lncRNA TUG1 regulates angiogenesis via the miR-204-5p/JAK2/STAT3 axis in hepatoblastoma

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Abstract. Hepatoblastoma is the most common malignant hepatic tumour type with hypervascularity in early childhood. In recent decades, emerging evidence has proven that long non-coding RNAs (lncRNAs) serve an important oncogenic role in the pathogenesis of hepatoblastoma. However, the underlying mechanism of lncRNA taurine upregulated 1 (TUG1) in the angiogenesis of hepatoblastoma remains unknown. The expression patterns of TUG1 and microRNA (miR)-204-5p were detected in hepatoblastoma tissues and cell lines via reverse transcription-quantitative PCR and were analysed using a Pearson's correlation test. A tube formation assay was performed using human umbilical vein endothelial cells to assess the vasculogenic activity of treated HuH-6 cells. ELISA was used to detect the level of the secretory proangiogenic factor VEGFA in the culture media of HuH-6 cells. A dual luciferase reporter assay was performed to validate the binding relationships of TUG1/miR-204-5p and miR-204-5p/Janus kinase 2 (JAK2). Moreover, western blotting was conducted to measure the protein expression levels of VEGFA, phosphorylated (p)-JAK2, JAK2, p-STAT3 and STAT3. It was identified that TUG1 was upregulated, while miR-204-5p was downregulated in hepatoblastoma tissues and cells. TUG1 knockdown inhibited angiogenesis induced by hepatoblastoma cells. Furthermore, miR-204-5p was identified as a target of TUG1. The results demonstrated that TUG1 attenuated the inhibitory effect of miR-204-5p on the JAK2/STAT3 pathway and promoted angiogenesis in hepatoblastoma cells. In summary, TUG1 was upregulated in hepatoblastoma and suppressed miR-204-5p, thereby activating the downstream signalling pathway of JAK2/STAT3 to facilitate angiogenesis. The present findings will provide novel targets for the treatment of hepatoblastoma.

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Abbreviations: lncRNAs, long non-coding RNAs; HUVECs, human umbilical vein endothelial cells; CM, conditioned media; 3'-UTR, 3'-untranslated region; p-, phosphorylated; miRNA/miR, microRNA; ceRNA, competitive endogenous RNA

Key words: angiogenesis, hepatoblastoma, lncRNA taurine upregulated 1, miR-204-5p

Introduction

Hepatoblastoma is one of the most common primary malignant liver tumour types in children, predominantly occurring in the first 2 years of life (1). The incidence rate is ~1.2/1,000,000 individuals, and it has been increasing in recent years, possibly due to the improved survival of premature and low-birth-weight infants (2,3). The current treatment strategy for hepatoblastoma includes chemotherapy, surgical resection and liver transplantation. Moreover, the application of platinum-based neoadjuvant chemotherapy has significantly improved the clinical outcome of hepatoblastoma (4). In the past three decades, the overall survival has increased from 30 to 70% (1). However, 25% of cases develop metastasis, and the prognosis remains unsatisfactory (5). To date, little is known regarding the molecular basis of the development of hepatoblastoma. Hence, further understanding of the underlying mechanisms is of great significance to identify novel diagnostic biomarkers and to enhance the therapeutic efficacy for patients with hepatoblastoma.

Long non-coding RNAs (lncRNAs) are a class of transcripts with lengths >200 nucleotides that have no or very limited potential to encode proteins (6). It has been reported that lncRNAs are widely involved in the development of a variety of diseases, especially cancer (6). Recently, studies have suggested that lncRNAs are often dysregulated, and may serve important roles in the pathogenesis of hepatoblastoma (7,8).

Taurine upregulated gene 1 (TUG1), a 7.1-kb lncRNA located at chromosome 22q12, has been shown to be upregulated and to contribute to the cell proliferation, migration and angiogenesis of hepatoblastoma, as well as negatively regulate its apoptosis (9,10), which provides a novel potential therapeutic marker for hepatoblastoma treatment. However, how TUG1 acts at the molecular level requires further investigation. In recent years, accumulating experimental evidence has indicated that TUG1 acts as a 'molecular sponge' of microRNAs (miRNAs/miRs) to indirectly participate in post-transcriptional processing and promote tumour development in certain types of cancer (11,12). In a study by He *et al* (13), TUG1 accelerated zinc finger E-box binding homeobox (ZEB) 1-mediated

epithelial-mesenchymal transition (EMT) acquisition by functioning as a competitive endogenous RNA (ceRNA) for miR-142-3p. Moreover, Yu *et al* (14) reported that TUG1 acted as a sponge of miR-204-5p by upregulating RUNX family transcription factor 2 in calcific aortic valve disease.

miRNAs represent a class of small non-coding RNA molecules that function in RNA silencing and gene expression regulation at the post-transcriptional level (3). miRNAs have emerged as novel players in different diseases, especially cancer (6). It has been revealed that miRNAs, such as the miR-100/let-7a-2/miR-125b-1 and miR-371-3 clusters, mediate hepatoblastoma pathogenesis, at least partially, by controlling the abnormally activated Myc or Wnt pathway (3). In addition, miR-204-5p is reported to be a tumour suppressor and is able to regulate cell proliferation and invasion in multiple cancer types, including breast cancer (15) and oesophageal squamous cell carcinoma (16). miR-204 was also found to be dysregulated in lung adenocarcinoma and control the biological behaviours of endothelial cells by targeting the Janus kinase 2 (JAK2)/STAT3 pathway (17). The JAK2/STAT3 pathway regulates the expression of genes associated with proliferation, migration, survival, invasion and angiogenesis (18). STAT3 is required for cancer initiation, development and progression by modulating oncogenes such as cyclin D2 and c-Myc (18). Moreover, Dong *et al* (19) reported that TUG1 expression was significantly upregulated in human hepatoblastoma samples and cell lines, and its upregulation caused VEGFA induction via miR-204-5p, thereby contributing to the hypervascularity of hepatoblastoma. Based on these previous studies, we hypothesized that high expression of TUG1 in hepatoblastoma cells possibly controls angiogenesis via the miR-204-5p/JAK2/STAT3 network.

In the present study, the molecular mechanism underlying TUG1 in hepatoblastoma was investigated and the effect of miR-204-5p was clarified. The present findings provided novel insight into the molecular basis of angiogenesis in hepatoblastoma and shed light on lncRNA-directed therapeutics.

Materials and methods

Tissue collection. All human specimens were collected from patients with diagnosed hepatoblastoma (n=10; 6 male and 4 female; age range, 4 months-4 years) at Hunan Children's Hospital (Changsha, China) between October 2018 and November 2019 with informed consent obtained from every patient's legal guardian. Hepatoblastoma tissues and non-cancerous adjacent tissues (the adjacent tumour tissues (normal controls) were defined as tissues resected ≥3 cm from the tumour margin that tested as normal by histopathologic assessments.) were obtained from the patients during biopsy and/or surgery. The present study was approved by the Hunan Children's Hospital Ethics Committee.

Cell culture. Human hepatic tumour cell lines, HuH-6 and HepG2, and the non-malignant liver cell line THLE-3 were obtained from the American Type Culture Collection, and human umbilical vein endothelial cells (HUVECs) were purchased from the Chinese Academy of Sciences Cell Bank. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Biological Industries), 100 IU/ml penicillin and 100 IU/ml streptomycin (Tianjin Hao Yang Biological Products Technology Co., Ltd.) in a humidified incubator with 5% CO_2 at 37˚C.

Cell transfection. To silence the expression of TUG1, short hairpin RNAs (shRNAs) against TUG1 (shRNA1, shRNA2, shRNA3) and negative control shRNA (shNC) were synthesized by GeneCopoeia, Inc. It was found that shRNA2 had the best silencing effect, and thus, shRNA2 was used for the subsequent experiments. The shRNA sequences used in the study are listed in Table SI. The miR-204-5p mimics (5'-UUCCCUUUGUCAUCCUAUGCCU-3'), inhibitor (5'-AGGCAUAGGAUGACAAAGGGAA-3'), NC (mimics NC, 5'-UUUGUACUACACAAAAGUACUG-3'; inhibitor NC, 5'-CAACGCUGCAUGGUACCAUGCU-3') and siNC (5'-GCGGUUAGCGUCUAUCUGAGU-3'), siJAK2 (5'-UUAAAGAGGAAGAUUUUUCUG-3') were purchased from Sangon Biotech Co., Ltd. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for shRNA and shNC (0.5 μg) transfection, while Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) was used for miR-204-5p (mimics, inhibitor and NC, 50 nM) and siJAK2/siNC (50 nM) transfection at 37˚C for 24 h. Cells were harvested after 48 h of transfection.

Preparation of conditioned media (CM). CM were collected after 3 days of cells being transfected with shNC or shRNA, centrifuged at 1,500 x g at 4˚C for 5 min and then concentrated using a 10-kDa ultrafiltration centrifuge tube (EMD Millipore) at 8,000 x g (4˚C) for 1 h. Subsequently, the CM were either used or stored at -80˚C for subsequent experiments.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA from cells or human samples was harvested using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) on ice according to the manufacturer's instructions, and then reverse-transcribed into cDNA using PrimeScript RT reagent kit (Takara Bio, Inc.). RT was performed in accordance with the Applied Biosystems TaqMan miRNA assay protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 85˚C for 5 sec, 37˚C for 10 min and 4˚C for 15 min, with U6 as the normalizer. qPCR and data collection were conducted with SYBR Green buffer (Takara Bio, Inc.) on an ABI 7500 Fast instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 95˚C for 5 min; followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. The relative RNA expression levels were calculated with the $2^{-\Delta\Delta Cq}$ (20) method and normalized to that of GAPDH (internal control). The primer sequences used in the study were as follows: TUG1 forward (F), 5'-ACGACTGAGCAAGCACTACC-3' and reverse (R), 5'-CTCAGCAATCAGGAGGCACA-3'; miR-204-5p F, 5'-GCCAGATCTGGAAGAAGATGGTGGTTAGT-3' and R, 5'-GGCGAATTCACAGTTGCCTACAGTATTCA-3'; JAK2 F, 5'-GGGAGGTGGTCGCTGTAAAA-3' and R, 5'-ACCAGCACTGTAGCACACTC-3'; GAPDH F, 5'-TGTGTCCGTCGTGGATCTGA-3' and R, 5'-CCTGCTTCACCACCTTCTTGA-3'; and U6 F, 5'-GCTTCGGCAGCACATATACTAA-3' and R, 5'-AACGCTTCACGAATTTGCGT-3'.

ELISA for VEGFA detection. A total of 5 days after culture of HuH-6 cells with shNC and shTUG1, the release of pro-angiogenic VEGFA from the respective medium samples (CM) was quantitatively detected using a human VEGFA ELISA kit (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. BMS277-2) in accordance with the manufacturer's protocols. Recombinant VEGFA was used as the standard. The optical density was measured using a microplate reader (Molecular Devices LLC) at a wavelength of 450 nm. The experiments were performed independently three times with three replicates each.

Tube formation assay. CM were collected from HuH-6 cells transfected with shTUG1 or shNC. A total of $8x10^4$ HUVECs were seeded in 96-well plates precoated with 50 μl per well Matrigel Basement Membrane Matrix (BD Bioscience; 45 min at 37˚C), and incubated with 100 μl per well of the indicated CM for 8 h at 37˚C. Images of tubular structures were captured using an inverted light microscope at x100 magnification. The cumulative tube length and total branching length were analysed and quantified using ImageJ software (National Institutes of Health; v1.8.0).

Plasmid construction. To knock down the expression of TUG1, shRNA1, shRNA2, shRNA3 and negative control shRNA (shNC) were constructed by GeneCopoeia, Inc. Packaging and amplification of lentiviruses were conducted in 293T cells (American Type Culture Collection) according to standard protocols. HuH-6 cells were infected with lentivirus carrying shRNA or shNC (0.5 μg) for 24 h at 37˚C and subsequently cultured under selection with 1 μg/ml puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) for 3 weeks.

Luciferase reporter assay. StarBase V2.0 (http://starbase.sysu.edu.cn) analysis was performed to predict the binding site of miR-204-5p with lncRNA TUG1 and JAK2. HuH-6 cells were co-transfected with pGL3 luciferase reporter (Promega Corporation) plasmids (2 μg) containing the human JAK2 (or TUG1) 3'-untranslated region (3'-UTR) with putative miR-204-5p binding sites or the corresponding mutant (MUT), and miR-204-5p mimics or the NC mimics (50 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 6 h. After 48 h, cells were collected and lysed with Passive Lysis Buffer. The levels of firefly luciferase were consecutively measured using the Dual Glo Luciferase Assay system (Promega Corporation) on the MultiScan MCC/340 system (Thermo Fisher Scientific, Inc.) following the manufacturer's manual, with *Renilla* luciferase activity as the internal control to eliminate the variability resulting from transfection efficiency.

Protein extraction and western blotting. Cells were collected and lysed on ice in RIPA buffer (Santa Cruz Biotechnology, Inc.) in the presence of 0.1% protease inhibitor PMSF (Thermo Fisher Scientific, Inc.). Total protein was quantified using Bradford assays (Bio-Rad Laboratories, Inc.). Equal amounts of protein samples (50 μg) were loaded and separated by denaturing 10% SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). After blocking with 5% non-fat milk for 4 h at 37˚C, the membranes were incubated with various primary antibodies at 4˚C overnight, and this was followed by incubation with HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized with Tanon 5200 using a ECL kit (Thermo Fisher Scientific, Inc.). Antibodies were diluted as follows: Anti-VEGFA (Invitrogen; Thermo Fisher Scientific, Inc.; 1:1,000; cat. no. MA5-12184), anti-phosphorylated (p)-JAK2 [Cell Signaling Technology, Inc. (CST); 1:1,000; cat. no. 3771S], anti-JAK2 (Santa Cruz Biotechnology, Inc.; 1:1,000; cat. no. sc-390539), anti-p-STAT3 (CST; 1:1,000; cat. no. 9145T), anti-STAT3 (Abcam; 1:1,000; cat. no. ab68153), anti-GAPDH (HUABIO; 1:5,000; cat. no. ER1706-83) and goat anti-rabbit or mouse secondary antibody (HUABIO; 1:5,000; cat. no. HA1019/HA1020). Protein bands were quantified using ImageJ software (v1.8.0; National Institutes of Health) with GAPDH as the loading control.

Statistical analysis. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc.). Data are presented as the mean ± SD. Statistical analyses were performed with unpaired Student's t-test (two-tailed) and one-way ANOVA following by Tukey's post hoc test. Pearson's analysis was used to analysis the corelation of TUG1 and miR-204-5p. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed three times independently.

Results

TUG1 is upregulated, while miR-204-5p is downregulated in hepatoblastoma tissues and cell lines. It has been previously reported that TUG1 is abnormally expressed in hepatoblastoma cell lines (17). The present study further collected 10 paired hepatoblastoma specimens and their paracancerous tissue specimens to detect the expression level of TUG1. As shown in Fig. 1A, TUG1 expression was significantly higher in tumour samples compared with in normal controls.

miR-204-5p was reported to participate in angiogenesis in ovarian cancer (21). The present study aimed to determine its role in hepatoblastoma. In contrast to TUG1 expression, miR-204-5p expression was found to be ~50% downregulated in hepatoblastoma tissues (Fig. 1B). Using Pearson's analysis, TUG1 was found to be negatively correlated with miR-204-5p (Fig. 1C). Consistent with the results in the tumour specimens, upregulation of TUG1 and downregulation of miR-204-5p were also observed in the human hepatoblastoma cell lines HuH-6 and HepG2 compared with the non-malignant liver cell line THLE-3 (Fig. 1D and E). Taken together, these data indicated the potential relationship between TUG1 and miR-204-5p in hepatoblastoma.

Knockdown of TUG1 suppressed angiogenesis in vitro. To investigate the role of TUG1 in tumour angiogenesis, HuH-6 cells were selected for further study as the expression of TUG1 was higher in HuH-6 cells. TUG1 expression was knocked down via transfection with shTUG1 plasmid (shRNA for TUG1, shRNA1, shRNA2, shRNA3) or a negative control shRNA (shNC), and shRNA2 had the best silencing effect (Fig. S1). Thus, shRNA2 was used for the subsequent experiments to knockdown TUG1 in HuH-6 cells (Fig. 2A), and then the CM were collected. The mRNA expression level of VEGFA, the key signalling molecule involved in pathological

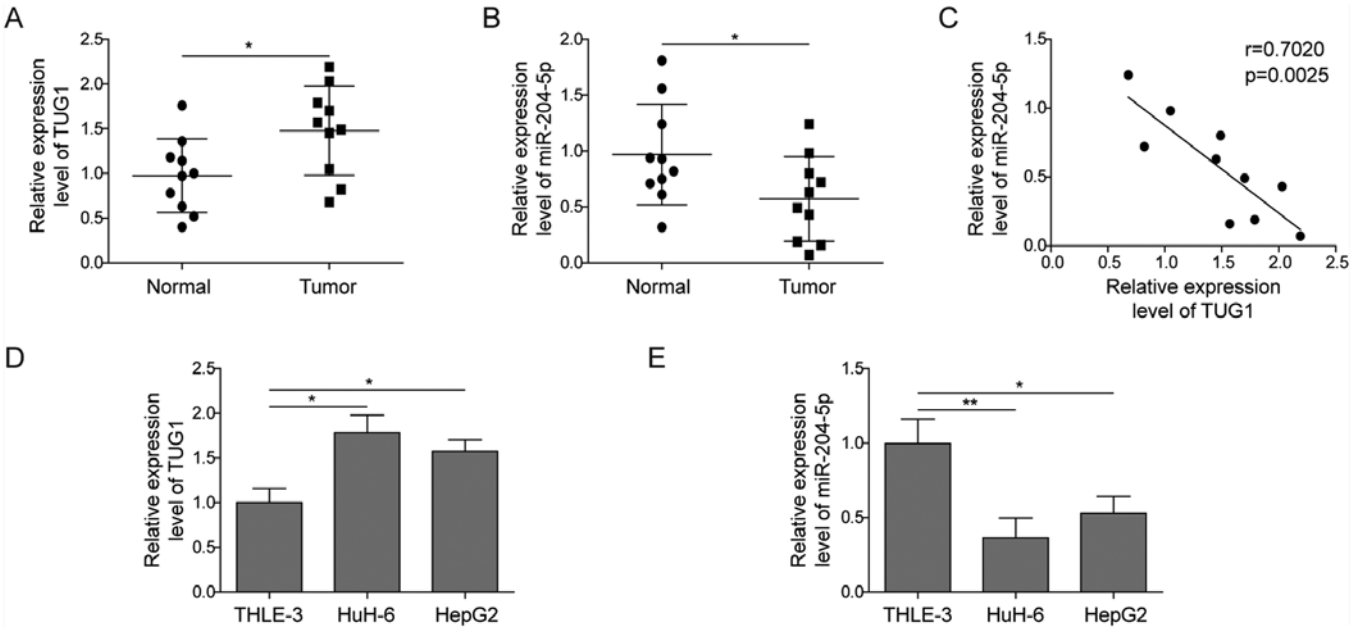

Figure 1. TUG1 expression is elevated, while miR-204-5p is decreased in hepatoblastoma tissues and cell lines. The expression levels of (A) TUG1 and (B) miR-204-5p in hepatoblastoma tissues and adjacent normal tissues (n=10) were detected via RT-qPCR. (C) Relationship between TUG1 and miR-204-5p expression levels was analysed using Pearson's correlation. (D) TUG1 and (E) miR-204-5p expression levels in the hepatoblastoma cell lines THLE-3, HuH-6 and HepG2 were detected via RT-qPCR. *P<0.05, **P<0.01. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; TUG1, taurine upregulated 1.

angiogenesis, was significantly decreased after transfection of HuH-6 cells with shTUG1 (Fig. 2B), and similar results were obtained for its protein expression (Fig. 2C). As presented in Fig. 2C, it was observed that, knockdown of TUG1 resulted in significantly decreased protein expression levels of VEGFA, p-JAK2, JAK2 and p-STAT3. Moreover, a Matrigel-based tube formation assay using HUVECs was also conducted. Reduced tube formation was observed in HUVECs cultured in the presence of CM derived from HuH-6 cells transfected with shTUG1 (Fig. 2D). These data indicated that TUG1 was potentially involved in angiogenesis in hepatoblastoma.

TUG1 acts as a sponge of miR-204-5p. Since cytoplasmic lncRNA was found to act as a natural miRNA sponge to interfere with miRNA-targeted genes at the post-transcriptional level (22), the present study further investigated the relationship between TUG1 and miR-204-5p. The sequence of TUG1 containing the putative binding site of miR-204-5p (Fig. 3A) (11) was cloned into the 3'-UTR of the luciferase reporter [Fluc-TUG1-wild-type (WT)] and transfected into HuH-6 cells along with miR-204-5p mimics or the NC mimics. As presented in Fig. 3B, luciferase activity was decreased in HuH-6 cells co-transfected with Fluc-TUG1-WT and miR-204-5p. However, when the putative binding site of miR-204-5p on the TUG1 sequence was mutated (Fluc-TUG1-MUT), there was no change in promoter activity compared with the control, which was designed to prevent non-specific binding. The effect of TUG1 on miR-204-5p was also demonstrated, indicating the binding of TUG1 with miR-204-5p (Fig. S2). Overexpression of TUG1 significantly decreased the expression of miR-204-5p, whereas its knockdown increased the expression of miR-204-5p (Fig. 3C). Collectively, these data suggested that miR-204-5p interacted with TUG1 in hepatoblastoma cells.

miR-204-5p directly targets JAK2 to regulate the JAK2/STAT3 pathway. A putative binding site between miR-204-5p and JAK2 was predicted using StarBase (Fig. 4A). To determine whether miR-204-5p directly targeted JAK2 and regulated the downstream signalling pathway in hepatoblastoma, a luciferase reporter assay was conducted in HuH-6 cells. As shown in Fig. 4B, it was found that co-transfection of miR-204-5p mimics inhibited the luciferase activity of the WT JAK2 3'-UTR, which contained the theoretical miR-204-5p binding site, but failed to suppress that of the MUT JAK2 3'-UTR. Transfection of miR-204-5p mimics increased the expression of miR-204-5p, while transfection of miR-204-5p inhibitor decreased the expression of miR-204-5p (Fig. 4C). In addition, overexpression of miR-204-5p resulted in a reduction in JAK2 mRNA expression (Fig. 4D), as well as in the protein expression levels of JAK2, p-JAK2 and p-STAT3 (Fig. 4E). Moreover, addition of the miR-204-5p inhibitor significantly increased JAK2 mRNA expression (Fig. 4D) and the protein expression levels of JAK2, p-JAK2 and p-STAT3 (Fig. 4E). These results demonstrated that endogenous JAK2 was targeted by miR-204-5p, which further affected the downstream STAT3 pathway in hepatoblastoma cells.

TUG1 promotes angiogenesis via the miR-204-5p/JAK2/STAT3 network in hepatoblastoma. Next, it was evaluated whether TUG1 could regulate the JAK2/STAT3 cascade via miR-204-5p. The mRNA expression of JAK2 was decreased following transfection with siJAK2 (Fig. S3). TUG1 knockdown increased the expression of miR-204-5p (Fig. 5A) and decreased the mRNA expression of JAK2 (Fig. 5B). The trend was reversed after co-transfection with shTUG1 and miR-204-5p. However, the mRNA expression of JAK2 was decreased following transfection with siJAK2. The expression level of the secretory proangiogenic factor VEGFA in the culture media of HuH-6

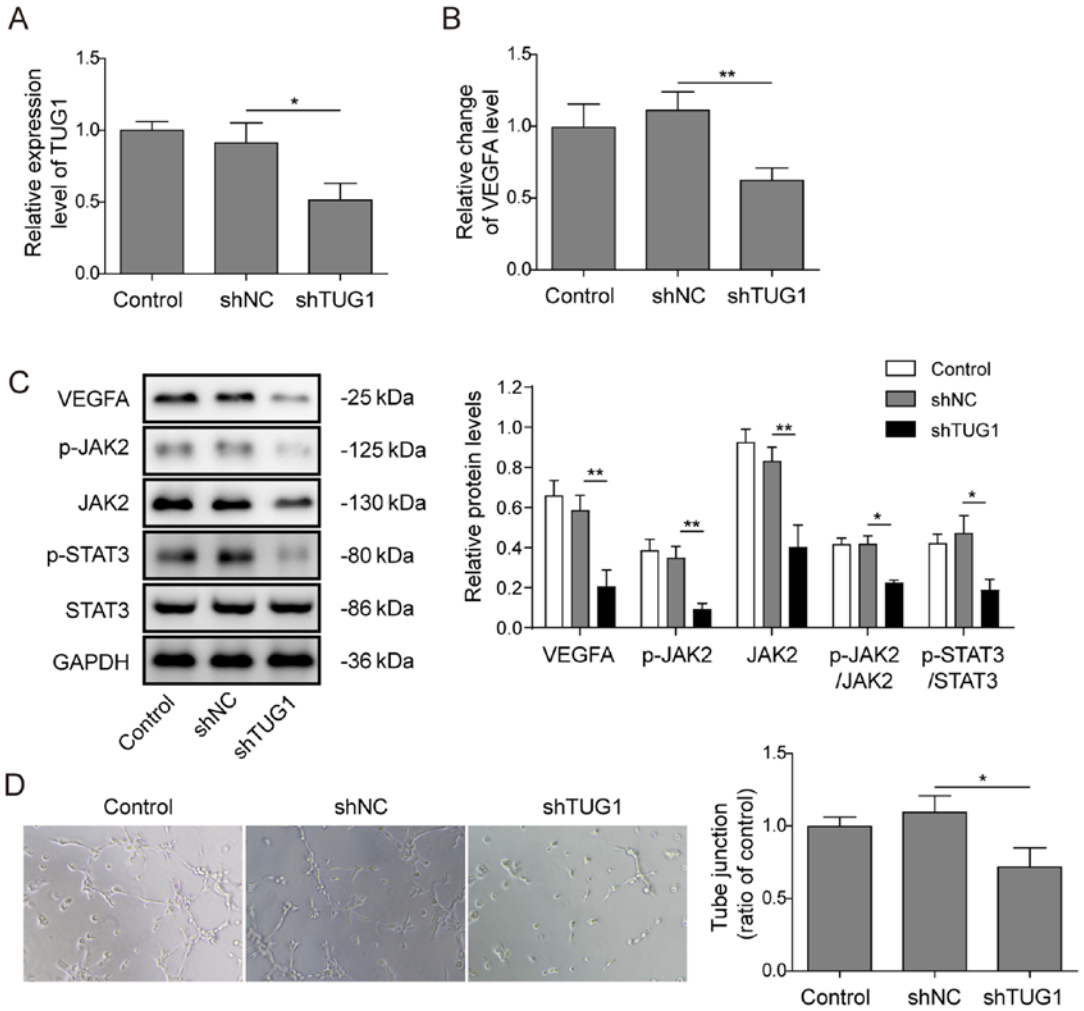

Figure 2. Knockdown of TUG1 inhibits angiogenesis *in vitro*. (A) Expression level of TUG1 after transfection with shTUG1 was detected via reverse transcription-quantitative PCR in HuH-6 cells. (B) The level of the secretory proangiogenic factor VEGFA in the culture media of HuH-6 cells was detected using ELISA. (C) Protein expression levels of VEGFA, p-JAK2, JAK2, p-STAT3 and STAT3 in HuH-6 cells were detected via western blotting. GAPDH was used as the internal control. (D) Human umbilical vein endothelial cells were cultured in 96-well plates precoated with Matrigel and conditioned media derived from shTUG1- or shNC-transfected HuH-6 cells. Representative images of tube formation in these groups were obtained using light microscopy at x100 magnification. *P<0.05, **P<0.01. sh, short hairpin RNA; TUG1, taurine upregulated 1; p, phosphorylated; NC, negative control.

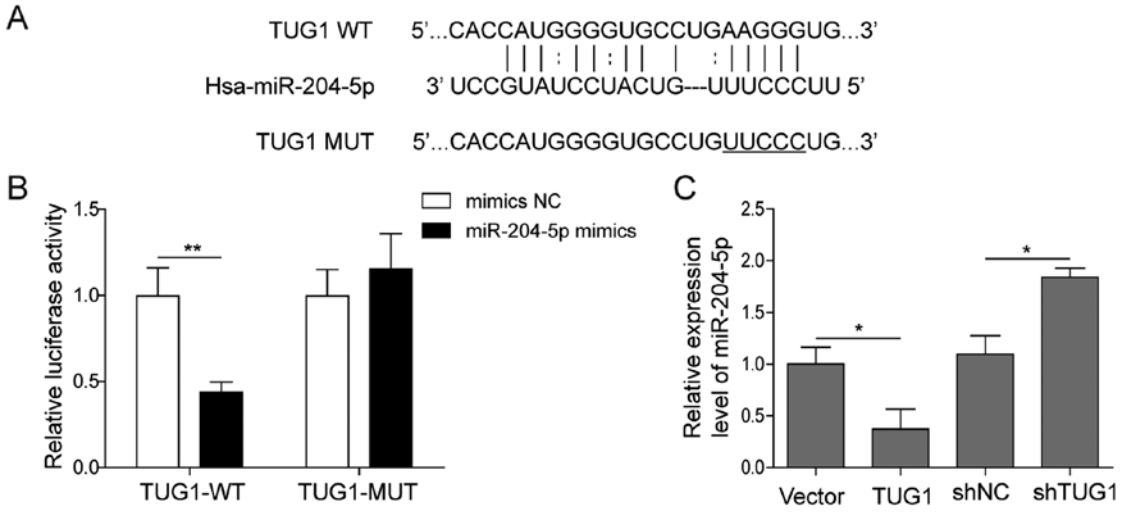

Figure 3. TUG1 acts as a sponge of miR-204-5p. (A) Putative binding site of TUG1 with miR-204-5p (9). (B) Fluc-TUG1-WT or Fluc-TUG1-MUT was co-transfected with miR-204-5p mimics or mimic NC. Luciferase activity was detected using a dual luciferase reporter assay system. (C) Relative expression of miR-204-5p after transfection of HuH-6 cells with TUG1 or shTUG1 was detected using reverse transcription-quantitative PCR. *P<0.05, **P<0.01. sh, short hairpin RNA; TUG1, taurine upregulated 1; p, phosphorylated; NC, negative control; miR, microRNA; WT, wild-type; MUT, mutant.

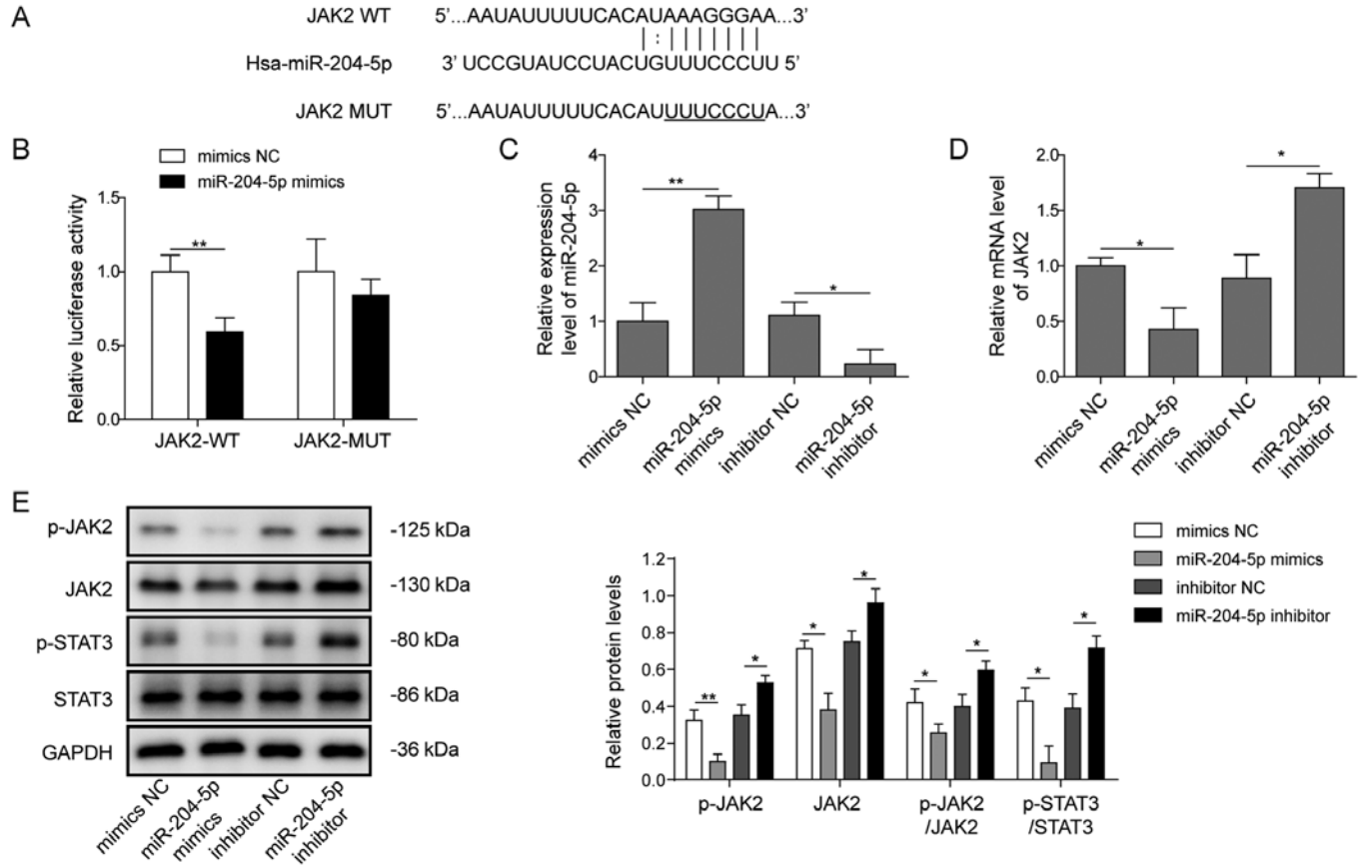

Figure 4. miR-204-5p targets JAK2 to regulate the JAK2/STAT3 pathway. (A) The putative binding site of miR-204-5p on JAK2 was predicted using StarBase. (B) Relative luciferase activity of JAK2-WT and JAK2-MUT co-transfected with mimics NC or miR-204-5p mimics was reported using a dual luciferase reporter assay. (C) miR-204-5p was detected via RT-qPCR after transfection of HuH-6 cells with miR-204-5p mimics or inhibitor. (D) Relative mRNA expression level of JAK2 after transfection with miR-204-5p mimics or inhibitor, as detected via RT-qPCR. (E) Representative western blotting images and semi-quantification of p-JAK2, JAK2, p-STAT3 and STAT3 protein expression levels after transfection with miR-204-5p mimics or inhibitor. *P<0.05, **P<0.01. p, phosphorylated; NC, negative control; miR, microRNA; WT, wild-type; MUT, mutant; JAK, Janus kinase 2; RT-qPCR, reverse transcription-quantitative PCR.

cells was decreased after transfection with shTUG1, while the expression level of VEGFA was increased after transfection with the miR-204-5p inhibitor. Moreover, VEGFA expression was restored after co-transfection of shTUG1 and the miR-204-5p inhibitor or miR-204-5p inhibitor and siJAK2 (Fig. 5C).

The protein expression levels of downstream effectors of the JAK2/STAT3 pathway were also detected. Knockdown of TUG1 significantly downregulated the protein expression levels of JAK2, p-JAK2, p-STAT3 and VEGFA (Fig. 5D). The same trend was observed in HepG2 cells (Fig. S4). However, the decrease was reversed by the miR-204-5p inhibitor, which suggested that TUG1 controlled the JAK2/STAT3 pathway via miR-204-5p.

To determine the role of TUG1 and miR-204-5p in the angiogenesis of hepatoblastoma, a tube formation assay was performed using HUVECs. It was found that tube junctions in HUVECs cultured in CM of shTUG1 HuH-6 cells were fewer compared with those in the control group, whereas the loss of CM angiogenic activity was countered by the addition of the miR-204-5p inhibitor to shTUG1-transfected HuH-6 cells (Fig. 5E). When siJAK2 was transfected into HuH-6 cells, the upregulation of VEGFA and the tube formation activity of the CM induced by the miR-204-5p inhibitor was partially suppressed, which confirmed that miR-204-5p regulated angiogenesis via JAK2/STAT3 signalling (Fig. 5D and E). Overall, TUG1 acted as a sponge of miR-204-5p, which activated the JAK2/STAT3 pathway and promoted the expression of VEGFA, leading to increased angiogenesis activity.

Discussion

Hepatoblastoma is a type of paediatric cancer arising from hepatic progenitors or hepatoblasts (23). In recent decades, there have been considerable improvements in the treatment of hepatoblastoma, such as the advances in cisplatin-based chemotherapy, and the overall 5-year survival has reached 70-80% (24). However, ~20% of children diagnosed with hepatoblastoma have pulmonary metastasis, which is associated with a very poor prognosis (25-50% survival rate) (25). Thus, it is of great clinical necessity to improve the early diagnosis and inhibit the progression of hepatoblastoma.

The dysregulation of lncRNAs and their correlation with the prognosis of patients with hepatoblastoma have been widely reported. Genome-wide analysis of lncRNA expression has also been conducted to evaluate lncRNAs that can serve as potential clinical targets or biomarkers for hepatoblastoma (26). The present study demonstrated that lncRNA

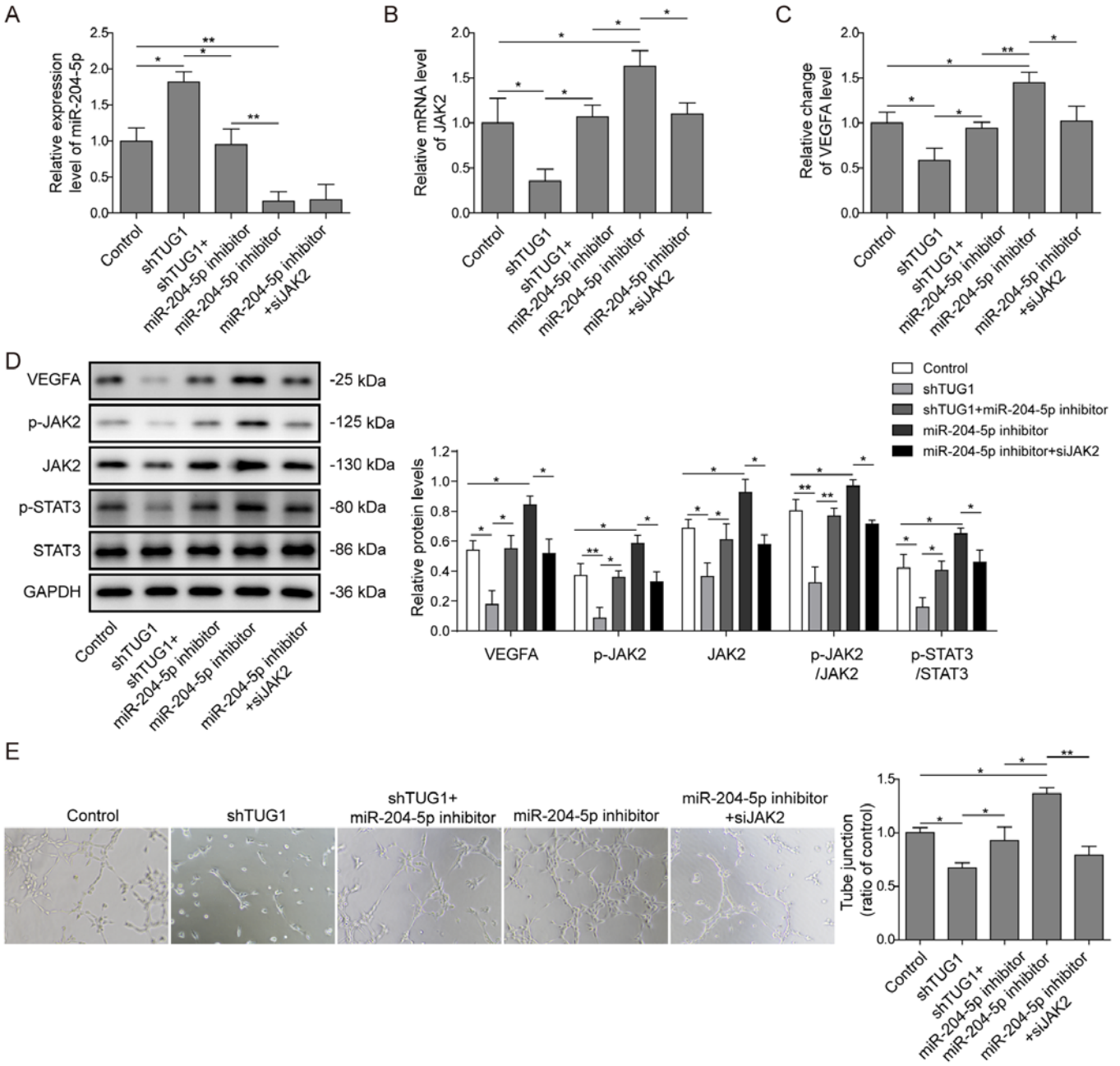

Figure 5. TUG1 modulates angiogenesis via miR-204-5p/JAK2/STAT3. HuH-6 cells were transfected with shTUG1 or miR-204-5p inhibitor or co-transfected with shTUG1 and miR-204-5p or siJAK2 and miR-204-5p. Relative expression levels of (A) miR-204-5p and (B) JAK2 mRNA were detected via reverse transcription-quantitative PCR. (C) The level of the secretory proangiogenic factor VEGFA in the culture media of HuH-6 cells was detected using ELISA. (D) Protein expression levels of VEGFA, p-JAK2, JAK2, p-STAT3 and STAT3 were measured using western blot analysis. (E) Human umbilical vein endothelial cells were cultured in 96-well plates precoated with Matrigel and conditioned media derived from treated HuH-6 cells. Representative graphs and tube junctions in these groups were obtained via light microscopy at x100 magnification. *P<0.05, **P<0.01. p, phosphorylated; miR, microRNA; JAK2, Janus kinase 2; sh, short hairpin RNA; TUG1, taurine upregulated 1; si, small interfering RNA.

TUG1 was highly expressed in hepatoblastoma tumour tissues and cell lines, which promoted the unusual hypervascularity.

Recent evidence has revealed the intricate interplay among diverse RNA transcripts, including mRNAs, lncRNAs and miRNAs. These molecules communicate and work together to co-regulate the expression of targeted genes, and have gained substantial attention (22). In the present study, the interaction between TUG1 and miR-204-5p could be explained by the ceRNA hypothesis. In normal hepatic cells, the expression levels of TUG1 and miR-204-5p maintained a balance. However, in hepatoblastoma cells, TUG1 was significantly upregulated, which attenuated the inhibitory effect of miR-204-5p on VEGFA and resulted in hypervascularity effects.

miRNAs are a class of small non-coding single-stranded RNA molecules containing 19-25 nucleotides that function as important post-transcriptional gene expression regulators via base pairing with specific targeted mRNAs (3). Importantly, via gene manipulation, miRNAs serve key roles in cell proliferation, invasion, differentiation, angiogenesis and metastasis, amongst other processes (27). Moreover, miRNAs were found to participate in the activation of angiogenesis by targeting the angiogenic factors VEGFA and MET (28). The present

study identified that miR-204-5p participated in the process of angiogenesis in hepatoblastoma by promoting the production of VEGFA, which is consistent with previous research (29). Furthermore, Tan *et al* (30) reported that TUG1/miR-145/ZEB2 was dysregulated in human bladder cancer and promoted EMT, as well as radio resistance, which indicated the tumour promoter role of TUG1 and was in accordance with the current findings. However, whether TUG1 can regulate hepatoblastoma through binding with other miRNA? It is needed to further exploration.

Aberration of the JAK2/STAT3 signalling pathway is involved in several oncogenic processes in solid tumours (31). Mutations in JAK1 and JAK2, which result in constitutive activation of STAT3, are frequently reported in various haematopoietic malignancies (32). The present study demonstrated that JAK2/STAT3 was involved in the angiogenesis of hepatoblastoma by regulating the expression of VEGFA, which is in accordance with the previously reported role of JAK2/STAT3 in cancer cells. For example, Xue *et al* (33) revealed that inhibition of JAK/STAT3 attenuated angiogenesis in an endothelial cell/adipose-derived stromal cell co-culture 3D model, and this process was mediated by decreasing VEGFA and cyclin D1 expression, which is consistent with the current observations. JAK inhibitors are already under clinical evaluation for the treatment of these diseases (33), and these may be expected to be used in the inhibition of angiogenesis of hepatoblastoma in further studies.

It is the limitations of the present study that TUG1 promote the angiogenesis of hepatoblastoma maybe through binding other miRNA and pathways, and miR-204-5p maybe have other target genes. However, we have only explored the TUG1/miR-204-5p/JAK2/STAT3 pathway, and other pathways was required further study.

In conclusion, the present study determined that TUG1 promoted the angiogenesis of hepatoblastoma via the miR-204-5p/JAK2/STAT3 axis. Further studies should investigate the possibility of developing effective drugs targeting this pathway in hepatoblastoma. Future studies will examine the molecular mechanism of TUG1 in hepatoblastoma in animal models. Whether TUG1 can regulate hepatoblastoma through other pathways requires further study.

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Availability of data and materials

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

Authors' contributions

MXY was the guarantor of integrity of the entire study, conceptualised and designed the study, defined the intellectual content and performed the literature search, as well as conducted the data/statistical analysis. MXY, CYJ, HQG, XYS, WXX and QY performed the clinical studies. MXY, CYJ, HQG, XYS and WXX performed the experimental studies and data acquisition. MXY and CYJ prepared and edited the manuscript. QY reviewed the manuscript. MXY and QY confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Hunan Children's Hospital Ethics Committee. All legal guardians of patients were informed of the study and signed the written consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yang T, Whitlock RS and Vasudevan SA: Surgical management of hepatoblastoma and recent advances. Cancers (Basel) 11: 1944, 2019.
2. Sharma D, Subbarao G and Saxena R: Hepatoblastoma. Semin Diagn Pathol 34: 192-200, 2017.
3. Cristóbal I, Sanz-Álvarez M, Luque M, Caramés C, Rojo F and García-Foncillas J: The role of microRNAs in hepatoblastoma tumors. Cancers (Basel) 11: 409, 2019.
4. Venkatramani R, Wang L, Malvar J, Dias D, Sposto R, Malogolowkin MH and Mascarenhas L: Tumor necrosis predicts survival following neo-adjuvant chemotherapy for hepatoblastoma. Pediatr Blood Cancer 59: 493-498, 2012.
5. Bell D, Ranganathan S, Tao J and Monga SP: Novel advances in understanding of molecular pathogenesis of hepatoblastoma: A Wnt/β-catenin perspective. Gene Expr 17: 141-154, 2017.
6. Huarte M: The emerging role of lncRNAs in cancer. Nat Med 21: 1253-1261, 2015.
7. Luo Z and Cao P: Long noncoding RNA PVT1 promotes hepatoblastoma cell proliferation through activating STAT3. Cancer Manag Res 20: 8517-8527, 2019.
8. Chen LJ, Yuan MX, Ji CY, Zhang YB, Peng YM, Zhang T, Gao HQ, Sheng XY, Liu ZY, Xie WX and Yin Q: Long non-coding RNA CRNDE regulates angiogenesis in hepatoblastoma by targeting the MiR-203/VEGFA axis. Pathobiology 87: 161-170, 2020.
9. Xie F, Zhang L, Yao Q, Shan L, Liu J, Dong N and Liang J: TUG1 promoted tumor progression by sponging miR-335-5p and regulating CXCR4-Mediated infiltration of pro-tumor immunocytes in CTNNB1-mutated hepatoblastoma. Onco Targets Ther 14: 3105-3115, 2020.
10. Li Q, Zhang J, Su DM, Guan LN, Mu WH, Yu M, Ma X and Yang RJ: lncRNA TUG1 modulates proliferation, apoptosis, invasion, and angiogenesis via targeting miR-29b in trophoblast cells. Hum Genomics 13: 50, 2019.
11. Lei H, Gao Y and Xu X: lncRNA TUG1 influences papillary thyroid cancer cell proliferation, migration and EMT formation through targeting miR-145. Acta Biochim Biophys Sin (Shanghai) 49: 588-597, 2017.
12. Zhou H, Gao Z and Wan F: Taurine-upregulated gene 1 contributes to cancers through sponging microRNA. Acta Biochim Biophys Sin (Shanghai) 51: 123-130, 2019.
13. He C, Liu Z, Jin L, Zhang F, Peng X, Xiao Y, Wang X, Lyu Q and Cai X: lncRNA TUG1-Mediated Mir-142-3p downregulation contributes to metastasis and the epithelial-to-mesenchymal transition of hepatocellular carcinoma by targeting ZEB1. Cell Physiol Biochem 48: 1928-1941, 2018.

14. Yu C, Li L, Xie F, Guo S, Liu F, Dong N and Wang Y: lncRNA TUG1 sponges miR-204-5p to promote osteoblast differentiation through upregulating Runx2 in aortic valve calcification. Cardiovasc Res 114: 168-179, 2017.
15. Hong BS, Ryu HS, Kim N, Kim J, Lee E, Moon H, Kim KH, Jin MS, Kwon NH, Kim S, *et al*: Tumor suppressor miRNA-204-5p regulates growth, metastasis, and immune microenvironment remodeling in breast cancer. Cancer Res 79: 1520-1534, 2019.
16. Tang J, Li Z, Zhu Q, Wen W, Wang J, Xu J, Wu W, Zhu Y, Xu H and Chen L: miR-204-5p regulates cell proliferation, invasion, and apoptosis by targeting IL-11 in esophageal squamous cell carcinoma. J Cell Physiol 235: 3043-3055, 2020.
17. Liu X, Gao X, Zhang W, Zhu T, Bi W and Zhang Y: MicroRNA-204 deregulation in lung adenocarcinoma controls the biological behaviors of endothelial cells potentially by modulating Janus kinase 2-signal transducer and activator of transcription 3 pathway. IUBMB Life 70: 81-91, 2018.
18. Wang X, Crowe PJ, Goldstein D and Yang JL: STAT3 inhibition, a novel approach to enhancing targeted therapy in human cancers (review). Int J Oncol 41: 1181-1191, 2012.
19. Dong R, Liu GB, Liu BH, Chen G, Li K, Zheng S and Dong KR: Targeting long non-coding RNA-TUG1 inhibits tumor growth and angiogenesis in hepatoblastoma. Cell Death Dis 7: e2278, 2016.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Chen X, Mangala LS, Mooberry L, Bayraktar E, Dasari SK, Ma S, Ivan C, Court KA, Rodriguez-Aguayo C, Bayraktar R, *et al*: Identifying and targeting angiogenesis-related microRNAs in ovarian cancer. Oncogene 38: 6095-6108, 2019.
22. Thomson DW and Dinger ME: Endogenous microRNA sponges: Evidence and controversy. Nat Rev Genet 17: 272-283, 2016.
23. Ranganathan S, Lopez-Terrada D and Alaggio R: Hepatoblastoma and pediatric hepatocellular carcinoma: An update. Pediatr Dev Pathol 23: 79-95, 2020.
24. Angelico R, Grimaldi C, Gazia C, Saffioti MC, Manzia TM, Castellano A and Spada M: How do synchronous lung metastases influence the surgical management of children with hepatoblastoma? An update and systematic review of the literature. Cancers (Basel) 11: 1693, 2019.
25. Meyers RL, Katzenstein HM, Krailo M, McGahren ED III and Malogolowkin MH: Surgical resection of pulmonary metastatic lesions in children with hepatoblastoma. J Pediatr Surg 42: 2050-2056, 2007.
26. Dong R, Jia D, Xue P, Cui X, Li K, Zheng S, He X and Dong K: Genome-wide analysis of long noncoding RNA (lncRNA) expression in hepatoblastoma tissues. PLoS One 9: e85599, 2014.
27. Kong YW, Ferland-McCollough D, Jackson TJ and Bushell M: MicroRNAs in cancer management. Lancet Oncol 13: e249-e258, 2012.
28. Hua Z, Lv Q, Ye W, Wong CK, Cai G, Gu D, Ji Y, Zhao C, Wang J, Yang BB and Zhang Y: miRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLoS One 1: e116, 2006.
29. Wu Q, Zhao Y and Wang P: miR-204 inhibits angiogenesis and promotes sensitivity to cetuximab in head and neck squamous cell carcinoma cells by blocking JAK2-STAT3 signaling. Biomed Pharmacother 99: 278-285, 2018.
30. Tan J, Qiu K, Li M and Liang Y: Double-negative feedback loop between long non-coding RNA TUG1 and miR-145 promotes epithelial to mesenchymal transition and radioresistance in human bladder cancer cells. FEBS Lett 589B: B3175-B3181, 2015.
31. Yoshikawa T, Miyamoto M, Aoyama T, Soyama H, Goto T, Hirata J, Suzuki A, Nagaoka I, Tsuda H, Furuya K and Takano M: JAK2/STAT3 pathway as a therapeutic target in ovarian cancers. Oncol Lett 15: 5772-5780, 2018.
32. Marotta LL, Almendro V, Marusyk A, Shipitsin M, Schemme J, Walker SR, Bloushtain-Qimron N, Kim JJ, Choudhury SA, Maruyama R, *et al*: The JAK2/STAT3 signaling pathway is required for growth of $CD44^{+}CD24^{-}$ stem cell-like breast cancer cells in human tumors. J Clin Invest 121: 2723-2735, 2011.
33. Xue C, Xie J, Zhao D, Lin S, Zhou T, Shi S, Shao X, Lin Y, Zhu B and Cai X: The JAK/STAT3 signalling pathway regulated angiogenesis in an endothelial cell/adipose-derived stromal cell co-culture, 3D gel model. Cell Prolif 50: e12307, 2017.