mRNA expression.

Protein extracts and western blot analysis

Protein samples were obtained from cells and homogenized tissues. The concentration of protein was determined using Bradford (Bio-Rad, Hercules, CA, USA). Then, 30 μ g of protein per sample and transferred onto PVDF membrane (Millipore, Billerica, MA, USA). 5% non-fat milk was used to block membranes, and were incubated overnight at 4 °C with the primary antibodies. The following antibodies were used: KIFC1 (sc-9553), E-cadherin (sc-3195), N-Cadherin (sc-13116), Snail (sc-3437), ZEB1 (sc-3396), MMP2 (sc-9126) and MMP9 (sc-2867) were provided by Cell Signaling Technology (Danvers, MA). ERK (sc-4659), p-ERK (sc-4660), JNK (sc-4334), p-JNK (sc-4335) and GAPDH (sc-166) antibodies were offered by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ALDH2 (sc-KGAA3d5), SOX2 (sc-KGAA5d1), p38 (sc-KGAA253) was purchased from KeyGen Biotechnology (KeyGen, China). Then incubate HRP-conjugated secondary antibodies at room temperature for 1 hour.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 software. Data are represented in the form of means + standard deviation (S.D). Student's t-test was used to compare the two groups. Multiple-group statistical analyses were performed by one-way analysis of variance (ANOVA). P value < 0.05 is considered with statistical significance.

Results

TRAF1 expression is frequently up-regulated in both PTC tissues and cell lines

Before experiments, bioinformatics analysis was conducted regarding human PTC datasets that were publicly available from TCGA and GEO. KIFC1 mRNA expression presented an up-regulation in PTC relative to the adjacent tissues in TCGA and GSE3678 (Fig. 1A). Furthermore, the KIFC1 expression levels of fresh PTC tissue from our center by real-time PCR. Just as the dataset analysis, KIFC1 presented an obvious up-regulation in PTC tissue compared with normal thyroid tissues (Fig. 1B). Then, we tested the KIFC1 expression in cell lines including PTC cell lines (KTC-3, NPA87) and Nthy-ori 3 - 1 by real-time PCR and Western blot. As show in Fig. 1C, the mRNA and protein levels of KIFC1 in PTC cells was higher than those in Nthy-ori 3 - 1.

The correlation between KIFC1 expression and clinicopathological features

To explore the clinical relevance of KIFC1 in PTC, we acquired 86 PTC clinicopathological information of patients from our center. As show in Table 1, The group with a low expression has 45 patients and the group with a high expression has 41 patients. KIFC1 expression exhibited a close relation to Lymph node metastasis ($p = 0.035$) and extrathyroidal invasion ($p = 0.026$). However, KIFC1 expression was not remarkably related to other clinical characteristics such as age, gender, unilateral or bilateral. The above findings confirmed that KIFC1 expression may paly function role in PTC progression.

Table 1
The correlation between KIFC1 and the clinicopathological features in the validated cohort.

| Clinicopathologic variables | KIFC1 expression | | | P-value |
|--|------------------|------------|------------|---------|
| | n | Low (%) | High (%) | |
| All patients | 86 | 45 | 41 | |
| Age (years) | | | | 0.683 |
| < 50 | 41 | 24 (58.5) | 17 (41.5) | |
| ≥ 50 | 45 | 21 (46.7) | 24 (53.3) | |
| Gender | | | | 0.537 |
| Male | 38 | 18 (47.4) | 20 (52.6) | |
| Female | 48 | 27 (56.25) | 21 (43.75) | |
| Tumor size | | | | 0.264 |
| < 2 cm | 25 | 19 (76.0) | 6 (24.0) | |
| ≥ 2 cm | 61 | 26 (42.6) | 35 (57.4) | |
| Unilateral or Bilateral | | | | 0.424 |
| Unilateral | 50 | 27 (54.0) | 23 (46.0) | |
| Bilateral | 36 | 18 (50.0) | 18 (50.0) | |
| Lymph node metastasis | | | | 0.035 |
| Yes | 54 | 21 (38.9) | 33 (61.1) | |
| No | 32 | 24 (75.0) | 8 (25.0) | |
| Extrathyroidal invasion | | | | |
| Yes | 46 | 18 (39.1) | 28 (60.9) | 0.026 |
| NO | 40 | 27(67.5) | 13 (32.5) | |
| Statistical analyses were performed by chi-square (χ^2) test. | | | | |

Knockdown of KIFC1 attenuates proliferation of PTC cells

In order to counteract the phenotypic changes by the KIFC1 overexpression in PTC cell lines, KIFC1 shRNA was transfected into NPA87 and KTC-3 cells, respectively. We first performed Western blot and real-time PCR to examine KIFC1 expression in PTC cells. As the results show, the KIFC1 shRNA inhibited the protein and mRNA expression of KIFC1 dramatically both in NPA87 and KTC-3 cells, whereas the NC shRNA had no effects (Fig. 2A). To investigate the effects of KIFC1 on PTC cells proliferation, CCK-8 and colony formation assays were performed. The CCK-8 assay led to results that knockdown of KIFC1 inhibits proliferation when compared to the shNC cells (Fig. 2B). Meanwhile, the colony formation assay made the results more convincing thanks to the same conclusions (Fig. 2C). These data demonstrated that KIFC1 expression is positively related to PTC cell proliferation and viability.

Knockdown of KIFC1 inhibits migration and invasion of PTC cells

We further investigated the biological role of KIFC1 in PTC cells. Migration and invasion assays were performed to determine whether the expression of KIFC1 was associated with the metastasis of NPA87 and KTC-3 cells. As the results show, knockdown of KIFC1 significantly inhibited the ability of invasion and migration of both NPA87 and KTC-3 cells (Fig. 3A). Based on the wound healing assay, the cell migration capacity of PTC cells was downregulated by KIFC1 silencing (Fig. 3B). Taken together, knockdown of KIFC1 by shRNA retarded PTC cells motility activity.

KIFC1 alters differentiation and stemness-related markers of PTC cells

We evaluated the effects of KIFC1 shRNA on cell differentiation markers retyroglobulin gene (Tg) and sodium/iodide symporter gene (Nis). We perform luciferase reporter assays that contain the promoter region of Nis (pNis 2.8) and Tg (pGI-Tg) genes promoter region in the upstream of luciferase gene (Fig. 4A). Moreover, our results showed that the expression level of mRNA of stemness markers ALDH2 and SOX2 (Fig. 4B, C) was increased by KIFC1 shRNA transfection in NPA87 cells.

Knockdown of KIFC1 suppresses the EMT process through MAPK/ERK signaling pathway

To gain a restored understanding of the effect of KIFC1 on migration and invasive ability exhibited by PTC cells, we further investigate the relationship between KIFC1 and EMT related transcription factors. As show in Fig. 5A, Western blot confirmed that the levels of N-cadherin, Snail and ZEB1 were markedly decreased, whereas E-cadherin was increased in PTC cells by KIFC1 silencing. Considering that MAPK/ERK pathway is one of the crucial signal pathways in tumor metastasis, which is involved in EMT. Accordingly, whether KIFC1 could inhibit PTC cells metastasis by regulating EMT through MAPK/ERK

pathway. Indeed, we found that knockdown of KIFC1 resulted in a reduction of p38, p-ERK, ERK, p-JNK and JNK expression in NPA87 cells (Fig. 4B). Additionally, real-time PCR analyses revealed a dramatical inhibition of MMP2 and MMP9 expression via MAPK/ERK downstream signaling when blocking KIFC1 in PTC cells (Fig. 4C). These suggested that knockdown of KIFC1 expression might be the key regulatory element in PTC development.

Discussion

Accumulating evidence demonstrated the up-regulation of KIFC1 expression in some malignant tissues, while KIFC1 is lowly or not expressed in human normal tissues [19, 20]. Xiao et al revealed that KIFC1 contributes to bladder cancer cell EMT and metastasis via AKT/GSK38 signaling [21]. Our earlier study showed that KIFC1 is up-regulated in hepatic cellular cancer (HCC), which was related to the progression of HCC metastatic both in vivo and vitro [22]. However, the role of KIFC1 and underlying mechanisms have not been comprehensively illuminated, especially regarding its proliferation and metastasis in PTC. Herein, we established that KIFC1 is a novel carcinogenic molecule, which could represent a suitable therapeutic target for PTC.

The bioinformatics analysis was firstly conducted on publicly available TCGA and GEO datasets to determine the KIFC1 mRNA expression [22, 23]. Findings were validated using clinical PTC specimens. The results indicated that the mRNA level of KIFC1 is overexpressed, which shows a consistence with the KIFC1 expression in other types of tumors. Next, the study focused on investigating the association between KIFC1 tissues and clinicopathological features from our PTC tissues. It's obviously that KIFC1 expression can cause lymph node metastasis and extrathyroidal invasion. Similarly, high levels of KIFC1 was closely correlated with the lymph positive node metastasis and poor survival prognosis in renal cell carcinoma (RCC) [24]. Moreover, we also caught sight of the high expression of KIFC1 in PTC cell lines compared with Nthy-ori 3 – 1. In line with these findings, the oncogenic role of KIFC1 has been confirmed in PTC, thus targeting that it may be beneficial.

In our study, KIFC1 silencing significantly decreased the protein and mRNA expression of KIFC. Based on the previous studies, KIFC1 silencing resulted in a dramatically inhibited tumor cell proliferation [25]. KIFC1 overexpression enhanced the cell proliferation of hepatocellular carcinoma [26]. The same principles applied to our result, which showed that knockdown of KIFC1 clearly inhibited proliferation of PTC cells. A significant next step is to determine whether KIFC1 overexpression is critical factor of PTC aggressiveness. Some studies have indicated that KIFC1 is a novel prognostic biomarker for ovarian cancer [27]. However, the role of KIFC1 in PTC cell metastasis have not been previously reported. Here, KIFC1 expression depletion suppressed the migration and invasive ability of PTC cells. Moreover, according to the wound healing assay, KIFC1 knockdown obviously suppressed metastasis of PTC cells. These findings are reminiscent of data observed in breast cancer cells, which further corroborating the concept that KIFC1 is a novel regulator in various cancer cells [28, 29]. The counterpart expression of specific thyroid genes Nis and Tg correlates inversely with cell differentiation and malignant phenotype [30, 31]. Thus, we evaluated whether knockdown of KIFC1 is associated with PTC cell differentiation.

Indeed, knockdown of KIFC1 increased expression of Nis and Tg. As expected, there was a decrease in the expression level exhibited by stemness markers ALDH2 and SOX2 following KIFC1 silencing. From the above, the results revealed that knockdown of KIFC1 suppressed the proliferation, migration and invasion of PTC cell lines. Therefore, a better understanding of the mechanisms by which KIFC1 modulates PTC is required.

EMT is characterized by switch E-cadherin to N-cadherin and Snail, thus greatly affecting the tumor metastasis [32, 33, 34]. As expected, we found that the knockdown of KIFC1 increased the expression of an E-cadherin, inhibited expression levels of EMT-related transcription factors N-cadherin, Snail and ZEB1, suggesting that KIFC1 is involved in the metastasis of PTC. This finding made a strong evidence for studying whether a more direct causal relationship between KIFC1 and PTC metastasis. The MAPK pathway includes the signaling protein kinases RET, ERK, JNK and MEK, participating in the control of cell proliferation, differentiation and metastasis [35, 36]. Some studies have addressed the result that MAPK signaling increase the levels of EMT-related transcription factors, whereas the decrease in E-cadherin expression can cause EMT [37, 38]. On that account, Western blot analysis assess the MAPK signaling cascades and downstream signaling to reveal metastasis mechanism of PTC. As revealed in the study, KIFC1 knockdown reduced the protein expression of ERK and JNK of PTC cells. We also confirmed that the phosphorylation level of p-ERK and p-JNK was strongly deregulated through KIFC1 silencing in PTC cells. In addition, we investigate the role of KIFC1 in regulating the level of MMP family proteins MMP2 and MMP9. Previous studies have shown that inhibition of the production and downregulation of MMP2 and MMP9 in colorectal cancer can play an anti-metastasis role [39]. We revealed that knockdown of KIFC1 resulted in a reduction of MMP2 and MMP9 of PTC cells, which agrees the results of previous studies [40, 41]. Collectively, these results suggest that knockdown of KIFC1 by shRNA transfection could suppress the ability of migration and invasion by inhibiting MAPK signaling in PTC cells.

Conclusion

In summary, this is the first study to investigate the relationship between KIFC1 and PTC. Our finding confirmed an up-regulation expression both in human PTC tissues together with metastasis and extrathyroidal invasion. More importantly, we also revealed that knockdown of KIFC1 alters the PTC cellular responses, including proliferation, differentiation and metastasis. Inhibition of KIFC1 downregulated the levels of EMT-related transcription factors through the MAPK signaling. These consequences provided a new perspective on KIFC1, which act as oncogenes in PTC tumorigenesis. Thus, therapeutic approaches designed to inhibit KIFC1 function might represent an attractive PTC therapeutic strategy.

Declarations

Ethics approval and consent to participate

The experiment was conducted in accordance with the Declaration of Helsinki. The study was approved by the Research Ethics Committees of Harbin Medical University Cancer Hospital. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable

Availability of data and material

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

Competing of interests

No conflicts of interest exist in the paper.

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Figures

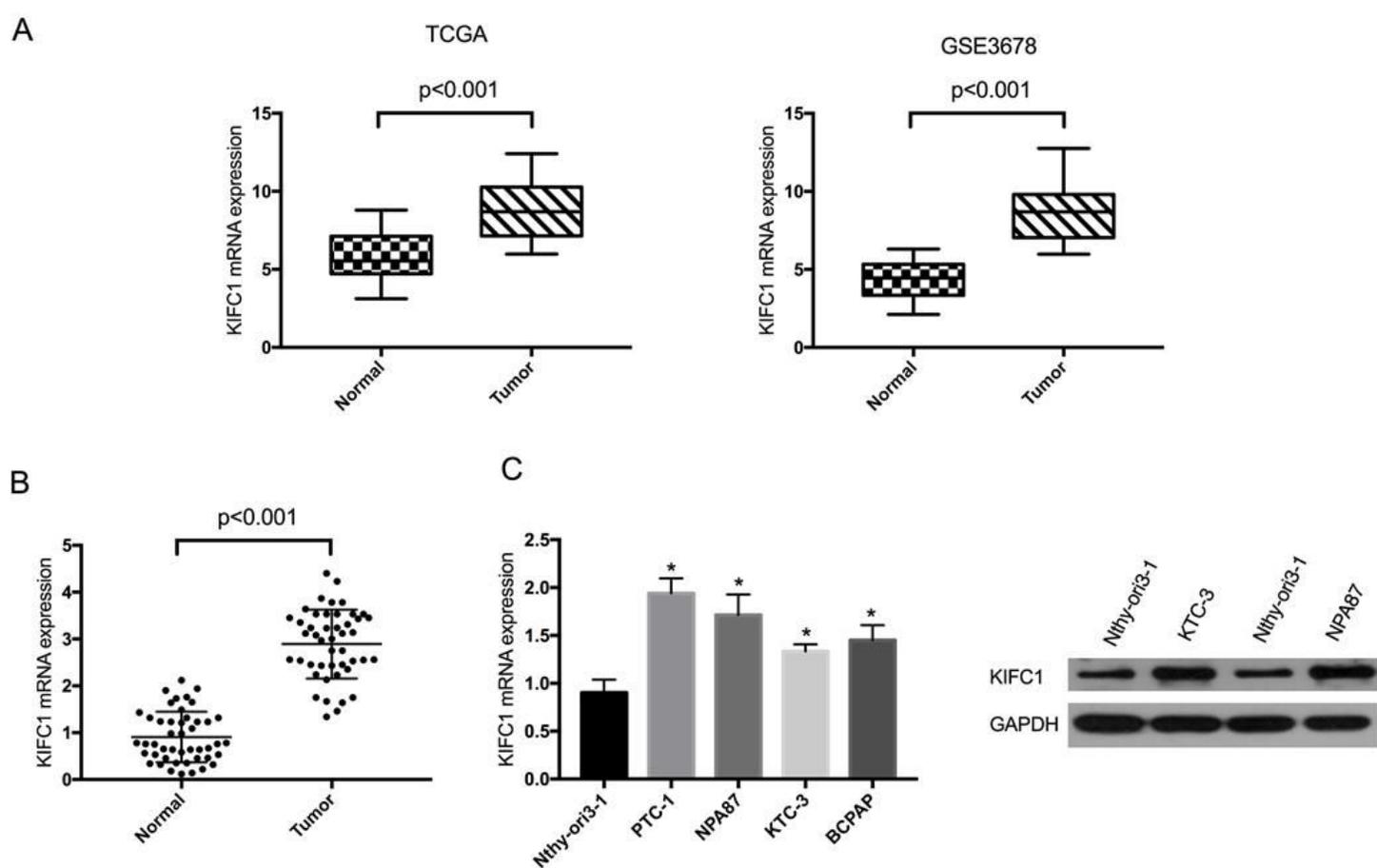


Figure 1

KIFC1 was overexpressed in PTC clinical specimen and cell lines. A. KIFC1 expression level of PTC tissues in TCGA and GEO datasets (GSE3678). B. mRNA expression levels of KIFC1 were detected by real-time PCR in 46 pairs of PTC tissues and the adjacent normal specimens. C. Western blot and qRT-PCR analyses showed that KIFC1 expression level was upregulated in PTC cell lines. Data represent mean \pm SD of three independent experiments. *P < 0.05 vs the shNC group.

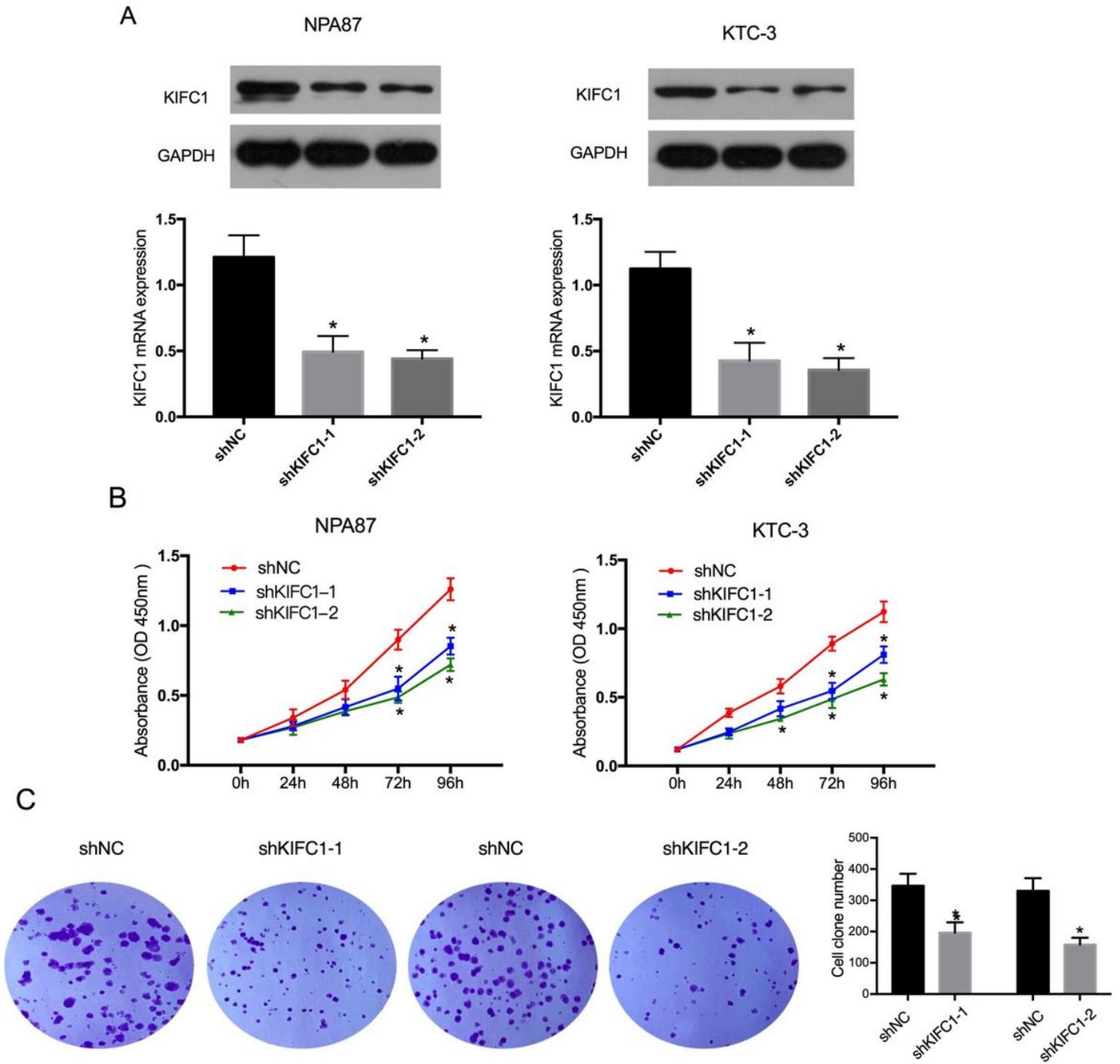


Figure 2

Knockdown of KIFC1 inhibits the proliferation of PTC cells. A. Western blot and real-time PCR analysis of KIFC1 expression in PTC cells. B. Growth curves obtained in proliferation (CCK-8) assays in PTC cells. C. The proliferation ability of PTC cells was determined by colony forming assays; The histogram showed the cell clone number. Data represent mean \pm SD of three independent experiments. * $P < 0.05$ vs the shNC group.

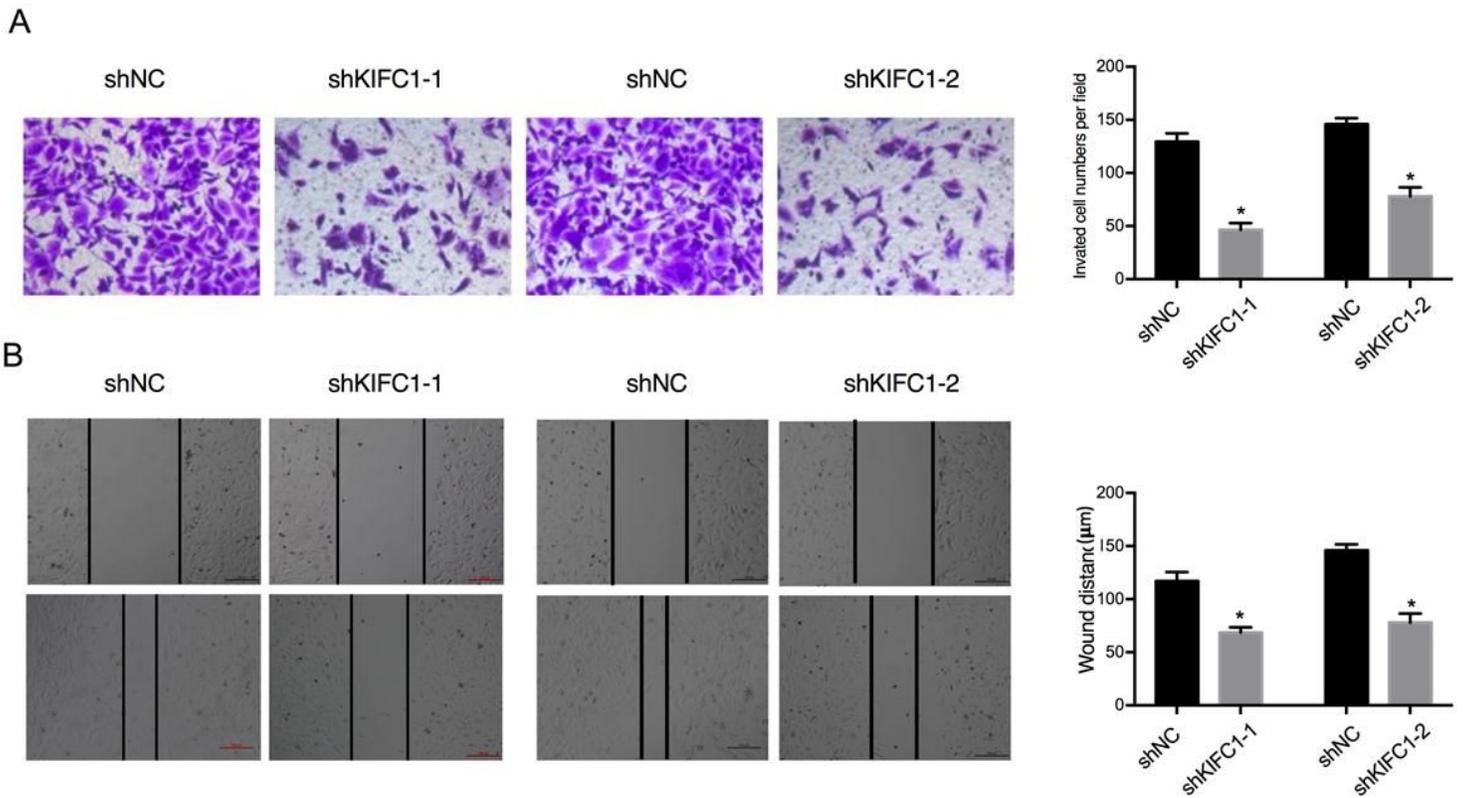


Figure 3

Knockdown of KIFC1 represses invasion and metastasis of PTC cells. A. Transwell migration of PTC cells was analyzed in transwell invasion assays; Counts of migrated and invaded PTC cells are shown in the right panel. B. Cell wound-healing assay revealed that knockdown of KIFC1 led to decreased of motility capability of PTC cells; The histogram showed the wound distance (μm). Data represent mean \pm SD of three independent experiments. * $P < 0.05$ vs the shNC group.

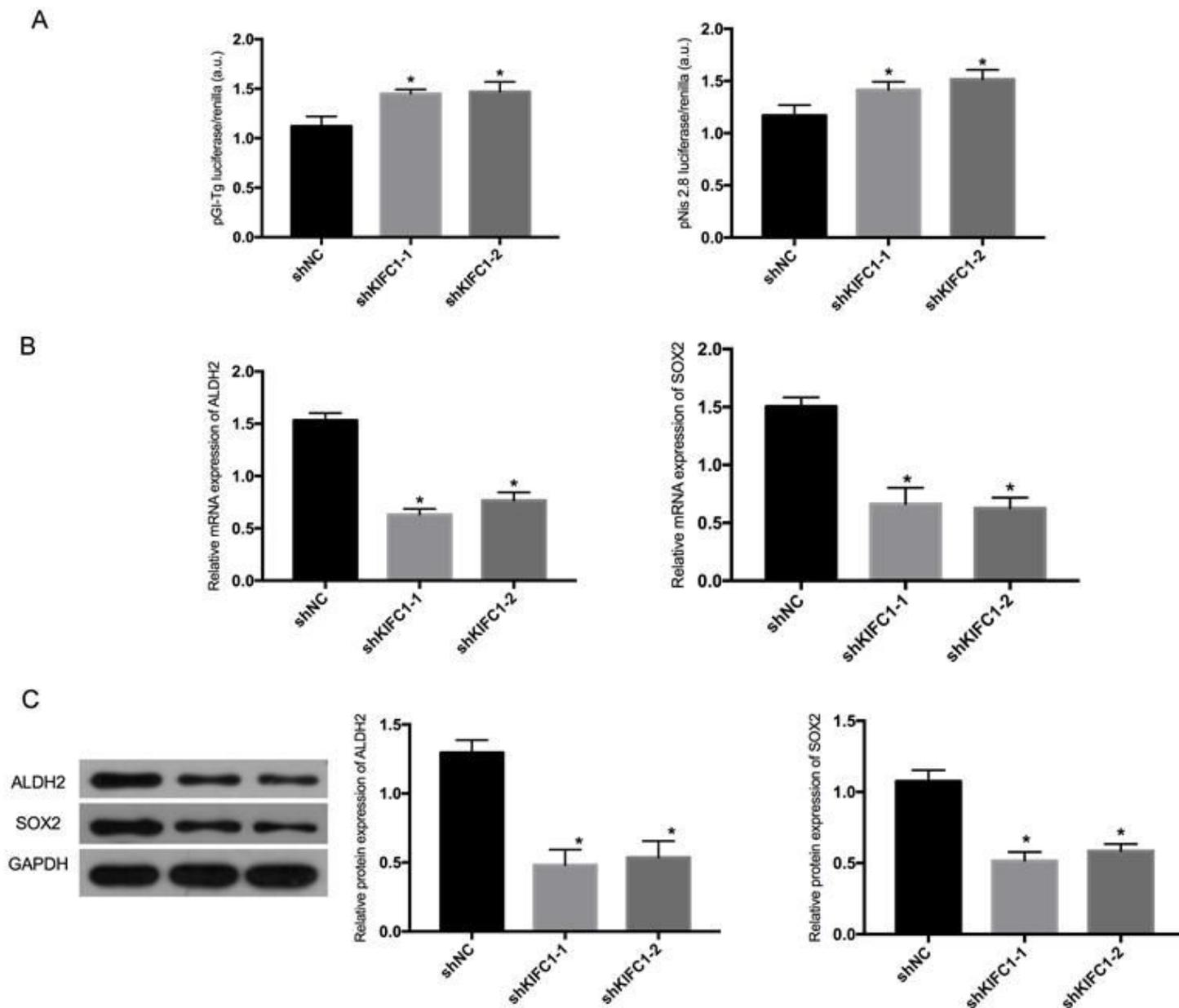


Figure 4

Knockdown of KIFC1 effects on thyroid differentiation and stemness markers of PTC cells. A. Transcriptional activity of Nis and Tg after transfection with pNis 2.8 luciferase plasmid and pGI-Tg luciferase plasmid was determined by Luciferase Reporter Assays. B. Relative mRNA expression of stemness markers ALDH2 and SOX2 analyzed by real-time PCR in PTC cells. C. Western blot analysis showed the protein expression of ALDH2 and SOX2. Data represent mean \pm SD of three independent experiments. * $P < 0.05$ vs the shNC group.

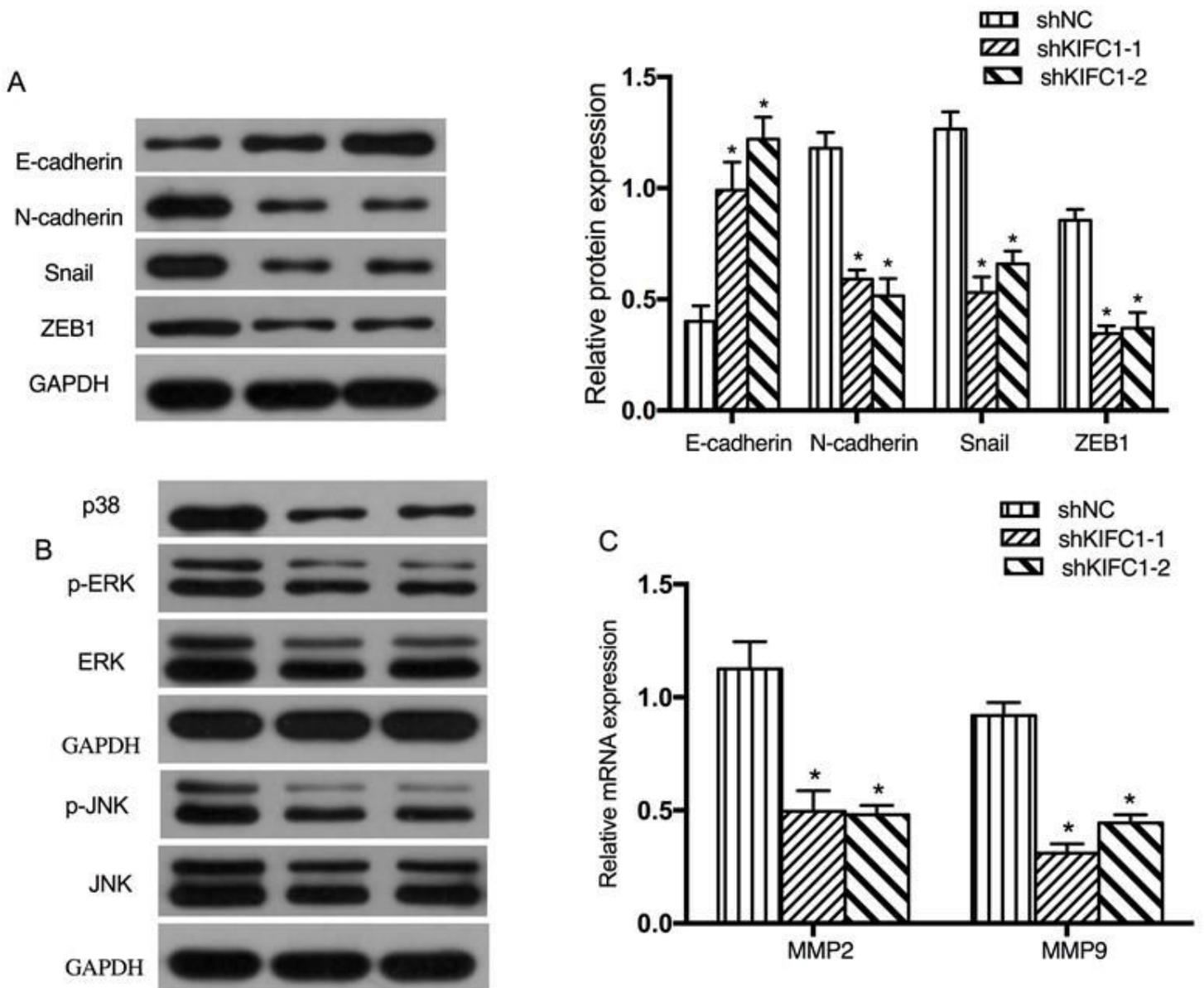


Figure 5

Knockdown of KIFC1 suppressed EMT progression through the MAPK signaling in PTC cells. A. Protein expression levels of N-cadherin, E-cadherin, Snail and ZEB1 were evaluated by Western blot. B. Protein expression levels of p-ERK, ERK, p-JNK and JNK were evaluated by Western blot. C. Relative mRNA expression of MMP2 and MMP9 was analyzed using real-time PCR. Data represent mean \pm SD of three independent experiments. * $P < 0.05$ vs the shNC group.