

TULP3 Silencing Suppresses Cell Proliferation, Migration and Invasion in Gastric Cancer Via the PTEN/Akt/Snail Signaling Pathway

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Abstract

Purpose

Tubby-like protein 3 (TULP3), a member of the tubby family, has been related to the development of nervous system by gene knockout researches. Nevertheless, the regulatory mechanism and role of TULP3 in the gastric cancer are not clear. Current research is the first probe into the regulatory effect of TULP3 in the gastric cancer.

Methods

Western blotting together with real time polymerase chain reaction (PCR) were employed for the quantitative detection of TULP3 expression in the gastric cancer and consecutive non-cancerous tissues, and gastric cancer cells. Kaplan-Meier method along with Log-rank test was exploited for the determination of the disease-free survival rate and overall survival time of patient containing with different expression of TULP3 in tumors. The roles of TULP3 in invasion, migration as well as proliferation of the gastric cancer cell *in vivo* and *in vitro* through utilizing colony formation test, MTT test, wound-healing test, transwell test and mouse xenograft model. Western blotting assay was implemented in order to clarify the potential molecular mechanisms. Furthermore, electron microscopy and western blot were evaluated TULP3 expression in gastric cancer patient extracted serum exosomes.

Results

TULP3 expression levels were remarkably up-regulated in the gastric cancer tissues and cells. Subsequent functional assays demonstrated that TULP3 downregulation suppressed invasion, migration as well as the proliferation of the gastric cancer cell. Mechanism assays depicted that the PTEN/Akt/Snail signaling pathway can inhibit invasion, migration as well as the proliferation of the gastric cancer cell via TULP3 silencing. Finally, we found that the expression of TULP3 could be determined in the extracted serum exons. The expression of TULP3 in gastric cancer group was higher in comparison with normal group.

Conclusion

Our outcomes reveal that TULP3 probably play a role in the diagnosis together with the prognostic biomarkers of gastric cancer.

Introduction

Gastric cancer is a kind of prevalent malignant tumor in the world and the third major cause of death in relation with cancer [1, 2]. Due to the mild symptoms and insidious onset, exceed 80 percent of patients

are in an advanced stage at the time of diagnosis, so the prognosis is usually poor [3, 4]. Delayed diagnosis has become the largest obstacle affecting the treatment and prognosis of gastric cancer [5, 6]. Hence, it is necessary to search for effective targets for screening and diagnosing gastric cancer as early as possible, thus improving the prognosis. Nevertheless, the latent molecular mechanism that regulates the invasive phenotype of the gastric cancer still is not clear [7]. As a result, it is significant to understand the gastric cancer molecular mechanism.

TULP3 belongs to a family of Tubby. There is a Tubby domain at the C-terminal with conservative nuclear loci, which can play a role as the transcription factor [8, 9]. Recent study of mammalian development showed that TULP3 gene plays an important role because its expression is widespread throughout the development of embryonic mice, TULP3 silencing results in embryonic lethality and the defects of neural tube [10, 11]. Some researches have exhibited the significant effect of TULP3 in the development of embryo. Nevertheless, few researches have explored its latent effect in carcinogenesis [12, 13, 14]. In recent, Qian et al. utilised radiogenomic test to determine the genes related to the glioblastoma multiforme risk prediction, in which TULP3 is also involved [15]. In another research, through utilizing electronic methods, up-regulated expression of TULP3 gene was related to poor prognosis in the patients with PDAC, but these outcomes have not been confirmed in the clinical samples [16]. Nonetheless, there exist no information on whether TULP3 is related to the invasion, migration and proliferation of the gastric cancer cells. In view of the essential effect of TULP3 in the regulation of cell process, it is momentous to explore the role and detail mechanism of TULP3 in invasion, migration as well as proliferation of the gastric cancer cells.

PTEN/Akt signaling pathway is one of the three major signalling pathways that have been identified as important in cancer [17]. Phosphatase and tensin homology (PTEN) that deleted on the chromosome 10 is a significant tumor inhibitor. After phosphatidylinositol-3,4,5-trisphosphate converted to the phosphatidylinositol-4,5-bisphosphate, it can antagonize the phosphatidylinositol-3-kinase (PI3K) effect and ultimately inhibits the activation of Akt [18]. In addition, PTEN was observed to be reduced in the gastric cancer, and its excessive expression can inhibit the gastric cancer cell lines growth through the Akt pathway inactivation [19]. The excessive expression of Akt can make gastric cancer cells enter a highly invasive and proliferative state [20]. PTEN regulates the bio-processes of cell through Akt (the target molecule) [21, 22]. Snail is a type of zinc finger transcription factor that can trigger EMT through suppressing the tight junction proteins expression, containing claudin-1, E-cadherin, ZO-1 together with occludin [23, 24, 25]. Snail is a principal mediator of the EMT and is related to the invasive gastric cancer phenotype [26].

In this paper, we revealed that the levels of TULP3 expression were markedly up-regulated in the gastric cancer tissues and cells. Subsequent functional assays demonstrated that TULP3 downregulation suppressed invasion, migration as well as proliferation of the gastric cancer cell. Mechanism assays depicted that the PTEN/Akt/Snail signaling pathway can inhibit the invasion, migration as well as proliferation of the gastric cancer cell by TULP3 silencing. Finally, we found that the expression of TULP3 could be determined in the extracted serum exons. The expression of TULP3 in gastric cancer group was

higher in contrast to normal group. As a result, TULP3 probably is a therapeutic target together with prognostic biomarker for the gastric cancer.

Materials And Methods

Human tissue samples

Sixty pairs of the gastric cancer samples as well as the adjacent non-neoplastic tissues were acquired from the Department of Gastrointestinal Surgery of The First Affiliated Hospital, Wannan Medical College (Wuhu, Anhui, PR China) from March 2019 to October 2020. In accordance with the guidelines of World Health Organization (WHO), all of the specimens were assessed by two pathologists. All the tissues were given at the time of surgery, which were stored directly in a liquid nitrogen. No systemic or local treatment was implemented to the patients prior to operation. The experiments and investigation have been agreed by patients and authorized by the Ethic Committee for Clinical Research of the First Affiliated Hospital of Wannan Medical College (No. 2020-52). The study was conducted in accordance with the Declaration of Helsinki (<https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/>). We did not provide any data that could help identify patients.

Cell line and cell culture

The cell lines of gastric cancer (BGC-823, AGS together with SGC-7901) and GES-1, the normal gastric mucosal epithelial cells were offered through the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cell viability, DNA fingerprinting, Mycoplasma as well as isozyme were detected to characterize cell lines. The last identification of cell was implemented on September 2017. Five cell lines were cultivated in Dulbecco's Modified Eagle medium with high glucose (DMEM; Invitrogen, Carlsbad, California, USA) containing antibiotics (penicillin (100 units/mL) and streptomycin (100 mg/mL)) together with fetal bovine serum (10%, FBS; Gibco, Bethesda, MD, USA). All of the cells were cultivated with 5% CO₂ under a temperature of 37 °C.

Cell transfection

The lentivirus establishing the TULP3 gene knockout was offered by genepharma (Shanghai, China). The cell lines of gastric cancer were inoculated in plates (6 wells) at 50 percent of confluence, which were then respectively infected by scramble control (called shCtrl) or TULP3 knockout lentivirus (called shTULP3) in BGC-823 and AGS cells. The stable transduction pools were produced via selecting with 4µg/ml puromycin for two weeks. The above cell transfection protocol shall comply with the instructions of manufacturer. After transfection, the cells were cultivated and gathered to conduct the subsequent studies.

Quantitative real-time polymerase chain reaction(qRT-PCR)

Trizol reagent (Sigma, Cat.No.T9424-100m) was utilized for the extraction of total RNA from the samples. qPCR (+gDNA wiper) (Vazyme, Cat. No. R123-01) was reverse transcribed through utilizing Hiscript QRT Supermix. In a VII7 Real-Time PCR System (Applied Biosystems, Weiterstadt, Germany), the products of cDNA could be amplified and then quantified through applying AceQ qPCR SYBR Green master mix (Vazyme, Cat. No. Q111-02). The TULP3 mRNA primers are composed of forward primer 5'-TTGAGGTGGAGTCTCGCTCTGTC-3' and reverse primer 5'-GAGGCAGGAGAATGGCGTGAAC-3'. The forward primer 5'-AGC CAC ATC GCT CAG ACA-3' together with reverse primer 5'-TGG ACT CCA CGA CGT ACT-3' were employed for the internal control of mRNA. The mRNA level was determined by the comparative Ct method ($\Delta\Delta Ct$).

Western blot

RIPA (Thermo Fisher, MA, USA) lysis buffer involving phosphatase suppressor (1%), PMSF (1%) and protease suppressor (0.1%) was utilized to dissolve cells. BCA Protein Assay Kit (Cat. # 23225, Thermo Fisher Scientific, MA, USA) was exploited for the assessment of total protein, and the same amount of proteins were transferred to PVDF membranes by SDS-PAGE Protein electrophoresis apparatus (Cat. # VE-180, TIANGEN, Shanghai, China). The membrane was blocked by skimmed milk (5%) in a Tris buffered saline involving Tween 20 (0.1%) for two hours and cultured overnight with the below primary antibodies under a temperature of 4 °C. The above primary antibodies contains: TULP3 (1:1000, ab254692, Abcam, Cambridge, MA, USA), PTEN(1:1000, #9188, Cell Signaling Technology, Danvers, MA, USA), p-PTEN(1:1000, #9554, Cell Signaling Technology, Danvers, MA, USA), Akt(1:1000, #2920, Cell Signaling Technology, Danvers, MA, USA), p-Akt(1:1000, #4060, Cell Signaling Technology, Danvers, MA, USA), Snail(1:1000, #3879, Cell Signaling Technology, Danvers, MA, USA), Alix(1:1000, #2171, Cell Signaling Technology, Danvers, MA, USA), HSP70(1:1000, #4876, Cell Signaling Technology, Danvers, MA, USA), GM130(1:1000, #553612480, Cell Signaling Technology, Danvers, MA, USA), Flotillin-1(1:1000, #18634, Cell Signaling Technology, Danvers, MA, USA), EpCAM(1:1000, #5532626, Cell Signaling Technology, Danvers, MA, USA), and β -Actin (1:3000, A1978, Sigma, Victoria, BC, Canada). The membrane was cleaned 3 times with TBST (0.1%) for five minutes each time, then cultured for two hours by anti-rabbit or anti-mouse secondary antibody combined with the horseradish peroxidase (Cell Signaling Technology, Danvers, MA, USA), and cleaned 3 times with TBST (0.1%) for five minutes each time. The reaction products were visualized with chemiluminescent ECL Plus reagents (Pierce, USA). And Tanon 5200 (Tanon, Shanghai, PR China) was employed to scan the membrane. Quantity One Software(Tanon, Shanghai, PR China) was applied for the measurement of band strength via densitometry. The levels of β -actin and protein were standardized. All of the experiments were conducted three times and reflected representative outcomes.

MTT assay

The shTULP3 and shCtrl cells were inoculated into the plates (96-well) (Cat. # 3599, Corning, NY, USA) overnight at 2×10^3 cells/well density. Before adding DMSO (150 μ l, Sigma-Aldrich), each well was added with the MTT (20 μ l, 5 mg/mL, Genview Inc., Santa Clara, California, U.S.), and then cultured for four

hours, after which Microplate Reader was conducted to determine the optical density (Cat. # M2009PR, Tecan infinite, Shanghai, China) at 490 nm. All of the studies were implemented for three times.

Colony formation assay

The transfected BGC-823 together with AGS cells were added to the plates (6 well) and kept in the medium under a standard condition. After incubation for 2 weeks under a temperature of 37 °C, rinse the above plates through using ice-cold phosphate buffer saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). Next, the colony cells were fixed with paraformaldehyde (4%, Sigma-Aldrich, St. Louis, MO, USA) for fifteen minutes, subsequently stained by 0.1 percent crystal violet (0.1%, Sigma-Aldrich, St. Louis, MO, USA) at RT. The optical microscope (Nikon, Japan) was used for imaging and calculating the colony cells number.

Transwell assay

Transwell chambers (BD Biosciences, USA) were used for the invasion and migration of cell. After two days of related treatment, in each group, BGC-823 along with AGS cells were cultured into upper Transwell chamber at 4×10^4 cells each well density (100 μ L medium involving 5 percent fetal bovine serum). In addition, the culture plate (24 well) in lower chamber was added with 100 μ L medium involving 10 percent of fetal bovine serum. After one day of the conventional culture, take out the chamber and wipe the cells on the upper microporous membrane layer through exploiting the cotton swab. Then, the cells were fixed in 4% solution of paraformaldehyde for ten minutes at RT and stained by crystal violet solution for fifteen minutes (0.5%, Sigma-Aldrich; Merck KGaA). In the end, five visual fields could be randomly chose and under the optical microscope (Nikon, Japan), they were subsequently observed to calculate the cells number invading the chamber microporous membrane sublayer.

Wound healing assay

Wound healing experiment was implemented for the observation of the cell migration. In short, when the BGC-823 and AGS cells after transfection remained in the 6-well plate and reached a confluence from 90 to 95%, the tip of a micropipette was utilized to create scratches. After scratching, X71 inverted microscope (Olympus, Tokyo, Japan) was applied for the observation of wound state at 0 and 24 hours.

Exosome isolation

In accordance with the instructions of manufacturer, ExoQuick precipitation solution (System Biosciences, Mountain View, CA, USA) was applied for acquiring exosomes from serum. Shortly, it will be ExoQuick solution (63 μ L) and serum (250 μ L) was mixed and incubated under a temperature of 4 °C overnight. After centrifugation for half an hour at 1500 \times g, suspending the pellets in PBS (50 μ L) and filtrated by 0.22 μ m of filter (EMD Millipore, Billerica, MA, USA). The separated ectoplasts were maintained under a temperature of -80 °C prior to use.

Transmission electron microscopy

A pipette was used to transfer 20 μ L of produced exosomes onto the copper grid coated with Formvar carbon, allow it to adsorb for ten minutes, and then discharge the excess liquid. The exosomes after absorbed were subsequently negatively stained by 2% (w/v) phosphotungstic acid (with a pH of 6.8) for five minutes, and electric incandescent lamp was subsequently employed to dry them, and transmission electron microscope (Fei TECNAI 12, Philips) was exploited to analyze them, bar = 200 nm.

Size analyses of exosomes

The separated exosomes were diluted in the PBS, NanoSight LM 10-HSBFT 14 Instrument (NanoSight, Malvern, UK) was employed for analyzing them based on the protocol. The exosomes size were subsequently analyzed studied with the software of Nanoparticle Tracking Analysis 2.0 (NTA 2.0).

Statistical analysis

All data were produced from three separated studies and they were described as mean \pm standard deviation. GraphPad Prism 8.0.1 for Windows (Graphpad Software Inc., La Jolla, California USA, www.graphpad.com) was utilized to conduct the statistical calculations. Between the two groups, the difference was calculated via proper unpaired Chi-square test or using Student's t-test, and there exist a statistical significance when $P < 0.05$.

Results

TULP3 is up-regulated in gastric cancer and has clinical value in gastric cancer

It can be found that the levels of TULP3 were remarkably enhanced in 156 patients with gastric cancer in comparison with five normal gastric tissues utilizing Cancer Genome Atlas (TCGA) database (Fig. 1a). qRT-PCR was implemented for the detection of the TULP3 expression in sixty paired normal tissues and gastric cancer tissues. Our outcomes exhibited that TULP3 was evidently enhanced in the gastric cancer tissues (Fig. 1b). In addition, in order to confirm the protein and the mRNA expression levels of TULP3 in different gastric cancer stages, the western blot together with real-time PCR was implemented for high-grade (WHO \geq II) and low-grade (WHO \leq I) gastric cancer groups. The results showed higher levels of TULP3 expression in high-grade (WHO \geq II) gastric cancers in comparison with the low-grade gastric cancers (Fig. 1c, d). Log-rank test and the Kaplan-Meier method indicated that the disease-free survival and total survival time of patients having high expression of TULP3 in tumors were obviously shorter than the patients containing low expression of TULP3 in gastric cancer (Fig. 1e, f).

High expression of TULP3 in gastric cancer cell lines and human gastric cancer tissues

Subsequently, the TULP3 expression levels in GES-1 and three cell lines of gastric cancer (namely, BGC-823, AGS as well as SGC-7901) were evaluated by Western blot detection and the real-time PCR. According to Figures 2A and 2B, the TULP3 expression in these three cell lines of the gastric cancer was higher than the expression in GES-1, and the level of expression was higher in the BGC-823 and AGS cells; Therefore, these lines are employed for the further studies of biological mechanisms and functions

(Fig. 2a, b). In addition, comparative research exhibited that the TULP3 expression level in four pairs of the human gastric cancer tissues was higher than the level in matched adjacent non-cancer tissues (Fig. 2c, d). In conclusion, these outcomes reflected that the expression of TULP3 is in relation with the clinical progression of the gastric cancer and may be a valuable gastric cancer prognostic marker.

Silencing of TULP3 expression in gastric cancer cells inhibits cell proliferation, migration and invasion in vitro

Afterwards, with the aim of clarifying the bio-function of TULP3 in the gastric cancer, we reduced the expression of TULP3 in BGC-823 and AGS cell lines through two specific lentiviral shRNAs of TULP3 (namely, shTULP3 #1 together with shTULP3 #2). Western blot detection together with real-time PCR was applied to observe the TULP3 expression was down-regulated approximately 85% after treating through shTULP3 #1 targeting TULP3 in comparison with negative control (Fig. 3a-d). Therefore, shTULP3#1 was selected to carry out the following experiments and the exploration of mechanism. With the aim of clarifying the effect of TULP3 in the gastric cancers, a sequence of *in vitro* experiments were performed. MTT assay demonstrated that tumor proliferation was remarkably suppressed in shTULP3 group in contrast to shCtrl group (Fig. 3e, f, Supplemental figures 1a, b). In AGS knockdown cells, the number of cell clones was deceased in shTULP3 group in contrast to shCtrl group. The same was true in BGC-823 cells (Fig. 3g). Transwell assay revealed that the invasion of BGC-823 together with AGS cells invasion was remarkably down-regulated after decrease of TULP3 (Fig. 3h). Eventually, the migration of cells were assessed through wound-healing assay, and reduced TULP3 expression evidently suppressed the BGC-823 and AGS cells migration (Fig. 3i, j). Taken together, the abovementioned datas indicated that *in vitro*, knockout of TULP3 can suppress the invasion, migration as well as proliferation of the gastric cancer cells.

Knockdown of TULP3 inhibits the tumorigenicity of gastric cells in vivo

A mouse xenotransplantation model was constructed to explore whether TULP3 could influence the occurrence of tumors *in vivo*. The nude mice was injected with shRNA or TULP3 shRNA AGS cells from the flank. The tumor volume and weight in TULP3 knockout group were reduced to about one-fourth and one-fifth of those in control group, respectively (Fig. 4a-c). As the result, TULP3 is very significant in the promotion of gastric carcinogenesis.

TULP3 regulates gastric cancer cell proliferation, migration and invasion via the PTEN/Akt/Snail signaling pathways

With the aim of comprehensively clarifying the mechanisms of TULP3 in regulating the invasion, migration as well as proliferation of the gastric cancer cell, we transfected with TULP3 shRNA in the cell lines of BGC-823 and AGS, and signaling pathways participated in tumor invasion, migration and proliferation, which might be activated through TULP3 were analyzed by examining the expression of snail and the phosphorylation forms of the PTEN/Akt through applying western blot detection. The outcomes exhibited that the expression of Snail was decreased, the phosphorylation of PTEN was up-

regulated and which of the AKT was remarkably decreased following TULP3 shRNA treatment compared to negative control (Fig. 5a, b). Together, these data indicated that TULP3 silencing attenuates the invasion, migration as well as proliferation of the gastric cancer cell through the PTEN/Akt/Snail signaling pathways.

Serum exosomal TULP3 level is upregulated in gastric cancer patients

Eventually, in our work, exosomes could be extracted from sixty serum samples of normal people and patients with gastric cancer. First of all, these vesicles were characterized through a variety of methods, like western blotting and electron microscopy (Fig. 6a, b). The presence of exome markers GM130, HSP70, Alix, EpCAM and Flotillin-1 demonstrated the purity of gastric cancer secretory exons isolated from serum. Our outcomes revealed that the expression of TULP3 could be determined in the serum exons after extraction, and the expression in gastric cancer group was higher than the expression in normal group (Fig. 6c). In conclusion, these outcomes suggest that in serum, the exosomal TULP3 is stable, which can be utilized as the latent biomarker in patients with gastric cancer.

Discussion

Gastric cancer is a malignant tumor with high mortality rate and incidence rate [27, 28]. Despite gastric cancer has been deeply investigated, its latent molecular mechanism has not been completely clarified [29]. In addition, there exist no effective prevention and the methods to treat gastric cancer [30]. As a result, there is an urgent demand to determine effective the predictors together with treatments.

The main result of this experiment is that TULP3 is markedly related to the gastric cancer progression. The invasion, migration as well as proliferation of the gastric cancer cells can be reduced by TULP3 silencing. We found that the deletion of TULP3 decreased the invasion, migration as well as proliferation of the gastric cancer cells through Snail, Akt and PTEN signaling pathway. This research gives strong evidence and novel insights for TULP3 to possess an essential effect in the gastric cancer tumorigenicity and its progression.

A bipartite transcription factor in tubby gene family was encoded by TULP3. The members of the above family have been found in invertebrates, vertebrates, and plants that share phosphatidylinositol-phosphate binding area and a conserved C-terminal DNA and a conserved N-terminal transcriptional activation area [31, 32, 33]. In plasma membrane, the phosphatidylinositol was bound with encoded protein through its C-terminal area and may utilize as the membrane-bound transcriptional regulator, for example, transporting it to nucleus through G-protein-coupled receptor signal-induced hydrolysis of phosphatidylinositol [13, 34, 35, 36]. It has an essential effect in the function and development of neurons. There are two transcriptional variants of this gene that encode different isoforms. The protein of TULP3 regulates negatively the Hedgehog pathway without a ligand of Sonic Hedgehog (Shh) in the primary cilia of mouse embryos, an extension of microtubule basement membrane employs as a sensory signal chamber [11]. TULP3 is also associated with vesicle transport because PIP2 (a component of the cell membrane) binds with the tubby domain binds [34]. Mukhopadhyay et al. emphasized the effects of

intraflagellar transport complex A (IFT-A) and TULP3 in the transport of G protein-coupled receptors (GPCRs) subsets to cilia in the mouse embryos [13]. In addition, Santagata et al. believe that TULP3 may play an effect in regulating gene expression and GPCR signal transduction of Neuro-2A cell [14].

In this experiment, the clinical significance and level of TULP3 expression in the gastric cancer was assessed through a relatively large number of clinical tissue samples. Western blot and real-time PCR were employed for detection the protein and mRNA expression levels of TULP3 in sixty gastric cancer tissue samples. These outcomes suggest that abnormally high expression of TULP3 may be related to poor gastric cancer prognosis. With the aim of clarifying the bio-behavior of TULP3 in the gastric cancer, the expression of TULP3 in three cell lines of gastric cancer was determined, established TULP3 lentiviral vector to statically knock out the TULP3, and utilized this vector to statically transduce BGC-823 together with AGS cell lines. Our outcomes suggest that the knockdown of TULP3 inhibits the invasion, migration, and proliferation of BGC-823 and AGS in vitro. Nevertheless, the TULP3 mechanism in the invasion, migration, as well as proliferation of gastric cancer cells has not been studied yet. In our work, we provided evidence that the knockdown of TULP3 could suppressed the Akt and PTEN phosphorylation, whereas the total of PTEN and Akt were no change. Aberrations in a variety of cellular signaling pathways help to regulate growth, the development of tumor, cell metabolism, cytoskeleton reorganization, metastasis and proliferation [37, 38]. The signaling pathway of PTEN/Akt is the basic cellular signaling cascade participated in the above processes. It is a key and deeply investigated intracellular signaling pathway in tumorigenesis process [39, 40]. A variety of activating mutations of oncogenes and the tumor inhibitor genes inactivation have been found in various malignant tumors of nearly all pathway members [41]. The signaling pathway of PTEN/Akt acts as a target for treating the cancer [42]. Recently, by the horizontal transfer of a variety of bioactive molecules for instance mRNAs and proteins, exons are becoming mediators of systemic and local intercellular carcinogenic information and possesses an essential effect in the progression of cancer [43]. Exosomes are secreted through many kinds of cells and involve in the intercellular communication through transmitting the intracellular goods [44]. Proteins can prevent degradation through exons in circulation, transfer between various cancer cells, and transmit phenotypes and signals through exons [45]. Nevertheless, the exosomal proteins effect come from the gastric cancer cells is unclear. Fascinatingly, it can be found that the expression of TULP3 could be determined in the serum exons after extraction, and the expression of TULP3 in gastric cancer group was higher than the expression in normal group.

To sum up, the present research exhibited that TULP3 is highly increased in cell lines and samples of gastric cancer patient, and the expression of TULP3 is positively associated with the poor gastric cancer clinical prognosis. TULP3 deletion suppresses the proliferation of gastric cancer cells through PTEN/Akt/Snail signaling pathway. What's more, TULP3 was determined in exosomes and the gastric cancer tissues, suggesting its latent application as biomarker and therapeutic target for the gastric cancer.

Declarations

Acknowledgment No applicable.

Authors' contributions Zhi Li and Rui Liu designed the study. Jun Song wrote the paper. Qingsheng Fu and Gang Liu performed the cellular, molecular experiments. Bing Wang performed the other all experiments. Zhi Li and Rui Liu analyzed the data. Chengxiong Zhang contributed reagents/materials. All authors approved the final version of the manuscript.

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Conflict of interest The authors report no declarations of interest.

Ethical approval This study, under approval of Ethics Committee of the First Affiliated Hospital of Wannan Medical College, was conducted with informed consent provided by all participants, strictly following the Declaration of Helsinki. The animal experiments was approved by the Animal Ethics Committee of the First Affiliated Hospital of Wannan Medical College.

Informed consent This study was conducted with informed consent from all participants.

Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

References

1. Rebecca L Siegel, Kimberly D Miller, Ahmedin Jemal. Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians*, 2019.
2. Takeshi Sano. Gastric cancer: Asia and the world. *Gastric Cancer*, 2017, 20(Suppl 1):1-2.
3. Den Hoed C M, Kuipers E J. Gastric Cancer: How Can We Reduce the Incidence of this Disease?. *Current Gastroenterology Reports*, 2016, 18(7):1-8.
4. Antonio Ponzetto, Natale Figura. Screening for Gastric Cancer. *American Journal of Gastroenterology*, 2019, 114(4):690.
5. Catherine de Martel, David Forman, Martyn Plummer. Gastric Cancer: Epidemiology and Risk Factors. *Gastroenterology Clinics of North America*, 2013, 42(2):219-240.
6. Lee Y Y, Derakhshan M H. Environmental and lifestyle risk factors of gastric cancer. *Archives of Iranian Medicine*, 2013, 16(6):358-365.
7. Arai H, Nakajima T E. Recent Developments of Systemic Chemotherapy for Gastric Cancer. *Cancers*, 2020, 12(5):1100.
8. Boggon TJ, Shan WS, Santagata S, et al. Implication of tubby proteins as transcription factors by structure-based functional analysis. *Science*, 1999, 286(5447): 2119–2125.

9. Sartor, Ivaine Taís Sauthier, Recamonde-Mendoza M, et al. TULP3: A potential biomarker in colorectal cancer? PLoS ONE, 2019, 14(1):e0210762.
10. Ikeda A, Ikeda S, Gridley T, et al. Neural tube defects and neuroepithelial cell death in Tulp3 knockout mice. Human Molecular Genetics, 2011, 10(12): 1325–1334.
11. Norman RX, Ko HW, Huang V, et al. Tubby-like protein 3 (TULP3) regulates patterning in the mouse embryo through inhibition of Hedgehog signaling. Human Molecular Genetics, 2009, 18(10): 1740–1754.
12. Cameron DA, Pennimpede T, Petkovich M. Tulp3 is a critical repressor of mouse hedgehog signaling. Developmental Dynamics, 2009; 238(5): 1140–1149.
13. Mukhopadhyay S, Wen X, Chih B, et al. TULP3 bridges the IFT-A complex and membrane phosphoinositides to promote trafficking of G protein-coupled receptors into primary cilia. Genes Development, 2010, 24(19): 2180–2193.
14. Santagata S, Boggon TJ, Baird CL, et al. G-protein signaling through tubby proteins. Science, 2001, 292(5524): 2041–2050.
15. Qian X, Tan H, Zhang J, et al. Identification of biomarkers for pseudo and true progression of GBM based on radiogenomics study. Oncotarget, 2016, 7(34): 55377–55394.
16. Sartor ITS, Zeida'n-Chulia' F, Albanus RD, et al. Computational analyses reveal a prognostic impact of TULP3 as a transcriptional master regulator in pancreatic ductal adenocarcinoma. Molecular Biosystem, 2014, 10(6): 1461–1468.
17. Aiqing L, Minzi Q, Hongbo Z, et al. PTEN, insulin resistance, and cancer. Current Pharmaceutical Design, 2017, 23(25):3667-3676.
18. Martini M, De Santis M C, Braccini L, et al. PI3K/AKT signaling pathway and cancer: an updated review. Annals of Medicine, 2014, 46(6):372-383.
19. Xu J , Liu D , Niu H , et al. Resveratrol reverses Doxorubicin resistance by inhibiting epithelial-mesenchymal transition (EMT) through modulating PTEN/Akt signaling pathway in gastric cancer. Journal of Experimental & Clinical Cancer Research, 2017, 36(1):1-14.
20. Jianbo, Xiong, Zhengrong, et al. PRL-3 promotes the peritoneal metastasis of gastric cancer through the PI3K/Akt signaling pathway by regulating PTEN. Oncology reports, 2016, 36(4):1819-1828.
21. Chappell WH, Steelman LS, Long JM, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget, 2011; 2:135-164.
22. Hyun T, Yam A, Pece S, et al. Loss of PTEN expression leading to high Akt activation in human multiple myelomas. Blood, 2000; 96:3560-3568.
23. Blanco M J, Moreno-Bueno G, Sarrio D, et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene, 2002, 21(20):3241-3246.
24. Ohkubo T, Ozawa M. The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. Journal of Cell science, 2004, 117(Pt 9):1675-1685.

25. Kim B J, Hancock B M, Bermudez A, et al. Bacterial induction of Snail1 contributes to blood-brain barrier disruption. *Journal of Clinical Investigation*, 2015, 125(6):2473-2483.
26. Masaru Katoh. Epithelial-mesenchymal transition in gastric cancer. *International Journal of Oncology*, 2005, 27(6):1677-1683.
27. Karimi P, Islami F, Anandasabapathy S, et al. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 2014, 23(5):700-713.
28. Marino V, Riccardo V, Theodoros R, et al. Gastric cancer: epidemiology, prevention, and therapy. *Helicobacter*, 2018;23 Suppl 1:e12518.
29. Robert S, Małgorzata S, Jerzy M, et al. Gastric cancer: epidemiology, prevention, classification, and treatment. *Cancer Management & Research*, 2018, 10:239-248.
30. Machlowska J, Baj J, Sitarz M, et al. Gastric Cancer: Epidemiology, Risk Factors, Classification, Genomic Characteristics and Treatment Strategies. *International Journal of Molecular Sciences*, 2020, 21(11):4012.
31. Mukhopadhyay S, Jackson P K. The tubby family proteins. *Genome Biology*, 2011, 12(6):225.
32. Carroll K, Gomez C, Shapiro L. Tubby proteins: the plot thickens. *Nature Reviews Molecular Cell Biology*, 2004, 5(1):55-63.
33. He W, Ikeda S, Bronson R T, et al. GFP-tagged expression and immunohistochemical studies to determine the subcellular localization of the tubby gene family members. *Molecular Brain Research*, 2000, 81(1-2):109-117.
34. Badgandi H B, Hwang S H, Shimada I S, et al. Tubby family proteins are adapters for ciliary trafficking of integral membrane proteins. *Journal of Cell Biology*, 2017, 216(3):jcb.201607095.
35. Nishina P M, North M A, Ikeda A, et al. Molecular characterization of a novel tubby gene family member, TULP3, in mouse and humans. *Genomics*, 1998, 54(2):215-220.
36. Victoria L P, Christine D, Anju P et al. Mouse hitchhiker mutants have spina bifida, dorso-ventral patterning defects and polydactyly: identification of Tulp3 as a novel negative regulator of the Sonic hedgehog pathway. *Human Molecular Genetics*, 2009, 18(10):1719-39.
37. Dreesen O, Brivanlou A H. Signaling Pathways in Cancer and Embryonic Stem Cells. *Stem Cell Reviews*, 2007, 3(1):7-17.
38. Hanahan D, Weinberg R A. The hallmarks of cancer. 2000, 100(1):0-70.
39. Lim H J, Crowe P, Yang J L. Current clinical regulation of PI3K/PTEN/Akt/mTOR signalling in treatment of human cancer. *Journal of Cancer Research and Clinical Oncology*, 2015, 141(4):671-689.
40. Mccubrey J A, Steelman L S, Kempf C R, et al. Therapeutic resistance resulting from mutations in Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR signaling pathways. *Journal of Cellular Physiology*, 2011, 226(11):2762-2781.

41. Luc G T, Timothy A C. Therapeutic targeting of tumor suppressor genes. *Cancer*, 2015, 121(9):1357-1368.
42. Renner O, Blanco-Aparicio C, Carnero A. Genetic modelling of the PTEN/AKT pathway in cancer research. *Clinical & Translational Oncology*, 2008, 10(10):618-627.
43. Kahlert C, Kalluri R. Exosomes in tumor microenvironment influence cancer progression and metastasis. *Journal of Molecular Medicine*, 2013, 91(4):431-437.
44. Schorey J S, Bhatnagar S. Exosome Function: From Tumor Immunology to Pathogen Biology. *Traffic*, 2008, 9(6):871-881.
45. Naohiro S, Kazunari A, Hiroshi S. Exosome-mediated regulation of tumor immunology. *Cancer Science*, 2018, 109 (10), 2998-3004.

Figures

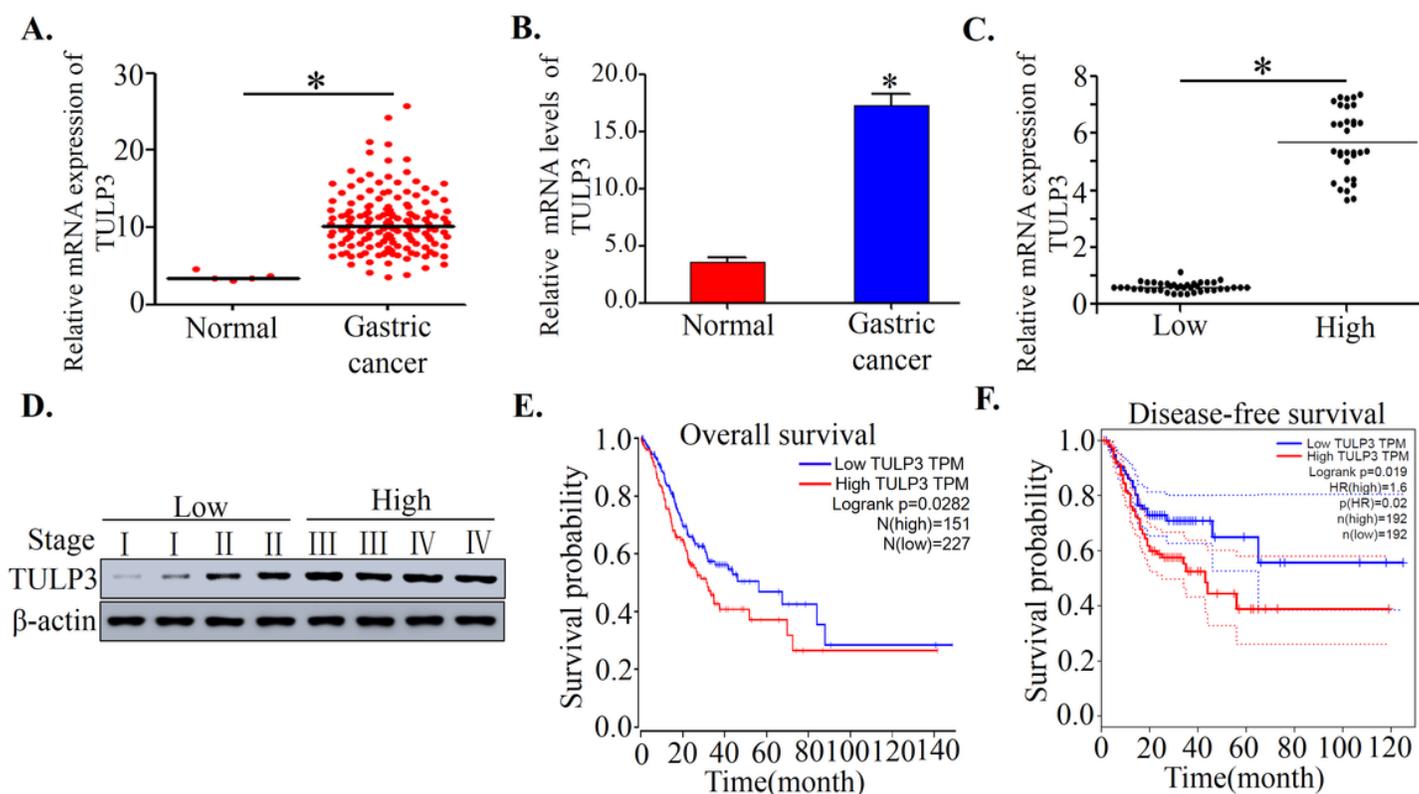


Figure 1

TULP3 is up-regulated in gastric cancer and has clinical value in gastric cancer. a Relative expression of TULP3 mRNA in the gastric cancer specimens (n=156) and normal gastric specimens (n=5) acquired from TCGA. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05. b Real-time PCR was employed for determining the relative expression of TULP3 mRNA in sixty paired normal tissues and gastric cancer tissues. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05. c Real-time PCR was employed for measuring the relative expression of TULP3 mRNA in

the gastric cancer specimens in high clinical stages together with low clinical stages. The data in bar graph are described with mean \pm SEM; *, P value is less than 0.05. d The western blot detection of expression of TULP3 in the gastric cancer specimens in high and low clinical stages. Utilizing β -actin as a loading control. e Kaplan-Meier univariate analyses (log-rank) survival curves of the overall survival for gastric cancer patients with high m-expression (n=151) and low TULP3 expression (n=227). The log-rank detection was employed for the comparison of differences between the two groups (P is equal to 0.0282). f Kaplan-Meier univariate analyses survival curves of the disease-free survival for gastric cancer patients with with high m-expression (n=192) and low TULP3 expression (n=192) tumors. The log-rank detection was employed for the comparison of differences between the two groups (P is equal to 0.019).

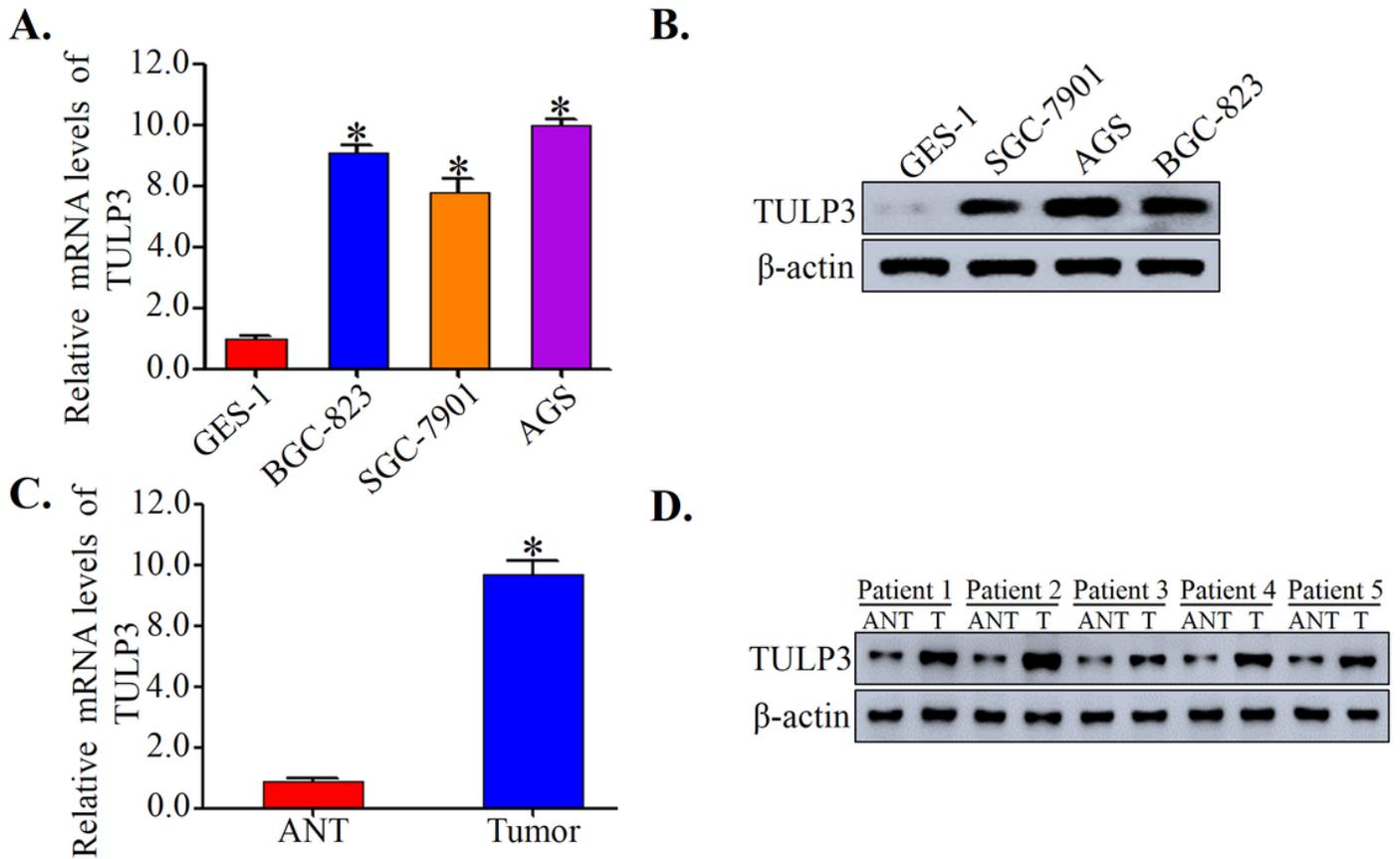


Figure 2

High expression of TULP3 in gastric cancer cell lines and human gastric cancer tissues. a Relative expression of TULP3 mRNA in GES-1 and in cell lines of the gastric cancer (namely, BGC-823, AGS together with SGC-7901) detected through real-time PCR. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05. b The western blot detection of TULP3 expression in GES-1 and in the cell lines of gastric cancer (that is, BGC-823, AGS as well as SGC-7901). Utilizing β -actin as the loading control. c Real-time PCR was conducted for detecting the relative expression of TULP3 mRNA in the matched primary gastric cancer tissues (T) together with the adjacent noncancerous tissues (ANT). The data in bar graph are described with mean \pm SEM; *, P value is less than 0.05. d Western blot assay for the expression of TULP3 in T together with ANT. Utilizing β -actin as the loading control.

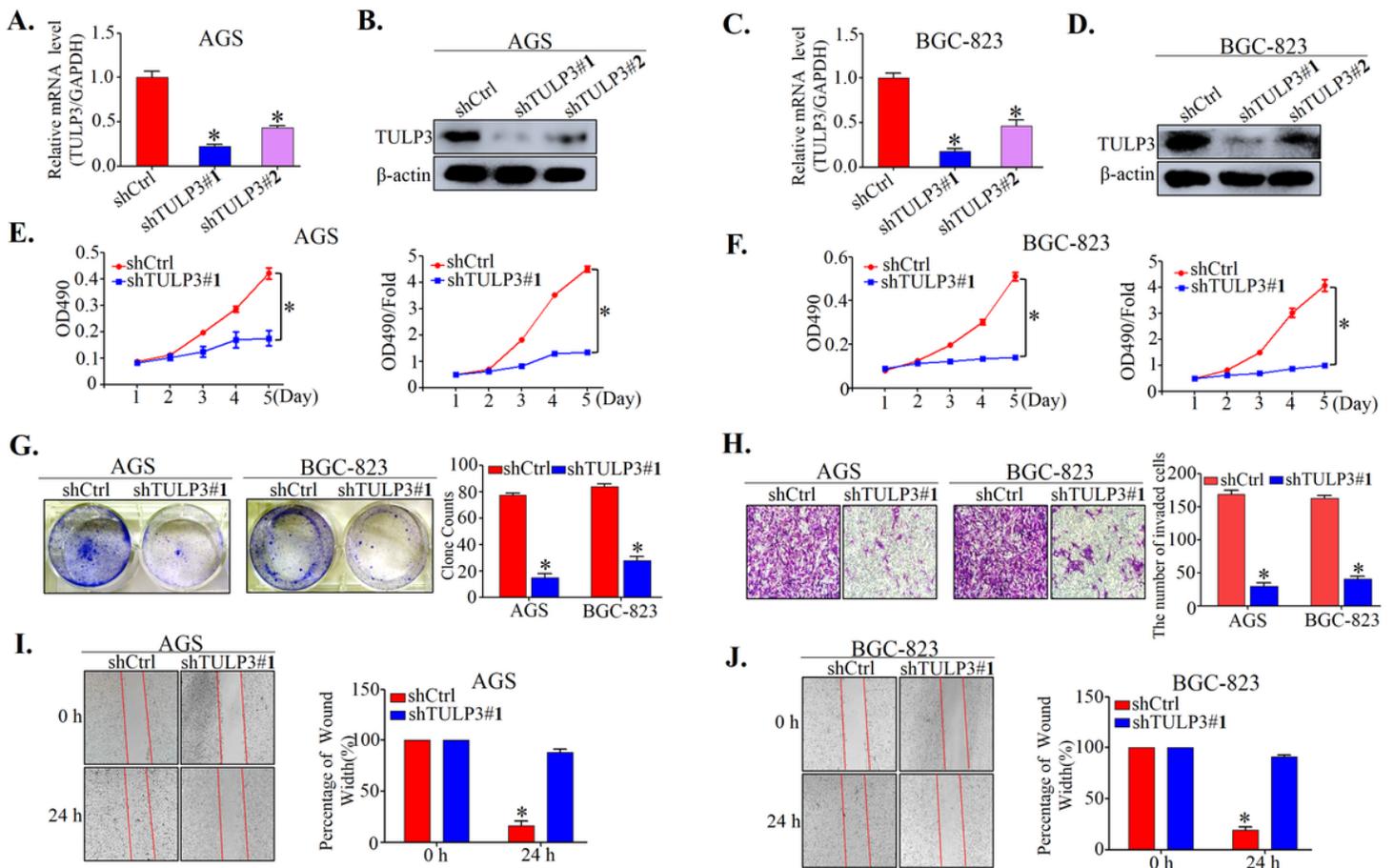


Figure 3

Silencing of TULP3 expression in gastric cancer cells inhibits cell proliferation, migration and invasion in vitro. a The relative expression of TULP3 mRNA in TULP3 shRNA expressed AGS cells was measured with real-time PCR. b The western blot detection for the expression of TULP3 in AGS cells silenced by TULP3. Utilizing β -actin as the loading control. The data in bar graph are described with mean \pm SEM; *, P value is less than 0.05. c Real-time PCR was applied for testing the relative expression of TULP3 mRNA in TULP3 shRNA expressed BGC-823 cells. d The western blot assay for the expression of TULP3 in AGS cells silenced by TULP3. Utilizing β -actin as the loading control. The data in bar graph are described with mean \pm SEM; *, P value is less than 0.05. e MTT tests were employed for investigating the proliferation rates in AGS cells silenced by TULP3. The representative images are exhibited at the bottom of panel. The data in bar graph are described with mean \pm SEM; *, P value is less than 0.05. f MTT tests were employed to explore the proliferation rates in BGC-823 cells silenced by TULP3. The representative images are illustrated at the bottom of panel. The data in bar graph are expressed with mean \pm SEM*, P value is less than 0.05. g The proliferation capacities of the BGC-823 and AGS cells silenced by TULP3 were explored by colony formation assay. The representative images are displayed on the left, and the colonies number was calculated on the right. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05. h The invasion capacities of the BGC-823 and AGS cells silenced by TULP3 were investigated by transwell test. The representative images are reflected on the left, and the metastasis number was calculated on the right. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05.

i The migration capacities of the AGS cells silenced by TULP3 was detected by wound healing test. The representative images are displayed on the left, and the width of wound was detected on the right. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05. j The migration capacities of the BGC-823 cells silenced by TULP3 was measured through wound healing test. The representative images are displayed on the left, and the width of wound was detected on the right. The data in bar graph are expressed with mean \pm SEM; *, P value is less than 0.05.

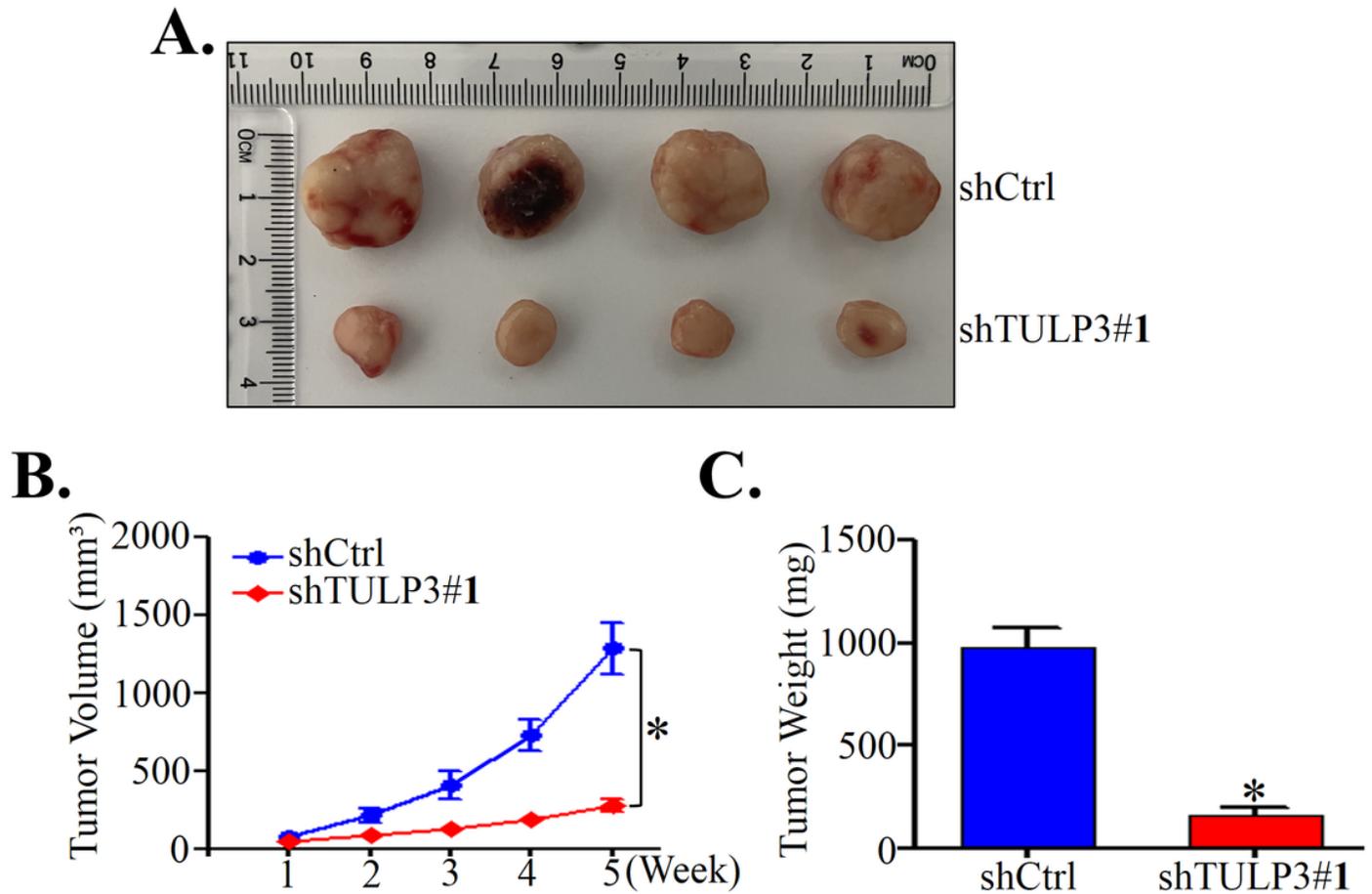


Figure 4

Knockdown of TULP3 inhibits the tumorigenicity of gastric cells in vivo. a Macroscopic view of tumor harvested of two indicates treatment groups (n=4). b Comparison of the tumor volume of two indicated treatment groups at the end-point (n=4). Bar graph data are presented as mean \pm SEM; *, P value is less than 0.05. c Comparison of the tumor weight of two indicates treatment groups at the end-point (n=4). Bar graph data are presented as mean \pm SEM; *, P value is less than 0.05.

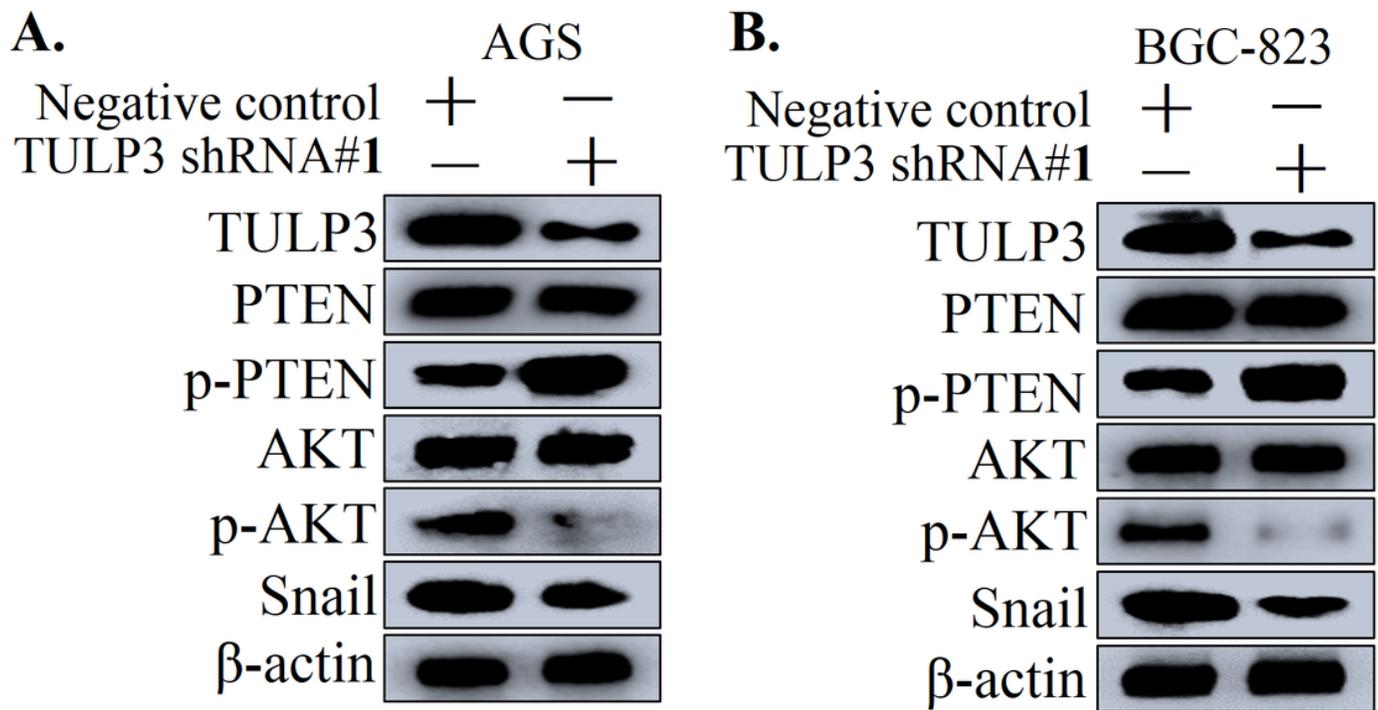
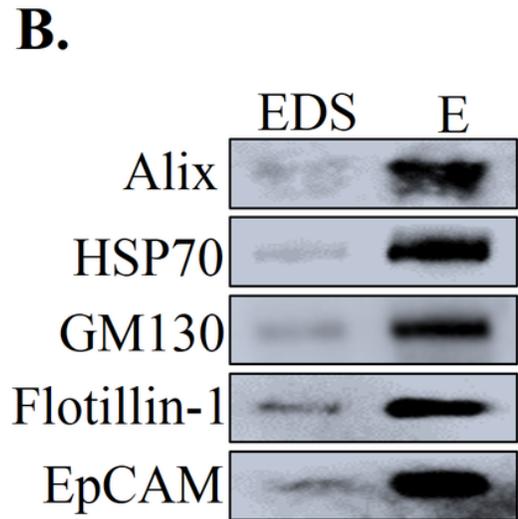
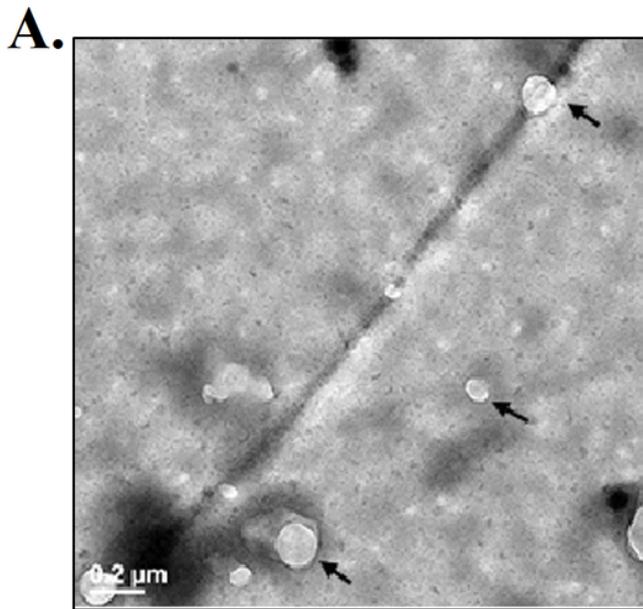


Figure 5

TULP3 regulates gastric cancer cell proliferation, migration and invasion via the PTEN/Akt/Snail signaling pathways. a Western blot assay for response gene protein expression downstream of Snail, Akt and PTEN signaling pathway in AGS cells silenced by TULP3. Utilizing β -actin as the loading control. b Western blot assay of response gene protein expression downstream of Snail, Akt and PTEN signaling pathway in BGC-823 cells silenced by TULP3. Utilizing β -actin as the loading control.



Exosome derived from serum

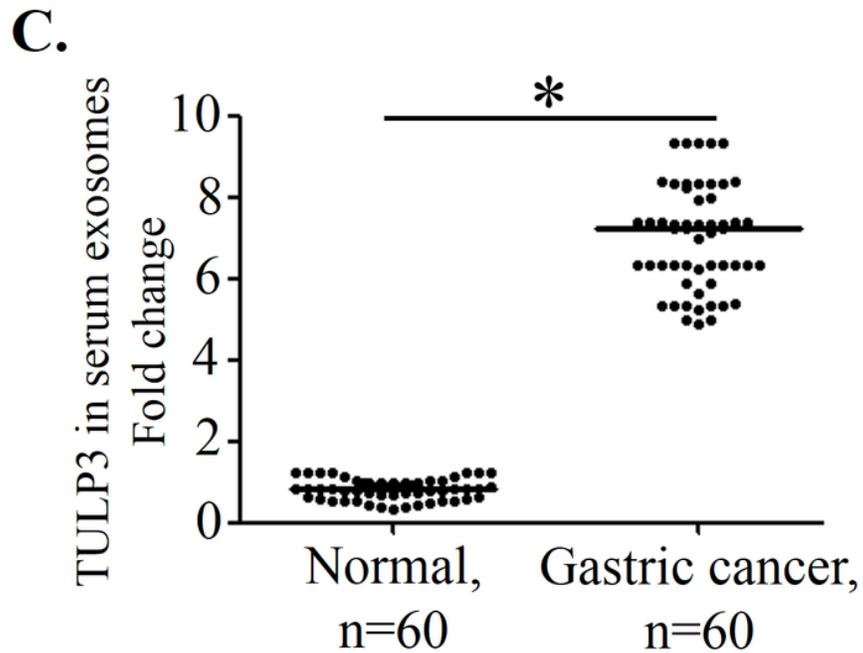


Figure 6

Serum exosomal TULP3 level is upregulated in gastric cancer patients. a The representative pictures of exosomes (black arrow) from serum of patients with gastric cancer examined by electron microscopy. b Western blot assay for the purified serum exosome markers from exosome-depleted supernatant (EDS) and exosome e. c Real-time PCR was applied for testing the relative expression of TULP3 mRNA in the serum exosomes of normal groups and patients with gastric cancer. The data in bar graph are described with mean \pm SEM;*, P value is less than 0.05.

Supplementary Files

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- [SupplementalFigure1.tif](#)