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Original Research Article

Honokiol inhibits gallbladder cancer proliferation and epithelial mesenchymal transformation by suppressing TMPRSS4-induced PI3K/AKT activation

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Abstract

Purpose: *To determine the role of TMPRSS4 in gallbladder cancer (GBC).*
Methods: *Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) and western blotting were used to evaluate the expression of genes, while CCK-8 kit and foci formation assay were used to assess cell growth in GBC-SD and non-obliterative zone (NOZ) cell lines. Cell apoptosis was evaluated by flow cytometry, while cell migration and cell invasion were determined by Transwell assay in GBC-SD and NOZ cell lines.*
Results: *TMPRSS4 was highly expressed in GBC cells, compared to normal cell, HIBEC. Overexpression of TMPRSS4 enhanced cell viability and 2D foci formation in GBC-SD (stratified) and NOZ; however, knockdown of TMPRSS4 reduced cell proliferation and colony formation in the cell lines ($p < 0.05$). TMPRSS4 deficiency increased cell apoptosis but its reinforced expression decreased cell apoptosis in GBC cell lines ($p < 0.05$). Moreover, TMPRSS4 positively regulated cell invasion and migration in GBC cells by upregulating TWIST1, vimentin and N-cadherin. TMPRSS4 also regulated the activation of PI3K/AKT signal pathway. Furthermore, honokiol inhibited gallbladder cancer proliferation and migration, and induced cell apoptosis by suppressing TMPRSS4-induced PI3K/AKT activation ($p < 0.05$).*
Conclusion: *TMPRSS4 plays an important role in regulating cell growth, apoptosis and metastasis of gallbladder cancer cells (GBC). Thus, TMPRSS4 might be a new biomarker in gallbladder cancer.*

Keywords: *Transmembrane Protease Serine 4, Gallbladder cancer, PI3K/AKT, Cell proliferation, Honokiol, Epithelial-mesenchymal transition*

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INTRODUCTION

Gallbladder cancer (GBC) is a relatively infrequent cancer that develops from the biliary tract and is extremely aggressive [1]. Almost all cases of GBC are diagnosed at a late stage due to the lack of early symptoms. To date, adjuvant therapy, including chemotherapy and radiotherapy is an indispensable option for the treatment of most GBC patients. However, the efficacy of these traditional therapies is short-lived, as most patients relapse rapidly with concomitant chemo- and radiotherapy resistance [2]. Hence, the overall prognosis of GBC is very

poor, with an average survival duration of 13.2 – 19.0 months [3]. Therefore, there is an urgent need for progress in GBC pathogenesis research to pave the way for new therapeutic approaches as well as discovery of new biomarkers for early diagnosis.

TMPRSS4 is currently known to be involved in two functions: embryonic development and cancer [4]. It localizes to the cell membrane, participating in regulating signal transduction between cells and their surroundings. It also increases The expression of Heparin-Binding EGF-like Growth Factor (HB-EGF) and the cleavage of its proteolytic form play a significant role in inducing angiogenesis in hepatocellular carcinoma [5]. Studies have shown that TMPRSS4 is highly expressed in a variety of solid malignant tumors, including pancreatic cancer, colorectal cancer, lung cancer, cervical cancer, and gallbladder cancer [6].

Twist1 belongs to the helix-loop-helix essential transcription factor family, and is one of the major transcription factors that induce EMT, cell migration and invasion in embryonic development and cancer cells. Reinforcement of Twist1 increases EMT and cancer stem-like cell properties in breast cancer cells [7]. TMPRSS4 upregulates the activation of STAT3 and expression of TWIST1, thus exacerbating prostate cancer migration [8]. Whether TMPRSS4 affects the migration and invasiveness of gallbladder cancer by regulating TWIST1 is not yet known.

EXPERIMENTAL

Cell lines and cell transfection

GBC-SD, HIBEC, and NOZ cells were purchased from the Academy of China (Shanghai, China). All cell lines cultured in DMEM medium were supplemented with 10 % FBS, 100U penicillin and streptomycin. The cell lines were consistently maintained under standard conditions preceding the commencement of the studies [9]. All experimental manipulations were performed as previously depicted [10]. For cell transfection, the indicated plasmids or siTMPRSS4 were transfected with lipofetamine2000 reagent following the instruction of the reagent manufacturer.

RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Ambion, CA, USA), then reverse-transcribed. The cDNA was used as template to measure TMPRSS4 expression at mRNA level. Gene expression levels were analyzed using the delta Ct method and normalized against β-actin. The following primers were used to evaluate related gene mRNA expression levels.

Table 1: Primers used in PCR

Gene	Sequence
TMPRSS4 forward	5'-TCCAAGGACCGATCCACACT-3'
TMPRSS4 reverse	5'-GTGAAGTTGTCGAAACAGGCA-3'
β-actin forward	5'-CATGTACGTTGCTATCCAGGC-3'
β-actin reverse	5'-CTCCTTAATGTCACGCACGAT-3'

Western blotting analysis

Western blotting was performed as previously reported [11], and immunoblotted with the following antibodies: TMPRSS4 (1:1000, Santa, sc-376415, USA), Bax (1:1000, Santa, sc-20067, USA), BCL-2 (1:1000, Santa, sc-73822, USA), p-AKT (1:1000, Santa, sc-377556, USA), AKT (1:1000, Santa, sc-5298, USA), E-cadherin (1:1000, Santa, sc-8426, USA), anti-mouse N-cadherin (1:1000, Santa, sc-8424, USA), TWIST1 (1:1000, Santa, sc-81417, USA), vimentin (1:1000, Santa, sc-6260, USA), ZO-1(1:1000, Santa, sc-33725, USA), and β-actin (1:1000, Santa, sc-8432, USA) which were purchased from Santa Cruze. PI3K (1:1000, ab191606, England) and p-PI3K (1:1000, ab182651, England) were purchased from Abcam, England.

Cell proliferation and 2D foci formation assays

Cell growth assessment was carried out as previously described [11], using 1×10^3 cells seeded in a 96-well plate in triplicate. The absorbance of these cells was used to determine cell growth rate with the aid of CCK-8 assay kit (Keygenbio, Nanjing, China). For the colony formation assay, 5×10^2 cells were seeded in a 60 mm dish to form 2D colonies. After 2 weeks, the colonies were stained, photographed, scored and counted.

Evaluation of cell apoptosis

For this determination, 2.5×10^5 cells were seeded into 6-well plates, then digested with trypsin, and collected, and then fixed with 70 % alcohol. After staining with PI and FITC, they were analyzed by flow cytometry.

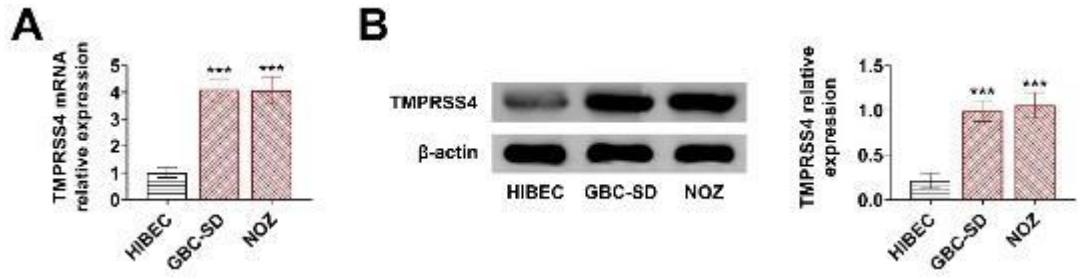

Figure 1: TMPRSS4 is upregulated expression in gallbladder cancer cells. (A) The mRNA of TMPRSS4 in GBC cells and normal biliary epithelial cell line; (B & C) Expression of TMPRSS4 in HIBEC, GBC-SD and NOZ cell. Error bars represent data from three independent experiments, mean ± SD. ***P < 0.001

Determination of cell migration and invasion

Transwell assay was used to conduct the migration and invasion of GBC cell lines. A quantity of the cells, 2.5 × 10^4 cells, were seeded into the upper chamber of the Transwell assay set-up, and after 24 h, cells attached to the filter were fixed and stained. Cell migration and invasion levels were measured as previously reported [11].

Statistical analysis

Student's t test was performed using SPSS 22.0 for Windows. Data are presented as mean ± SEM of three independent experiments. P < 0.05 considered statistically significant.

RESULTS

TMPRSS4 was upregulated in gallbladder cancer cells

The results are shown in Figure 1. TMPRSS4 was highly expressed in GBC-SD and NOZ compared with HIBEC cells (Figure 1 A). Furthermore, TMPRSS4 protein was also overexpressed in GBC-SD and NOZ when compared with HIBEC cells (Figure 1 B).

TMPRSS4 promotes the proliferation of gallbladder cancer cells

As shown in Figure 2 A, TMPRSS4-overexpression or -deficient cell lines were successfully modelled in gallbladder cancer cells with the control group (Figure 2 B), while the knockdown of TMPRSS4 decreased cell proliferation in GBC cell lines compared to negative control cells (Figure 2 B). Furthermore, TMPRSS4 upregulation facilitated foci formation in GBC cell lines compared with the control group. However, TMPRSS4 deficiency suppressed colony formation in GBC cell lines when compared with control group (Figure 3). Taken together, therefore, TMPRSS4 positively regulated cell growth in gallbladder cancer cells.

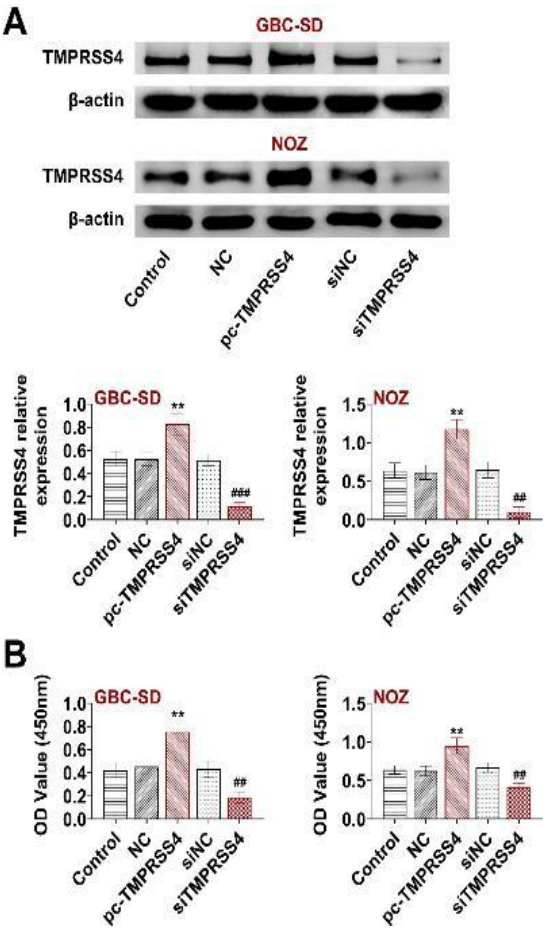

Figure 2: TMPRSS4 enhances the proliferation of gallbladder cancer cells. (A) GBC-SD and NOZ cells were transfected with pc-TMPRSS4, siTMPRSS4, and their respective control plasmids; the expression of TMPRSS4 was assessed by western blotting. (B) Cell viability of TMPRSS4-overexpressing or knockdown GBC-SD and NOZ cells. Error bars denote data from three independent experiments, presented as mean ± SD. **P < 0.01, ##p < 0.01; * indicates comparison with the negative control group, # indicates comparison with the siNC group

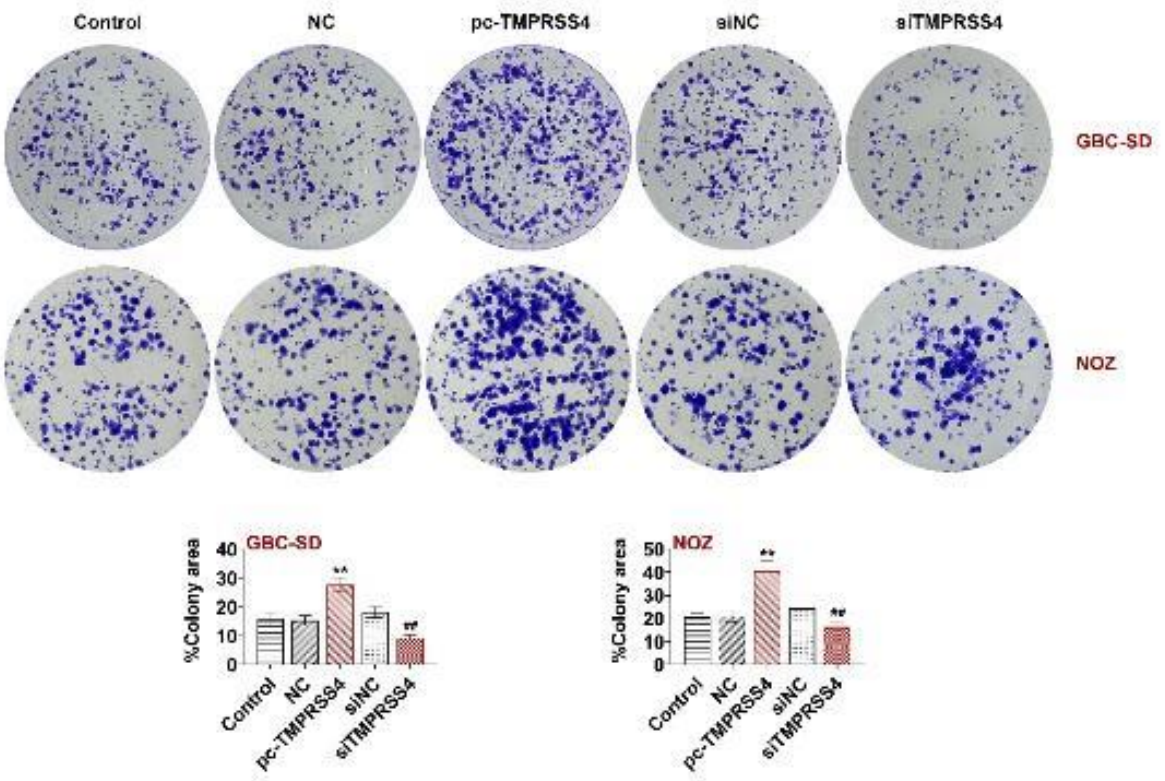

Figure 2: TMPRSS4 enhances the proliferation of gallbladder cancer cells. Cell proliferation of TMPRSS4 knockdown/overexpression GBC-SD and NOZ cell lines. Error bars represent data from three independent experiments, mean ± SD. ***P < 0.01, ##p < 0.01, *compared with negative control group, #compared with siNC group

TMPRSS4 inhibits apoptosis of gallbladder cancer cells

As shown in Figure 4, Bax was suppressed while BCL-2 was increased in TMPRSS4 overexpression of GBC-SD and NOZ cell lines. In contrast, knockdown of TMPRSS4 enhanced the expression of Bax and inhibited BCL-2 expression in GBC cell lines. In addition, TMPRSS4 overexpression reduced cell apoptosis, whereas TMPRSS4 deficiency increased cell apoptosis in GBC-SD and NOZ cell lines when compared with control group (Figure 5).

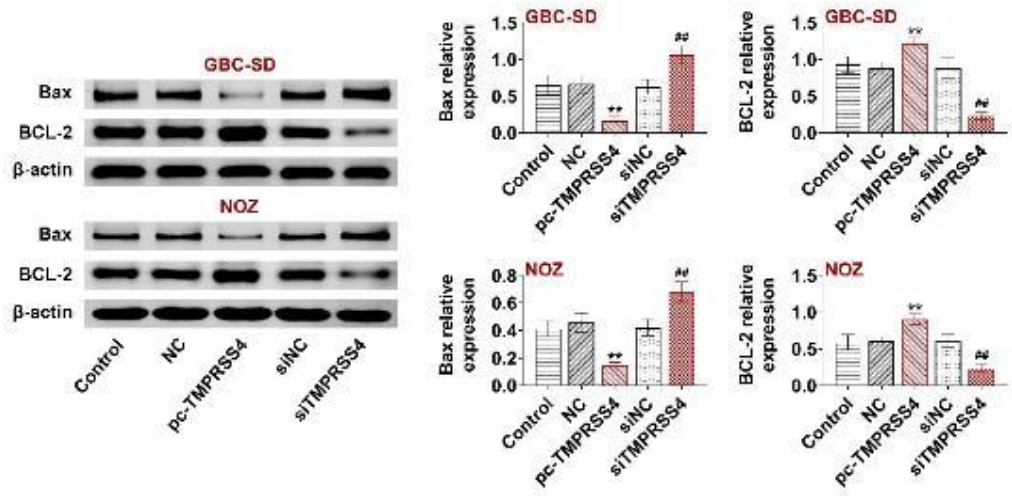

Figure 4: TMPRSS4 impedes the apoptosis of gallbladder cancer cells. The expression levels of Bax and BCL-2 in TMPRSS4 knockdown/overexpression GBC-SD and NOZ cell lines are depicted. Error bars denote data from three independent experiments, presented as mean ± SD. **P < 0.01, ##p < 0.01; *compared with negative control group, #compared with siNC group

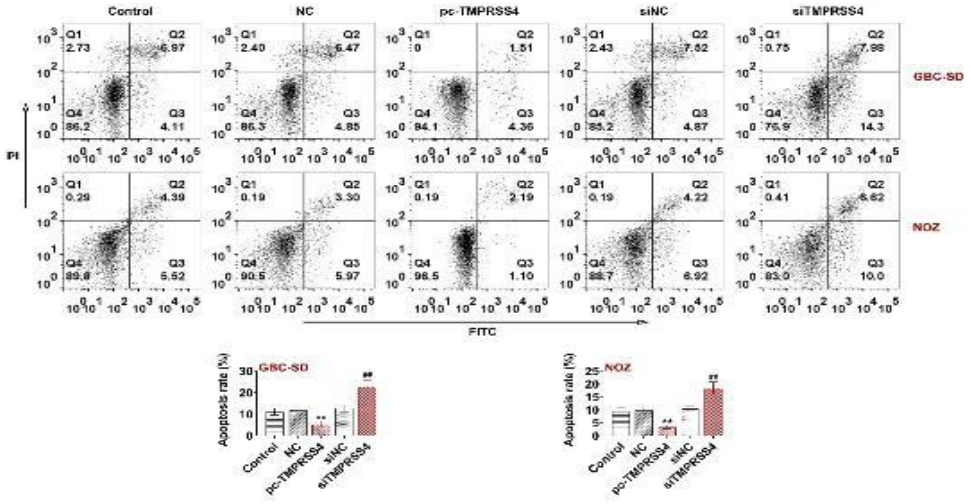

Figure 5: TMPRSS4 impedes the apoptosis of gallbladder cancer cells. The impact on cell apoptosis in TMPRSS4 knockdown/overexpression GBC-SD and NOZ cell lines was measured by flow cytometry. Error bars represent data from three independent experiments, mean ± SD. ***P* < 0.01, ##*p* < 0.01, *compared with negative control group, #compared with siNC group

TMPRSS4 increased the migration, invasion and mesenchymalization of gallbladder cancer cells by upregulating TWIST1

TMPRSS4 overexpression increased cell migration and invasion in GBC-SD and NOZ cell lines when compared with the control group. TMPRSS4 deficiency exerted an opposite effect (Figure 6). The results show that Twist, Vimentin, and N-cadherin were upregulated by TMPRSS4 overexpression but suppressed by TMPRSS4 inhibition. E-cadherin and ZO-1 were inhibited in GBC-SD and NOZ cells by TMPRSS4 overexpression. The knockdown of TMPRSS4 decreased the expression of Twist, Vimentin, and N-cadherin, but enhanced the expression of E-cadherin and ZO-1 in GBC-SD and NOZ cell lines (Figure 7). Thus, TMPRSS4 positively regulated cell migration and invasion via upregulation of TWIST.

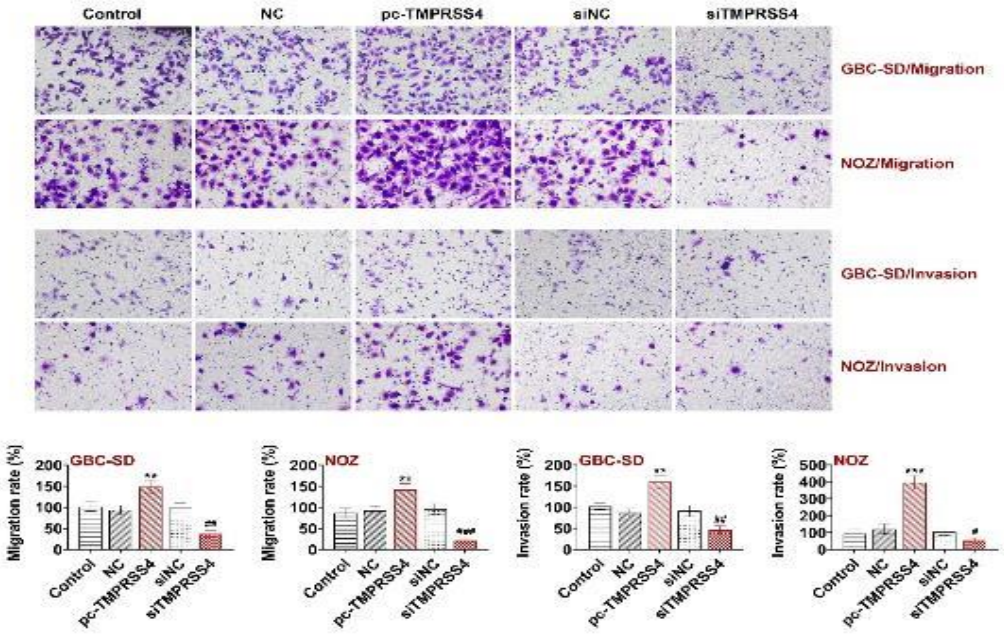

Figure 6: TMPRSS4 promotes the migration, invasion and mesenchymalization of gallbladder cancer cells by upregulating TWIST1. Cell migration in TMPRSS4 knockdown/over-expression GBC-SD and NOZ cell lines. Error bars represent data from three independent experiments (mean ± SD). ***P* < 0.01, ##*p* < 0.01 *compared with negative control group, #compared with siNC group

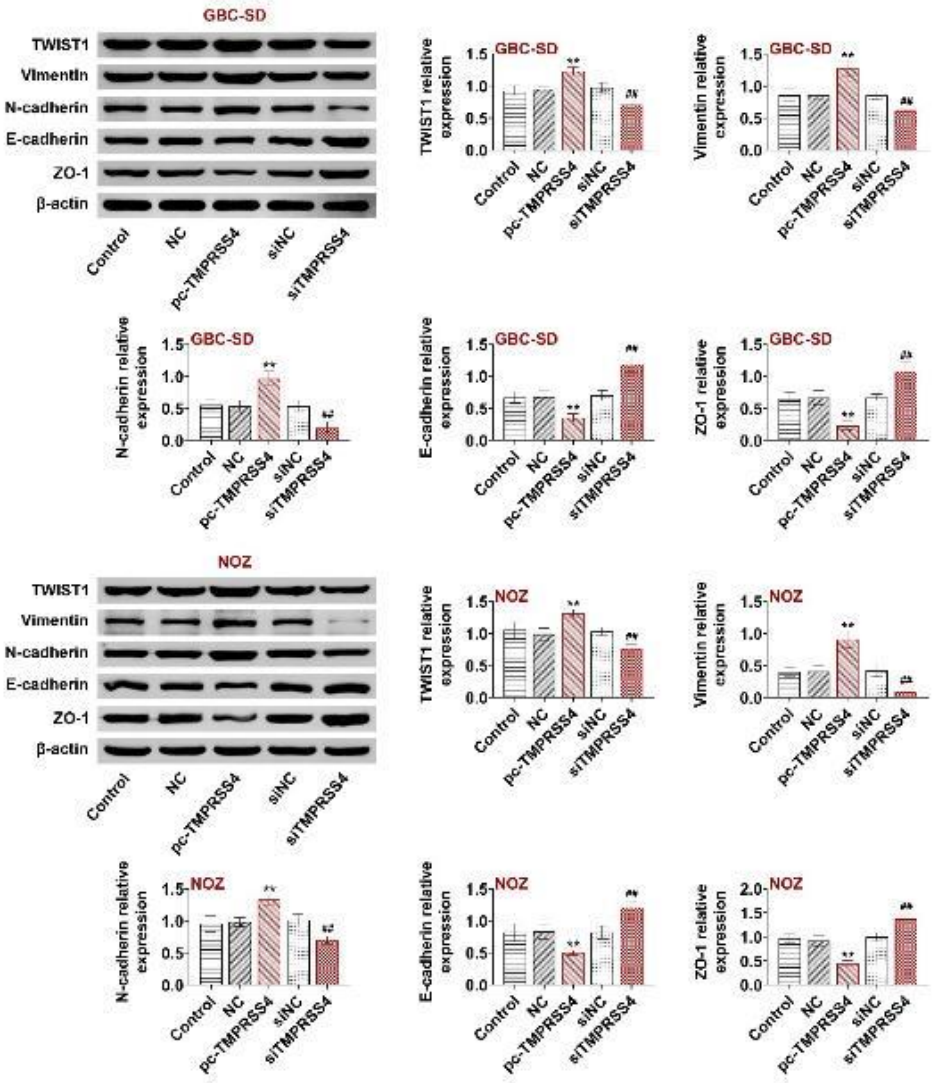

Figure 7: TMPRSS4 promotes the migration, invasion and mesenchymalization of gallbladder cancer cells by upregulating TWIST1. Expression of EMT related proteins (TWIST1, Vimentin, N-cadherin, E-cadherin and ZO-1) in TMPRSS4 knockdown/over-expression GBC-SD and NOZ cell lines. Error bars represent data from three independent experiments, mean ± SD. ***P* < 0.01, $^{\#\#}p$ < 0.01. *compared with negative control group, #compared with siNC group

TMPRSS4 activates PI3K/AKT signaling

The effect of TMPRSS4 on the activation of PI3K/AKT signaling was also investigated. As shown in Figure 8, both p-AKT and p-PI3K showed increased expressions in GBC-SD and NOZ cells, following TMPRSS4 upregulation. In contrast, knockdown of TMPRSS4 reduced the expression of p-AKT and p-PI3K in GBC cell lines.

Honokiol inhibits gallbladder cancer proliferation and migration, and induces cell apoptosis

Different doses of HNK was used to treat GBC-SD and NOZ cell line, as shown in Figure 9 A, cell growth was significantly inhibited by 15 and 30 μM HNK. Similarly, cell colony formation was also suppressed dose-dependently by HNK in GBC-SD and NOZ cell line (Figure 9 B). Moreover, cell apoptosis was increased by HNK treatment (Figure 9 C). Furthermore, HNK treatment reduced cell migration and invasion in GBC-SD and NOZ cell lines when compared with the control group (Figure 10). In addition, expression of TMPRSS4 was decreased by HNK treatment in GBC-SD and NOZ cells, while both p-AKT and p-PI3K expressions were inhibited in GBC-SD and NOZ cells(Figure 9 C and Figure 11).

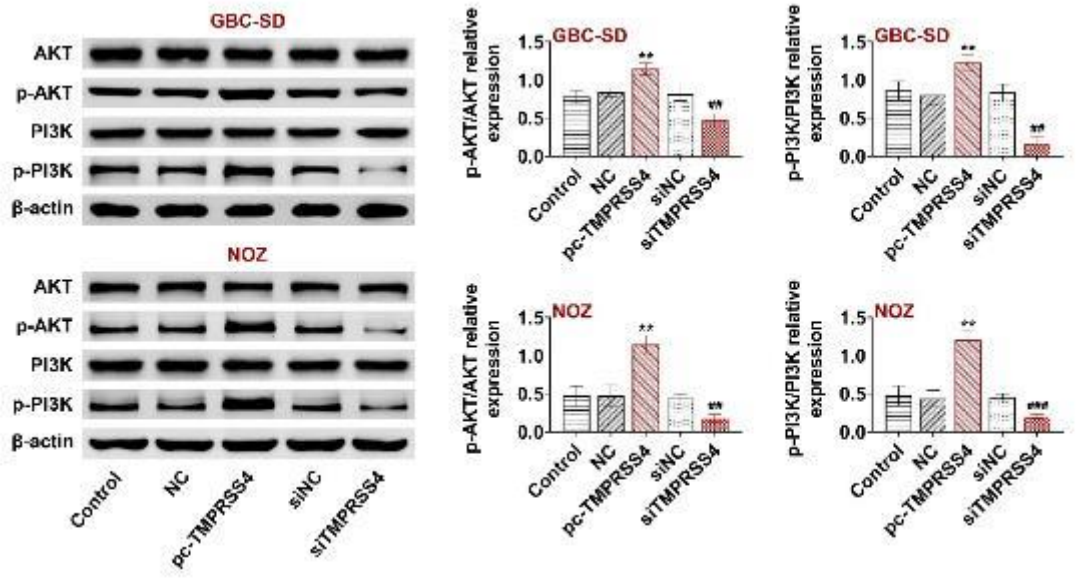

Figure 8: TMPRSS4 activates PI3K/AKT pathway signaling. Error bars represent data from three independent experiments, mean ± SD. ***P* < 0.01, ##*p* < 0.01 *compared with negative control group, #compared with siNC group

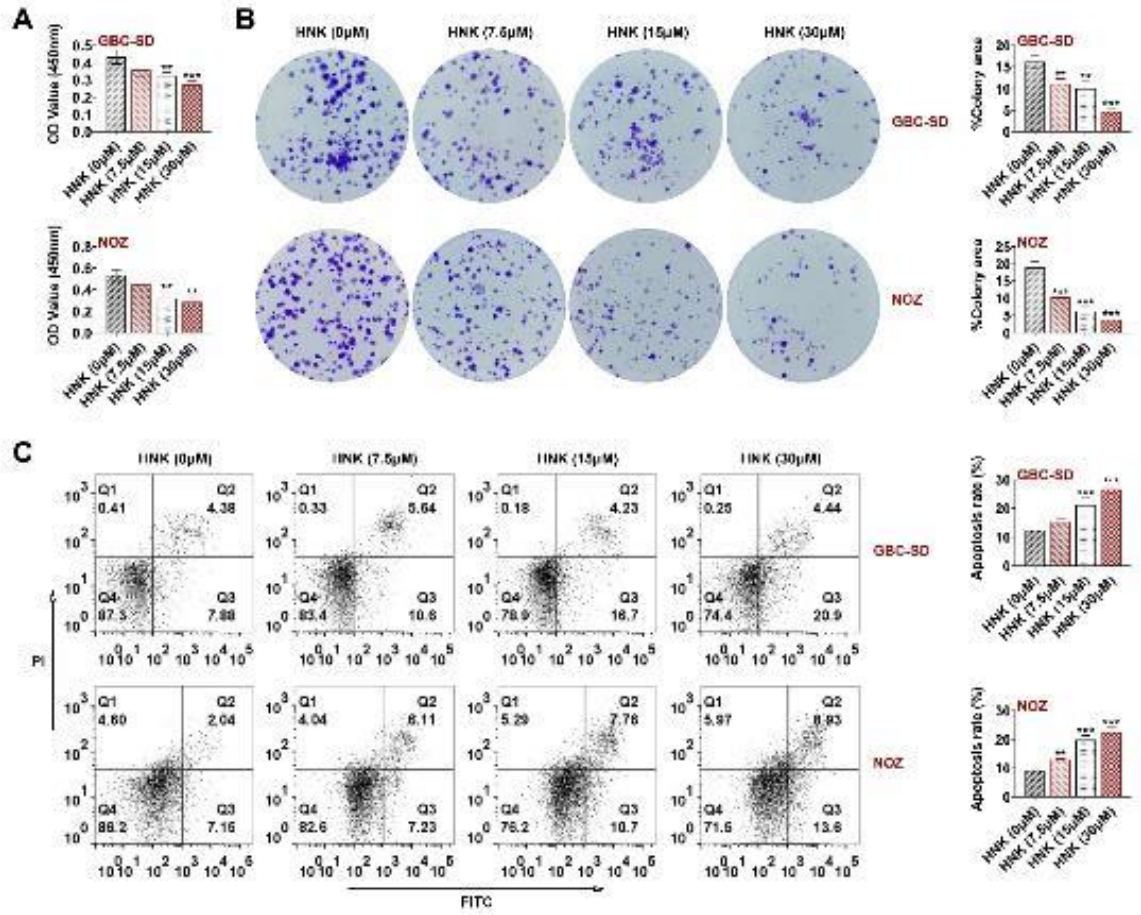

Figure 9: Honokiol inhibits the proliferation and migration of gallbladder cancer cells and promotes apoptosis. Error bars represent data from three independent experiments, presented as mean ± standard deviation (SD). ***P* < 0.01, * represents the treatment group compared to the control group

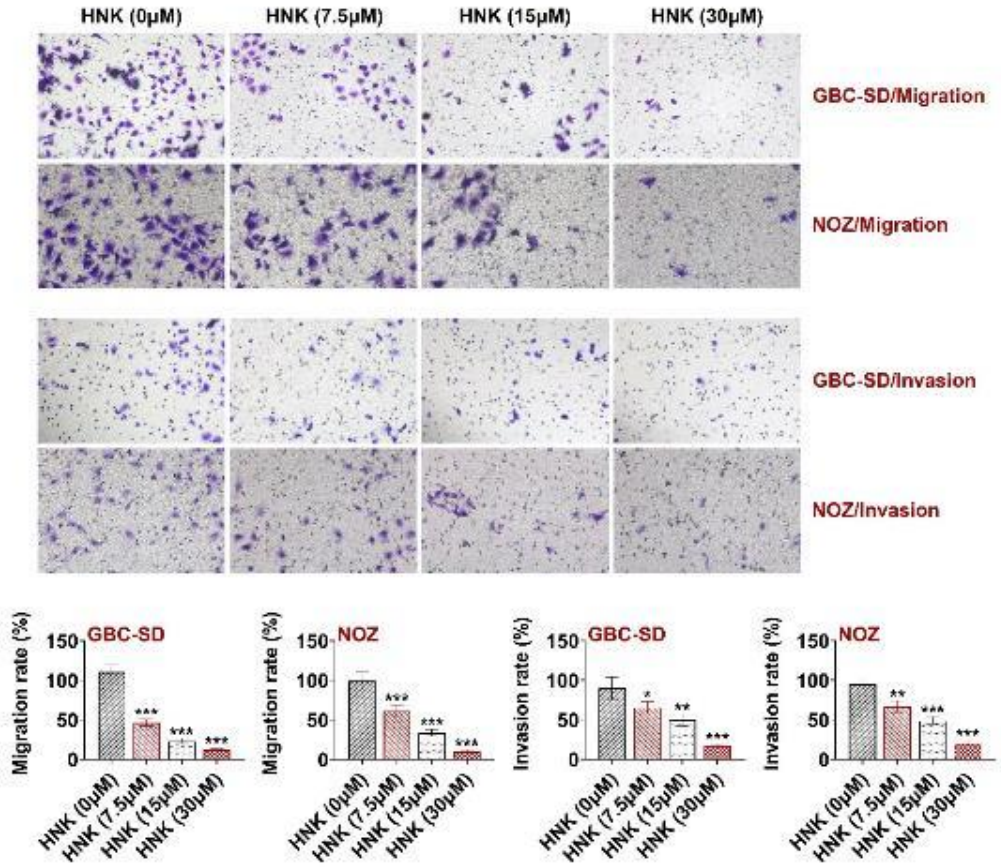

Figure 10: Honokiol inhibits the proliferation and migration of gallbladder cancer cells and promotes apoptosis. The levels of PI3K signaling pathway-related proteins (AKT, p-AKT, PI3K, and p-PI3K) in GBC-SD and NOZ cell lines with TMPRSS4 knockdown/overexpression were assessed using western blotting. Error bars represent data from three independent experiments, presented as mean ± standard deviation (SD). ***P* < 0.01, *treatment group compared to the control group

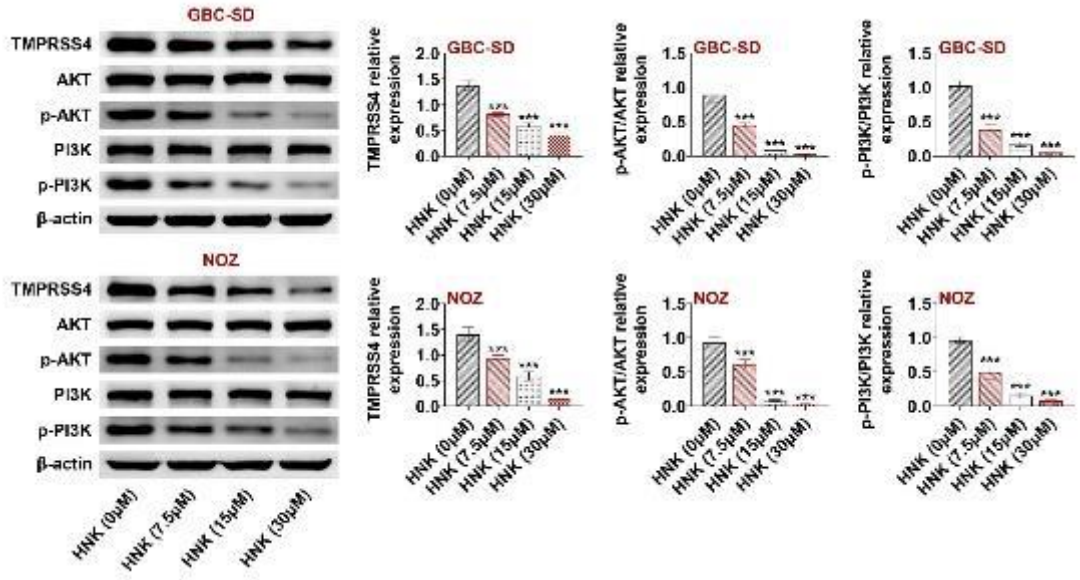

Figure 11: Honokiol inhibits PI3K/AKT pathway signaling. Error bars represent data from three independent experiments, mean ± SD. ***P* < 0.01, $^{\#\#}p$ < 0.01 *compared with negative control group, #compared with siNC group

DISCUSSION

Gallbladder cancer (GBC) is a fatal disease with a low incidence but poor prognosis. Clinically, it occurs mainly in women, elderly patients, and Native Americans. Surgical resection is the only possible treatment, but success depends on staging, tumor biology, and the integrity of the resection. There are significant differences in survival rate among patients at different stages, with a 5-year survival rate of 50 % for stage I cancer and 3 % for stage IV cancer [3]. Incidental gallbladder cancer (IGBC) is usually diagnosed at an early stage and therefore has a higher survival rate than non-IGBC. Chemotherapy may be considered as a strategy to increase survival [4]. Therefore, it is essential to identify the key genes in the development and progression of GBC. Over the past two decades, increasing number of studies have focused on the underlying mechanisms of GBC, including MAPK/ERK, PI3K/AKT/mTOR, and Notch signaling pathways [12]. However, the functional regulatory mechanisms of GBC are not fully understood.

Since TMPRSS4 is up-regulated in GBC cells, therefore, the overexpression of TMPRSS4 may be crucial to the development and progression of GBC. TMPRSS4 positively regulated cell growth and 2D colony formation, and cell apoptosis was negatively regulated by TMPRSS4 through the downregulation of Bax. Moreover, TMPRSS4 has been identified as a positive regulator of both cell migration and invasion, exerting its effects through the induction of TWIST and Vimentin. Furthermore, TMPRSS4 exhibits a positive regulatory influence on the activity of the PI3K/AKT signaling pathway. Notably, honokiol demonstrates inhibitory effects on gallbladder cancer by suppressing TMPRSS4-induced PI3K/AKT activation, leading to the inhibition of proliferation, migration, and the induction of apoptosis in cancer cells.

A previous study reported that TMPRSS4 is highly expressed in gallbladder cancer (GBC) tissue, when compared to adjacent normal tissues, and that the expression of TMPRSS4 is negatively correlated with the survival rate of patients [13]. The data from this study, showed the similar expression of TMPRSS4 in GBC cells. However, it is noteworthy that the analysis of TMPRSS4 expression in GBC tissue specimens is limited due to the current constraints on sample collection. TMPRSS4 has been demonstrated to play important roles in a diversity of cancers, including breast cancer, prostate cancer, colorectal cancer, hepatocellular carcinoma, gastric cancer, lung cancer, and pancreatic cancer. Min reported that TMPRSS4 upregulated uPA expression to facilitate cell invasion in lung and prostate cancers via activation of JNK signaling pathway. TMPRSS4 was correlated with cell proliferation and aggressiveness in breast cancer [14]. Additionally, Guan *et al* demonstrated that TMPRSS4 increased thyroid cancer proliferation through the activation of CREB [15]. These studies all reported the positive function of TMPRSS4 in regulating cell growth, Similarly, in the present study, TMPRSS4 played the same role in the regulation of cell proliferation in GBC cell lines, thus indicating that TMPRSS4 may be a significant marker in tumor cell growth. However, the underlying functional regulatory mechanisms need to be further investigation.

In prostate cancer, TMPRSS4 positively mediated cell growth and cell invasion by upregulating Slug and cyclin D1 [16]. Another study also demonstrated that TMPRSS4 induced the expression of TWIST1 to promote cell migration by the activation of STAT3 in prostate cancer [8]. Moreover, Lee showed that TMPRSS4 positively regulates the stem-like properties of cancer by upregulating of SLUG and TWIST1 in prostate cancer [17]. In the present study, it was found that TMPRSS4 is associated with the expression of TWIST, Vimentin, E-cadherin, and ZO-1 in regulating cell viability and EMT. Recently, Gu reported that TMPRSS4 induces cell proliferation and reduces cell apoptosis by activating ERK1/2 signaling pathway in PDAC [18]. Similarly, in the present study, TMPRSS4 increased cell proliferation and foci formation through the upregulation of TWIST, and suppressed cell apoptosis by reducing Bax.

A study has reported that TMPRSS4 induces cell proliferation, cell invasion and EMT by activation of MAPK and AKT in endometrial carcinoma cells. Additionally, previous studies also demonstrated that PI3K/AKT pathway exerts a crucial role in carcinogenesis. The PI3K/AKT pathway correlated with cell growth, metastasis and invasion through the suppression of GPER1 [19]. In this study, the role of TMPRSS4 in cell growth and EMT may be regulated by the activation of PI3K and AKT. In order to further confirm the critical role of TMPRSS4 in GBC, function rescue assay is required in a future study.

CONCLUSION

TMPRSS4 positively regulates cell growth, cell migration and cell invasion via the upregulation of TWIST1, and negatively regulates cell

apoptosis by Bax inhibition. Honokiol inhibits gallbladder cancer proliferation and migration, and induces cell apoptosis by suppressing TMPRSS4-induced PI3K/AKT activation. These findings offers insight into a key molecular mechanism in the progression of GBC and, hence a possible approach to the treatment of GBC.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Conglin Lin designed the study and supervised the data collection; Congren Wang analyzed and interpreted the data; Mingzhu Li and Zhibing Cai prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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