miR-373-3p inhibits EMT via regulation of TGF β R2 in choriocarcinoma

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Abstract

Background: Previous studies have indicated that early metastasis is a major cause of mortality in patients with choriocarcinoma. However, what determines whether early metastasis of choriocarcinoma has occurred is unknown. The emerging role of miRNA in regulating cancer development and progression has been recognized. MiR-373-3p has been shown to play pivotal roles in tumorigenesis and metastasis. However, whether miR-373-3p functions to promote choriocarcinoma metastasis is not clear. The purpose of this study is to determine the function of miR-373-3p in the progression of choriocarcinoma.

Methods: In this study, we first compared EMT-related markers, which are inversely correlated with miR-373-3p expression, in trophoblast and choriocarcinoma cell lines. Using PCR and western blot, the upregulation of miR-373-3p was observed to inhibit EMT progression. Similarly, gain-and loss-of-function studies revealed that ectopic miR-373-3p overexpression inhibited the metastasis of choriocarcinoma cells.

Results: Our results revealed that miR-373-3p functions as an inhibitor in JEG-3 and JAR cells; this is due to its mediation of the TGF- β signalling pathway, which is responsible for EMT. The bioinformatic analysis and dual-luciferase reporter gene assays were employed to verify that miR-373-3p might interact with the 3' untranslated region of TGF β R2 mRNA. Further western blot results showed miR-373-3p reversed the increases of TGF β R2 and inhibited EMT.

Conclusions: In light of our observations, miR-373-3p upregulation partly accounts for TGF β R2 downregulation and leads to a restraint of EMT and metastasis. MiR-373-3p may, therefore, serve as a valuable target in potential anticancer strategies to treat choriocarcinoma.

Background

Gestational trophoblastic disease is a series of cellular proliferation arising from the placental villous trophoblast. Choriocarcinoma is one of the main clinicopathologic forms of gestational trophoblastic disease, which demonstrates early progression, invasion and metastasis [1]. Choriocarcinoma easily metastasizes at an early stage, and cancer metastases are responsible for the majority of cancer-related deaths [2]. In one study, choriocarcinoma had a mortality rate of almost 100% when metastases were present and a mortality rate of approximately 60% even when uterectomy was performed for apparent nonmetastatic disease [3]. Therefore, it is especially important to explore the pathogenesis of the early invasion and metastasis of choriocarcinoma.

MiRNAs are non-coding RNAs that target mRNAs post-transcriptionally and have been reported to possess many important functions in the regulation of the invasiveness and metastasis of tumours [4, 5]. The miR-520/373 family consists of three different miRNA clusters that share an identical seed region: miR-371/372/373, miR-520 and miR-302/367 [6]. The miR-520/373 family can inhibit tumour progression, metastasis and inflammation in oestrogen receptor-negative breast cancer by targeting the

NF- κ B and TGF- β signalling pathways [7]. A decrease in miR-373-3p can alter the expression of TGF β R2/p-Smad3, which increases prostate cancer metastasis [8].

The importance of epithelial-mesenchymal transition (EMT), which describes the change in cell phenotype from an epithelial to a mesenchymal state, in the pathogenesis of tumour invasion and metastasis has been increasingly recognized. Stability of cell-cell contacts is essential for maintenance of the epithelial phenotype. Conversely, mesenchymal cells are characterized by a unique spindle morphology and enhanced invasive potential [9]. Trophoblasts undergo EMT and gain the ability to invade and migrate throughout normal development, and the dysregulation of EMT is associated with the pathological processes of preeclampsia (PE) and foetal growth restriction (FGR) [10]. Nevertheless, whether EMT occurs during choriocarcinoma metastasis and cancer progression is unclear. Multiple extracellular signals can initiate an EMT programme, and members of the transforming growth factor- β (TGF β) family of cytokines are the primary and the best characterized inducers of EMT [11, 12]. The TGF- β signalling pathway is activated through the heteromeric complex of the TGF- β RI and TGF- β RII. TGF- β binds to T β RII and induces the phosphorylation and activation of T β RI. After interacting with T β RI, phosphorylated Smad2/3 dissociates to form a heterotrimeric complex with Smad4 after which it translocates to the nucleus, where it regulates gene transcription [13]. Thus, TGF- β RII plays a crucial role in the TGF- β signalling pathway.

In this study, we compared EMT-related markers, which are inversely correlated with the miR-373-3p expression, in trophoblast and choriocarcinoma cell lines. An increase or decrease in miR-373-3p can release or reduce the repression of its direct target TGF β RII, respectively. TGF β RII is also part of the TGF β signalling pathway, which is the molecular mechanism that mediates EMT. Our data show that miR-373-3p plays an inhibitor role in choriocarcinoma, which is due to its specific mediation of the TGF β RII signalling pathway and inhibition of EMT progression.

Methods

Reagent

DMEM medium, foetal bovine serum and 0.25% Trypsin-EDTA were purchased from Gibco (St. Louis, MO, USA). A Hairpin-it Real-Time PCR Kit was purchased from GenePharma (Shanghai, China) and Quantitative polymerase chain reaction (qPCR) master mix was purchased from Promega (Madison, WI, USA). Primers used in this study were synthesized by Sangon Biotech Technology (Shanghai, China). The Epithelial-Mesenchymal Transition Antibody Sampler Kit was purchased from Abcam (Cambridge, MA, UK). The anti-TGF β R2 rabbit monoclonal antibody anti- β -actin mouse monoclonal antibodies were purchased from Abcam (Cambridge, MA, UK). Peroxidase-conjugated AffiniPure goat anti-mouse IgG and peroxidase-conjugated AffiniPure goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (CA, USA). The luciferase activity was measured by the Dual-Luciferase® Reporter Assay System. (Promega, Madison, WI, USA)

Cell culture

Human placental choriocarcinoma cell lines (JEG-3 and JAR) were obtained from the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. HTR8-SVneo was purchased from Jennio Biotech Co., Ltd (GuangZhou, China). Cells were cultured in DMEM supplemented with 10% FBS in an incubator with an atmosphere of 5% CO₂ at 37°C. Experiments were performed using cells at a logarithmic growth phase.

Paraffin-embedded human choriocarcinoma tissue

We included ten formalin-fixed paraffin-embedded (FFPE) choriocarcinoma samples from the pathology archive of the Affiliated Hospital of Chengde Medical College, Chengde Third Hospital and the Second Affiliated Hospital of Hebei Medical University. Ten women with normal early pregnancy and a single gestational sac were randomly selected, and the gestational villus was obtained at Chengde Women's and Children's Hospital.

miRNA microarray analysis

Microarray analyses were performed by OE biotech (Shanghai, China). Total RNA in paraffin-embedded sections was extracted using a Recover All™ Total Nucleic Acid Isolation Kit, Ambion-1975. The human miRNA microarray, Release19.0, 8×60K (Agilent Technologies) was used to detect the miRNA expression in total RNA samples containing small RNAs. Images of hybridized arrays were automatically analyzed using a DNA microarray scanner and were quantified with Feature Extraction Software (Agilent Technology). GeneSpring GX software (Agilent Technology) was used to normalize the data and perform variation analysis. The selection of miRNAs were screened out according to fold change (FC) > 2.

Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was extracted using TRIzol reagent. 2 µg of total RNA was used to synthesize first-strand cDNA according to the MMLV FirstStrand Synthesis System kit (Promega). The primers used for gene amplification were shown in Table1. Real-time PCR reactions were performed using the GoTaq qPCR Master Mix (Promega) according to the manufacturer's [instructions](#) in an ABI 7500 Real-Time PCR system. The results were analysed using the comparative threshold cycle method and normalized by GAPDH.

Total RNA from FFPE samples were isolated using Qiagen RNeasy FFPE protocol. For analysis of miRNA expression, miRNA-specific reverse transcription was performed using the MicroRNA Reverse Transcription Kit (GenePharma). Quantitative Real-time PCR reactions were performed using GenePharma PCR Master Mix and U6 small nuclear RNA was used as a standardized control.

Western blot analysis

Total proteins were isolated from each group using ice-cold lysis buffer. The concentration of proteins was quantified using a BCA kit (Thermo Scientific). Protein samples were separated by SDS-PAGE and transferred to polyvinylidenedifluoride (PVDF) membranes. After blocking with 5% skim milk in TBST at room temperature for 1h, the blots were probed overnight at 4°C with the appropriate primary antibodies. The antibodies used included antibodies against E-cadherin (1:1000 dilution), N-cadherin (1:1000 dilution), snail (1:1000 dilution), slug (1:1000 dilution), Vimentin (1:1000 dilution), α SMA (1:1000 dilution), TGF β R2 (1:1000 dilution) and β -actin (1:500 dilution). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit; 1:1000 dilution) at room temperature for 2h. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) and imaged by Tanon6100 Chemiluminescent Imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China)..

In vitro migration, invasion and wound scratch assays

The cells were collected and resuspended in serum-free DMEM at a concentration of 4×10^5 cells/ml, as determined by cell counts. Then, the cell suspension solution was seeded into the upper chambers (200 μ l/well), and the bottom chambers were filled with DMEM containing 10% FBS (1ml/well). We used CORNING (USA) Transwell chambers (Lot 3422, 6.5mm Diameter Inserts; 8- μ m pore size; polycarbonate membrane) to quantify cell migration. For the Transwell invasion assay, CORNING (USA) Transwell chambers (Lot 354480, polycarbonate membrane, Matrigel-coated) were used. After they were cultured at 37°C for 24h, the cells that had not penetrated the polycarbonate membrane were wiped off with a cotton swab. The membrane was fixed in 4% paraformaldehyde and stained with crystal violet solution. Cell migration and invasion were determined by counting five random high-power fields (Olympus Corp, Tokyo, Japan).

For the wound scratch assay, cells were plated and grown until confluence after which the cells were scratched using sterile tips. Cellular migration was assessed after 24h.

Transient transfection

Hsa-miR-373-3p inhibitor (miR-373i), hsa-miR-373 mimics (miR-373) and a negative control (NC) were purchased from GenePharma (Shanghai, China). (Please look at the RNA sequence in Table 1) Cells were transiently transfected using jetPRIME® in vitro DNA & siRNA transfection reagent (Polyplus Transfection) according to the manufacturer's instructions.

Luciferase assay

In the luciferase reporter vector, the wild-type or mutant 3'-UTR of human TGF β R2 was cloned into the downstream of the renilla luciferase gene in pGL3 vectors (GenePharma, Shanghai, China). Cells were transfected with the firefly luciferase reporter plasmid including either the wild-type (WT) or mutant (MU) 3'-UTR of TGF β R2 and the pRL-TK Renilla luciferase reporter. The cells were then cotransfected with miR-373-3p mimics or negative control. After transfection for 24h, cells were lysed with 1 $\times$ reporter lysis buffer, and the luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was standardized to renilla activity as a control.

Statistical analysis

Each experiment was repeated three times, unless otherwise indicated. Data are presented as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and Student's t test were used to analyze the difference in two groups and multiple groups. A P-value <0.05 was considered statistically significant and is indicated by an asterisk in the figures, and P-value <0.01 was indicated by two asterisk.

Results

MET occurs in choriocarcinoma cells but not in trophoblasts

We found an interesting phenomenon in that MET occurred in choriocarcinoma cells but not in trophoblasts. As shown in Fig.1A, the mRNA level of the epithelial marker E-cadherin (CDH1) was upregulated, while the levels of mesenchymal markers, including N-cadherin (CDH2), ZEB1 and vimentin, were dramatically downregulated in JAR and JEG-3 cells compared with trophoblast HTR8-SVneo cells. To confirm these findings, the protein levels of epithelial and mesenchymal markers were evaluated. As shown in Fig.1B, the protein levels of the epithelial marker E-cadherin were upregulated, while the levels of mesenchymal markers, including N-cadherin, vimentin, α -SMA, snail and slug, were downregulated in JAR and JEG-3 cells. These results suggest that trophoblast cells exhibit stronger mesenchymal characteristics. Conversely, choriocarcinoma cells exhibit relatively more epithelial characteristics.

Trophoblasts have strong invasion and migration potential

To further detect if the EMT phenotype is the main reason through which cells obtain the capacity to migrate and invade, a wound healing assay was performed to examine whether choriocarcinoma and trophoblast cells have different abilities to migrate into a scratched area. The scratch wound assay indicated that the choriocarcinoma cell lines showed a weaken ability to migrate compared with the trophoblast line, as demonstrated by quantification and representative images shown in Fig.2A. This indicates that trophoblasts exhibit a strong migration ability. A Transwell chamber assay was then performed to analyse choriocarcinoma cell migration and invasion. Compared with trophoblast cells, the

numbers of migrated and invasive cells were downregulated in choriocarcinoma JAR and JEG-3 cells. (Fig.2B)

miR-373-3p expression is upregulated in choriocarcinoma tissue and cell lines and is inversely correlated with EMT marker expression

Three choriocarcinoma tissue samples and three gestational villus tissues were included in a microarray analysis, and the fold change method was employed to conduct a differential analysis of the miRNA expression levels in these two groups. Most members of the miR-520/373 family demonstrated expression changes that attracted our attention. Compared with gestational villus tissues, the average expression level of miR-373-3p was significantly increased (Fig.3A), yet relevant research on choriocarcinoma is largely unreported. To further identify the role of miR-373-3p in choriocarcinoma cells and trophoblasts, we analysed the expression of miR-373-3p by qRT-PCR in HTR-8, JAR and JEG-3 cell lines. Compared with HTR-8 cells, the average expression level of miR-373-3p was significantly increased in JAR and JEG-3 cells (Fig.3B). Our results suggest that miR-373-3p is upregulated in choriocarcinoma and that the upregulation of miR-373-3p is inversely correlated with EMT, which implies an EMT-inhibiting function of miR-373-3p in the transition of trophoblast cells to choriocarcinoma.

miR-373-3p inhibits EMT in choriocarcinoma

To further detect the function of miR-373-3p in choriocarcinoma, the following experiments were performed. After transfecting miR-373-3p mimics and a miR-373-3p inhibitor into the human choriocarcinoma cell lines JAR and JEG-3, qRT-PCR and WB experiments were performed to explore the effects of miR-373-3p on EMT in choriocarcinoma cells. As shown in Fig.3C and Fig.3D, the mRNA levels of the epithelial marker E-cadherin (CDH1) were upregulated, while the levels of mesenchymal markers, including N-cadherin (CDH2), ZEB1 and vimentin were dramatically downregulated in JAR and JEG-3 cells transfected with miR-373-3p mimics. On the contrary, the mRNA levels of the epithelial marker E-cadherin were downregulated, while the levels of the mesenchymal markers N-cadherin, ZEB1 and vimentin were upregulated in JAR and JEG-3 cells transfected with a miR-373-3p inhibitor. To confirm this hypothesis, the protein levels of epithelial markers and mesenchymal markers were evaluated. As shown in Fig.3E and Fig.3F, the protein levels of the epithelial marker E-cadherin were upregulated and the levels of mesenchymal markers, including N-cadherin, vimentin, α -SMA, snail and slug, were downregulated in cells transfected with miR-373-3p mimics. Conversely, the protein levels of the epithelial marker E-cadherin were downregulated and the levels of the mesenchymal markers were upregulated in JAR and JEG-3 cells transfected with a miR-373-3p inhibitor. These results suggest that miR-373-3p may inhibit the transition of cells from an epithelial to a mesenchymal phenotype.

miR-373-3p inhibits the invasion and migration ability of JEG-3 and JAR cells

In vitro gain-of-function analyses were performed to explore the effects of miR-373-3p function by transfecting miR-373-3p mimics and a miR-373-3p inhibitor into JAR and JEG-3 cells. Transwell migration experiments were then performed to explore the effects of miR-373-3p on migration ability. It was shown that miR-373-3p mimics can significantly inhibit the migration ability of JEG-3 cells. On the contrary, a miR-373-3p inhibitor can promote the migration ability of JEG-3 and JAR cells. (Fig.4A-4B) Consistent with this hypothesis, Matrigel-coated Transwell assays showed that miR-373-3p overexpression can significantly inhibit the invasion ability of these two cell lines. However, low miR-373-3p expression can enhance the invasion ability of JEG-3 and JAR cells (Fig.4C-4D). Collectively, our data suggest that miR-373-3p may inhibit the invasion and migration ability by influencing EMT in JEG-3 and JAR cells.

TGF- β signalling induces EMT in choriocarcinoma

Since TGF- β signalling is one of the most important pathways that functions to maintain an epithelial cell phenotype and proper cell-cell junctions, we sought to understand whether TGF- β affected the EMT process. TGF- β treatment decreased the mRNA levels of epithelial markers and increased the levels of mesenchymal markers in a dose-dependent (Fig.5A) and time-dependent manner (Fig.5B) in JEG-3 cells. To confirm these findings, the protein levels of epithelial markers and mesenchymal markers were evaluated. TGF- β treatment decreased the protein levels of epithelial markers and upregulated the protein levels of mesenchymal markers in JEG-3 (Fig.5C) and JAR (Fig.5D) cells in a dose-dependent manner. TGF- β treatment decreased the protein levels of epithelial markers and enhanced the protein levels of mesenchymal markers in JEG-3 (Fig.5E) and JAR (Fig.5F) cells in a time-dependent manner. These results suggest that TGF- β signalling induces EMT in choriocarcinoma cells.

miR-373-3p targets TGF β R2, which influences EMT in choriocarcinoma

A bioinformatic analysis using 3 miRNA target gene databases (targetscan.org, miranda.org and mirdb.org) showed that TGF β R2 mRNA is a presumed target of miR3733p. The potential target region for miR3733p in the TGF β R2 mRNA 3'UTR are shown in Fig.6A. To verify that miR3733p can regulate TGF β R2 mRNA, we investigated TGF β R2 protein expression in JEG-3 cells transfected with amiR3733p mimic and a miR3733p inhibitor. The expression of TGF β R2 protein was downregulated in successful transfection of the miR3733p mimic group (Fig.6B). Dual luciferase reporter assays were implemented to validate whether regulation exists. Dual luciferase reporter vectors containing either the mutant or wildtype 3'UTR of TGF β R2 mRNA were then constructed and cotransfected into JEG-3 (Fig.6C) and JAR (Fig.6D) cells together with the miR3733p mimic or NC. The results showed that cells transfected with

pmirGLOwtTGFβR2 group was significantly responsive to miR3733p overexpression and that these cells also exhibited a weaker reaction to the NC because both cell lines express miR373. However, no significant differences were observed in the relative luciferase activity between the miR3733p mimic group and the NC group of cells cotransfected with pmirGLOmutTGFβR2 (Fig.6C–6D). The results imply that miR3733p acts directly on the 3'UTR of TGFβR2 mRNA and that it negatively regulates TGFβR2 expression.

We further explored the functional significance of miR–373–3p and found that it activates TGFβ signalling, which increases EMT in choriocarcinoma cells. Recent studies and our previous data [14, 15] showed that TGFβ signalling can active EMT in choriocarcinoma [16, 17]. Given the active roles of TGFβ in regulating EMT, we then questioned whether TGFβR2 is a potential regulator involved in this process. JEG–3 (Fig.6E) and JAR (Fig.6F) cells were pretreated for 12h with TGFβ before transfection with the miR3733p mimic for 6h. The expression levels of TGFβR2 and EMT markers were evaluated by Western blot. Our results indicate that TGFβ stimulation resulted in a significant upregulation of TGFβR2 and further induced EMT, whereas miR3733p reversed the increases in TGFβR2 and inhibited EMT (Fig.6E–6F).

Discussion

EMT is a highly conserved process by which cells lose epithelial characteristics and acquire a [mesenchymal](#), migratory phenotype. EMT is involved in the process of tissue and organ development at the embryonic stage. In recent years, most scholars focus on the EMT process as it relates to tumour progression and metastasis. Most tumours may undergo EMT, which is a hall mark of aggressive cancer; they may also acquire invasive and metastatic potential, which leads to a poor patient prognosis. Different from other tumours, trophoblast cells and their tumours are special. Several studies have reported that trophoblast cells and malignant cells share many similar phenotypic properties including proliferation, migration and invasion of neighbouring tissues. The cellular mechanisms of EMT in trophoblasts are poorly understood, but attempts have been made to use trophoblast cells to understand the EMT process from a cancer perspective because of the high invasive capacity of trophoblasts [18]. In this study, we present an exciting discovery that trophoblast cells have a stronger mesenchymal phenotype and greater ability to invade and migrate, which is different from the phenotype of most other cancers. Indeed, many reports have indicated that trophoblast cells undergo EMT, which allows them to migrate and infiltrate into the maternal decidua and vessels to ensure normal gestation [19, 20]. Few studies on epithelial-to-mesenchymal transition in choriocarcinoma cells have been published. One study even suggests that gestational trophoblastic neoplasms do not display EMT features [21].

Based on our findings, it appears that trophoblast cells exhibit strong expression of mesenchymal markers and that choriocarcinoma cells exhibit strong expression of epithelial markers. However, in this study, we describe EMT as an immotile, polarized process through which epithelial cells undergo a number of biochemical changes to attain mesenchymal cell characteristics. Actually, EMT is a dynamic and somewhat unstable procedure, and thus future research should use dynamic and changeable

methods to further understand the EMT process in the occurrence and transformation of trophoblast cell malignancy. EMT is closely related to invasion, and shallow invasion is a characteristic feature of foetal growth restriction, while abnormal deep invasion is associated with placenta accrete, and uncontrolled invasion is associated with choriocarcinoma [22, 23]. Hence, clarifying the potential regulatory mechanism of EMT in trophoblast cells and tumour development may provide novel diagnostic and therapeutic perspectives of malignancy.

Recent work suggests that miRNAs are important molecular regulators that can modulate EMT [24]. Hence, clarifying the potential regulatory mechanism of miRNAs in choriocarcinoma development and the invasive characteristics of trophoblast cells may provide novel diagnostic and therapeutic perspectives for trophoblastic malignancies. In this study, q-PCR validation revealed that miR-373-3p expression was enhanced in choriocarcinoma cells compared with trophoblasts and was inversely correlated with the occurrence of EMT. MiR373 has been shown to serve as a tumour-suppressing miRNA in gastric cancer because it downregulates vimentin [25]. One study suggested that miR373p acts as an oncogenic miRNA, which when upregulated, targets CD44 to promote invasion and metastasis in breast cancer [26]. However, the role of miR373p remains controversial because the expression of miR373p is abnormal in various types of tumours, which influences proliferation, invasion and metastasis.

TGF β may induce EMT through multiple distinct signalling mechanisms, including regulation of tight junction formation by certain cytoplasmic proteins and phosphorylation by ligand-activated receptors of SMAD transcription factors. TGF β RII can directly phosphorylate both SMAD2 and SMAD3 proteins [27–29]. Our studies indicated a direct connection between TGF β 1 and EMT in choriocarcinoma. According to our observations, TGF β 1 recognized and activated the receptor TGF β R2, which subsequently activated its downstream EMT mediator.

Given the roles of miR373p in regulating EMT and inhibiting invasion and metastasis activity, we hypothesized that miR373p may also regulate TGF β signalling, which regulates EMT in choriocarcinoma cells. Our results established TGF β R2, a direct target regulator of TGF β signalling, as a direct functional effector of miR-373-3p, which is in agreement with many studies showing that TGF β R2 is downregulated in various cancers. Apart from this, increased expression of miR-373-3p in choriocarcinoma cells was significantly associated with downregulated expression of TGF β R2 and a less aggressive [mesenchymal](#) phenotype.

In conclusion, miR-373 was upregulated in choriocarcinoma, and miR-373 overexpression inhibited the EMT process as well as invasion/migration in vitro. These data suggest that miR-373 over expression may have potential in the development of a new therapy for choriocarcinoma. Finally, our results provide a first step in understanding the pathology of these rare gestational neoplasms and reveal novel molecular mechanisms. However, trophoblasts are unique since they undergo migration and invasion to establish their roles in the placenta. During the process in which trophoblasts transform into choriocarcinoma cells, intrinsic and extrinsic mechanisms precisely control this transition. In particular, EMT is a dynamic equilibrium [course](#), and more research is needed on the variable process.

Conclusion

The study show the phenomenon of MET occurs in choriocarcinoma cells but not in trophoblasts, and further demonstrated the mechanism of miR3733p in regulating EMT and inhibiting invasion and metastasis activity via targeting TGFβR2.

Abbreviations

EMT: epithelial-mesenchymal transition; *MET*: mesenchymal-epithelial transition; *FBS*: foetal bovine serum; *FFPE*: formalin-fixed paraffin-embedded; *DMEM*: dulbecco's modified eagle medium; *RIPA*: Radio Immunoprecipitation Assay; *EDTA*: Ethylene Diamine Tetraacetic Acid; *MMLV*: Moloney Murine Leukemia Virus; *qRT-PCR*: quantitative real-time polymerase chain reaction; *SD*: standard deviation; *miR-373i*: Hsa-miR-373-3p inhibitor, *miR-373*: hsa-miR-373 mimics; *NC*: negative control.

Declarations

Ethics approval and consent to participate:

FFPE choriocarcinoma samples from the pathology archive of the Affiliated Hospital of Chengde Medical College, Chengde Third Hospital and the Second Affiliated Hospital of Hebei Medical University. The work was performed in accordance with a protocol for archival tissue collection and use, which was approved by the Institutional Review Board at all institutions. Gestational villus was obtained at Chengde Women's and Children's Hospital.

Consent for publication

All patients signed informed consent before the surgery to allow use of specimens for research and publication of the research results.

Availability of data and materials

All datasets generated or analyzed during this study are included in this published article and its information files.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

YL and XL contributed equally to this work, who performed the research and wrote the paper; YL as the corresponding author designed the study and contributed to the reagents; YZ, QX, LL, HW and LC contributed to analyse data; YZ, WF and YL carried out experiments.

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Table

Table1. Primers and RNA sequence used in this study

Transcript	Primers sequence
GAPDH	-TGAAGGTCGGAGTCAACGGAT (Sense); -CTGGAAGATGGTGTGATGGGATT (Antisense)
CDH1	-GATAGAGAACGCATTGCCACATA (Sense); - ACCTTCCATGACAGACCCCTTAA (Antisense)
CDH2	-TGTTTGTCTTACTGTTGCTGC (Sense); -TTCTTCTTGGCGAATGATCTTAG (Antisense)
Vimentin	-GACCTGCTCAATGTTAAGATGGC (Sense); -CAGAGGGAGTGAATCCAGATTAGTT (Antisense)
ZEB1	-GCAGTCTGGGTGTAATCGTAAAT (Sense); -CTTGCAGTTTGGGCATTCATAT (Antisense)
has-miR373 mimics (5' to 3')	GAAGUGCUUCGAUUUUGGGGUGU ACCCCAAAAUCGAAGCACUUCU
has-miR373-3p inhibitor (5' to 3')	ACACCCCAAAAUCGAAGCACUUC
Negative control	-UUCUCCGAACGUGUCACGUTT (Sense); -ACGUGACACGUUCGGAGAATT (Antisense)

Figures

Figure 1

Figure 1

MET occurred in choriocarcinoma cells but not in trophoblasts (A) qRT- (B) PCR analysis of EMT marker expression in HTR-8 trophoblast cells and JAR and JEG-3 choriocarcinoma cells. The results showed that the mRNA levels of the epithelial marker E-cadherin (CDH1) was significantly upregulated in JEG-3 and JAR cells, while the levels of mesenchymal markers, including N-cadherin (CDH2), ZEB1 and vimentin, were dramatically regulated in JAR and JEG-3 cells compared with HTR-8 cells. (B) The protein levels of the epithelial marker E-cadherin were upregulated, while the levels of mesenchymal markers, including N-cadherin, vimentin, α -SMA, snail and slug, were downregulated according to Western blot. β -actin was used as a loading control. Our data revealed that the protein levels of E-cadherin were upregulated and that the protein levels of N-cadherin, vimentin, α -SMA, snail and slug were downregulated in JAR and JEG-3 cells. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).

Figure 2

(A) Representative images and quantification analysis of the scratch wound assay. While the wound was healed in $77.5 \pm 4.6\%$ of the baseline area in the control group after 24 h, the wound area was still $15.7 \pm 3.2\%$ of the baseline area in JEG-3 cells and $9.0 \pm 3.6\%$ in JAR cells. This indicates that trophoblasts have strong migratory properties. (B) Representative images on the left showed that cell migration and invasion were downregulated in choriocarcinoma JAR and JEG-3 cells compared with trophoblast cells. Transwell migration and invasion analyses were performed and the quantification of five randomly selected fields was shown on the right. Data are presented as the mean $\pm$ SD (n=5 for each group, *p<0.05, **p<0.01).

Figure 3

Figure 3

miR-373-3p is highly expressed in choriocarcinoma tissue and cell lines and inhibits EMT in choriocarcinoma (A) Heat maps of differentially expressed miRNAs in three choriocarcinoma tissue specimens (C1,C2,C3) and three gestational villus tissues (N1,N2,N3). Red represents higher expression and green represents lower expression. (B) The expression of miR-373-3p by qRT-PCR in HTR-8, JAR and JEG-3 cell lines. miR-373-3p levels increased remarkably in JEG-3 and JAR cell lines compared with HTR-

8 cells. (C-F) JEG-3 and JAR cells were transfected with a hsa-miR-373-3p inhibitor (miR-373i), hsa-miR-373 mimics (miR-373) and a negative control (NC). The expression levels of the indicated molecules were examined by real-time PCR. The results showed that miR-373 inhibited the expression of mesenchymal markers and promoted epithelial marker expression, while miR-373i inhibited the expression of epithelial markers and promoted mesenchymal marker expression in JEG-3 cells (C). Similarly, miR-373 inhibited EMT marker expression in JAR cells (D). miR-373 inhibited the protein expression of EMT markers in JEG-3 cells (E) and JAR cells (F). The relative protein intensities of EMT markers with the β -actin loading control are shown. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).

Figure 4

miR-373-3p inhibits the invasion and migration ability of JEG-3 and JAR cells (A-D). JEG-3 and JAR cells were transfected with an hsa-miR-373-3p inhibitor (miR-373i), hsa-miR-373 mimics (miR-373) and a negative control (NC). Representative images on the left show that cell migration was downregulated when JEG-3 cells (A) and JAR cells (B) were transfected with hsa-miR-373 mimics. However, in the miR-373 inhibitor group, the inhibition of migration was reversed. Similarly, cell invasiveness was downregulated when JEG-3 cells (C) and JAR cells (D) were transfected with hsa-miR-373 mimics. Transwell migration and invasion analyses were performed, and the quantification of five randomly selected fields is shown on the right. Data are presented as the mean $\pm$ SD (n=5 for each group, *p<0.05, **p<0.01).

Figure 5

Figure 5

TGF- β signalling induces EMT in choriocarcinoma Real-time PCR analyses of the mRNA levels of EMT markers in JEG-3 cells after TGF- β stimulation. TGF- β treatment decreased the mRNA levels of the epithelial marker E-cadherin (CDH1) and increased the levels of the mesenchymal markers N-cadherin (CDH2), ZEB1 and vimentin in a dose-dependent (A) and time-dependent manner (B) in JEG-3 cells. The protein levels of epithelial and mesenchymal markers were evaluated. TGF- β treatment decreased the

protein levels of E-cadherin and upregulated the levels of mesenchymal markers, including N-cadherin, vimentin, α -SMA and snail in JEG-3(C) and JAR (D) in a dose-dependent manner. TGF- β treatment decreased the protein levels of epithelial markers and enhanced the levels of mesenchymal markers in JEG-3(E) and JAR (F) in a time-dependent manner. The relative protein intensities of EMT markers compared with the β -actin loading control are shown. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).

Figure 6

Figure 6

miR-373-3p targets TGF β R2, which influences EMT in choriocarcinoma (A) A bioinformatic analysis using 3 miRNA target gene databases showed that TGF β R2 mRNA is a presumed target of miR 373 3p (target site: 218-224, 602-608, 912-918, 2423-2430). (B) The expression of TGF β R2 protein was downregulated in the miR 373 3p mimic group. miR 373 3p significantly inhibited wild-type but not mutated TGF β R2 3'UTR luciferase reporter activity in JEG-3 (C) and JAR (D) cells, and it also exhibited a weaker reaction to the NC because both cell lines express miR 373 3p. JEG-3 (E) and JAR (F) cells were pretreated for 12 h with TGF β before transfection with the miR 373 3p mimic for 6 h. The protein levels of the indicated molecules were examined by Western blot analysis. TGF β stimulation resulted in a significant upregulation of TGF β R2 and further induced EMT, whereas miR 373 3p reversed the increases in TGF β R2 and inhibited EMT. The relative protein intensities compared with the β -actin loading control are shown. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).