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 has been shown to play pivotal roles in tumorigenesis and metastasis. However, whether miR-373 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 this cancer.

Methods: In this study, we first compared epithelial-to-mesenchymal transition (EMT)-related markers, which were inversely correlated with miR-373-3p expression, in trophoblast and choriocarcinoma cell lines. Using PCR and Western blot, 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 acted as an EMT inhibitor in JEG-3 and JAR cells; this was due to its mediation of the TGF- β signalling pathway, which was responsible for EMT. Bioinformatic analysis demonstrated that miR-373-3p interacted with the 3' untranslated region of TGF β R2 mRNA, and then Western blot and dual-luciferase reporter gene assays verified this interaction.

Conclusions: Our findings suggest that 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 potential target in the treatment of 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 hysterectomy 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-302/367, miR-371/372/373 and miR-520 [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 dysregulation of EMT is associated with 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 best characterized inducers of EMT [11, 12]. TGF- β signalling pathway is activated through heteromeric complex of TGF- β RI and TGF- β RII. TGF- β binds to T β RII and induces phosphorylation and activation of T β RI. After interacting with T β RI, phosphorylated Smad2/3 dissociate to form a heterotrimeric complex with Smad4; and then the complex 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 were inversely correlated with miR-373-3p expression, in trophoblast and choriocarcinoma cell lines. An increase or decrease in miR-373-3p can release or reduce 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 showed that miR-373-3p played an inhibitor role in choriocarcinoma, which was due to its specific mediation of the TGF β RII signalling pathway and inhibition of EMT progression.

Methods

Reagents

DMEM medium, foetal bovine serum (FBS), 0.02% EDTA and 0.25% trypsin were purchased from Gibco (St. Louis, MO, USA). Quantitative polymerase chain reaction (qPCR) master mix was purchased from Promega (Madison, WI, USA) and a Hairpin-it Real-Time PCR Kit was purchased from GenePharma (Shanghai, China). The Epithelial-Mesenchymal Transition Antibody Sampler Kit was purchased from Abcam (Cambridge, MA, UK). The anti-TGF β RII rabbit monoclonal antibody and anti- β -actin mouse monoclonal antibody 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 (Santa Cruz, CA, USA). The Dual-Luciferase® Reporter Assay System was purchased from Promega (Madison, WI, USA).

Cell culture

Human placental choriocarcinoma cell lines (JAR and JEG-3) were obtained from the State Key Laboratory of Reproductive Biology (SKLRB), Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS). HTR8-SVneo was purchased from Jennio Biotech Co., Ltd (Guang Zhou, China). Cells were

cultured in DMEM supplemented with 10% FBS in an incubator with an atmosphere of 5% CO₂ at 37°C. The cells were then subcultured using 0.25% trypsin and 0.02% EDTA when the cells were approximately 80% confluent. 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 pathological archives 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 were obtained at Chengde Women's and Children's Hospital.

miRNA microarray analysis

Total RNA in formalin-fixed paraffin-embedded (FFPE) sections was extracted using a Recover All™ Total Nucleic Acid Isolation Kit (Ambion-1975). MiRNA expression analysis was conducted on total RNA samples containing small RNAs using the human miRNA microarray, Release19.0, 8x60K (Agilent Technologies). For control and test RNAs, the synthesis of target miRNA probes and hybridization was performed using a miRNA Labeling Reagent and Hybridization Kit (Agilent Technology) according to the manufacturer's instructions. The hybridized microarrays were washed according to the manufacturer's washing protocol (Agilent Technology). Images of hybridized arrays were obtained using a DNA microarray scanner (Agilent Technology) and were quantified with Feature Extraction Software (Agilent Technology). Data normalization and the selection of genes with a fold change (FC) > 2 were performed using GeneSpring GX software (Agilent Technology).

Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was isolated using the RNeasy® Total RNA Isolation System (Qiagen) with DNase I (Invitrogen) treatment. Then, 2 µg RNA, oligo (dt) 20 primers and the M-MLV First-Strand Synthesis System kit was used to synthesize cDNA. Real-time PCR was performed using the SYBR® Green I on GoTaq® qPCR Detection System (Promega) according to the manufacturer's instructions. The results were analysed using the comparative threshold cycle method with GAPDH as an internal control. The results were normalized to GAPDH levels using the formula ΔCt (Cycle threshold) = Ct of target gene – Ct of GAPDH. The mRNA level of the control group was used as the baseline; therefore, $\Delta\Delta Ct$ was calculated using the formula $\Delta\Delta Ct = \Delta Ct$ of target gene – ΔCt of the baseline. The fold change in the mRNA level was calculated as fold = $2^{-\Delta\Delta Ct}$. Primers used in this study were synthesized by Sangon Biotech Technology (Shanghai, China) (see Table 1).

FFPE samples were cut into 1-3 mm cores. Total RNA was isolated using the Qiagen RNeasy FFPE protocol. For analysis of miRNA expression, miRNA-specific reverse transcription was performed with a GenePharma MicroRNA Reverse Transcription Kit according to the manufacturer's instructions.

Quantitative RT-PCR was performed using GenePharma PCR Master Mix; U6 small nuclear RNA was used as an internal control. All reactions were performed in triplicate.

Western blot analysis

Total proteins were extracted from each group using RIPA buffer (Thermo Scientific, Rockford, IL, USA) and were quantified using a BCA kit (Thermo Scientific). The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes. After blocking with 5% bovine serum albumin, the blots were probed with the appropriate primary antibodies overnight at 4°C. 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 smooth muscle actin (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 anti-rabbit; 1:1000 dilution). Immunoreactive bands were detected using enhanced chemiluminescence (ECL) substrate (Pierce, Rockford, IL, USA) and imaged using the Image Quant LAS4000 system (GE Company, Pittsburgh, PA, USA).

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 removed, 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 (toward the scratched area) was assessed after 24 h.

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). The miRNA sequences are shown in Table 1. Cells were transiently transfected using jetPRIME® in vitro DNA & siRNA transfection reagent (Poly plus) 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 seeded onto 24-well plates (1×10^5 cells per well) the day before transfection. Cells were then transfected with the firefly luciferase reporter plasmid including either the wild-type or mutant 3'-UTR of TGF β R2 (50 ng per well) and the pRL-TK Renilla luciferase reporter (10 ng per well). The cells were then transfected with miR-373-3p mimics or negative control (20 μ M) using jetPRIME® in vitro DNA & siRNA transfection reagent (Poly plus). After 24 h of transfection, cells were lysed with 1 $\times$ reporter lysis buffer, and firefly and renilla luciferase activities were measured using the Dual-Luciferase® Reporter Kit (Promega) according to the manufacturer's instructions. Firefly luciferase activity was standardized to renilla activity as a control.

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Student's *t* test. A *P*-value <0.05 was considered statistically significant and is indicated by asterisks in the figures.

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to the manufacturer's instructions. Firefly luciferase activity was standardized to renilla activity as a control.

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Student's t test. A *P*-value <0.05 was considered statistically significant and is indicated by asterisks in the figures.

Results

mesenchymal-epithelial transition occurs in choriocarcinoma cells but not in trophoblasts

We found an interesting phenomenon that mesenchymal-epithelial transition (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-C, 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 simultaneously indicated that the choriocarcinoma cell lines JEG-3 and JAR showed a weaker 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 stronger 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 less 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 Western blot 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-3G and Fig.3F-3H, 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. The same results can be seen in JAR cells (Fig.5C-D). 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.6A-B) and JAR (Fig.6C-D) 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.6E-F) and JAR (Fig.6G-H) 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 miR-373-3p. The potential target region for miR-373-3p in the TGF β R2 mRNA 3'UTR are shown in Fig.7A. To verify that miR-373-3p can regulate TGF β R2 mRNA, we investigated TGF β R2 protein expression in JEG-3 cells transfected with a miR-373-3p mimic and a miR-373-3p inhibitor. The expression of TGF β R2 protein was downregulated in successful transfection of the miR-373-3p mimic group (Fig.7B). Dual-luciferase reporter assays were implemented to validate whether regulation exists. Dual-luciferase reporter vectors containing either the mutant or wild-type 3'UTR of TGF β R2 mRNA were then constructed and cotransfected into JEG-3 (Fig.7C) and JAR (Fig.7D) cells together with the miR-373-3p mimic or non-specific control (NC) miRNA. The results showed that cells transfected with pmirGLO-wt-TGF β R2 group was significantly responsive to miR-373-3p overexpression and that these cells also exhibited a weaker reaction to the NC because both cell lines express miR-373. However, no significant differences were observed in the relative luciferase activity between the miR-373-3p mimic group and the NC group of cells cotransfected with pmirGLO-mut-TGF β R2 (Fig.7C-7D). The results imply that miR-373-3p acts directly on the 3'-UTR of TGF β R2 mRNA and that it negatively regulates TGF β R2 expression.

We further explored the functional significance of miR-373-3p and found that it activated TGF β signalling, which increased EMT in choriocarcinoma cells. Recent studies and our previous data [14, 15] showed that TGF β signalling can activate EMT in choriocarcinoma [16, 17]. Given the active roles of TGF β in regulating EMT, we then questioned whether TGF β R2 is a potential regulator involved in this process. JEG-3 and JAR cells were pretreated for 12h with TGF β before transfection with the miR-373-3p mimic for 6h. The expression levels of TGF β R2 and EMT markers were evaluated by Western blot. Our results indicate that 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 in JEG-3 (Fig.7E-F) and JAR (Fig.7G-H).

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. MiR-373 has been shown to serve as a tumour-suppressing miRNA in gastric cancer because it downregulates vimentin [25]. One study suggested that miR-373-3p acts as an oncogenic miRNA, which when upregulated, targets CD44 to promote invasion and metastasis in breast cancer [26]. However, the role of miR-373-3p remains controversial because the expression of miR-373-3p 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 indicate a direct connection between TGF β 1 and EMT in choriocarcinoma. According to our observations, TGF β 1 recognizes and activates the receptor TGF β R2, which subsequently activates its downstream EMT mediators.

Given the roles of miR-373-3p in regulating EMT and inhibiting invasion and metastasis activity, we hypothesized that miR-373-3p may also regulate TGF β signalling, which regulates EMT in choriocarcinoma cells. Our results demonstrated that TGF β R2, an apical 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-373-3p was upregulated in choriocarcinoma and miR-373-3p overexpression inhibited the EMT process and invasion/migration in vitro. These data suggest that miR-373-3p overexpression may have potential in the development of a new therapy for choriocarcinoma. Finally, our results provide a first step in understanding the pathogenesis 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 to understand the variable process.

Conclusion

This study shows that phenomenon of MET occurs in choriocarcinoma cells but not in trophoblasts and further demonstrates the mechanism of miR-373-3p in regulating EMT and inhibiting invasion and metastasis activity via targeting TGF β R2.

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. This work was performed in accordance with a protocol for archival tissue collection and use, which was approved by the Institutional Review Board (IRB) at all institutions. Gestational villus tissues were obtained at Chengde Women's and Children's Hospital. All patients signed informed consent before the surgery to allow use of the de-identified specimens for research.

Consent for publication

All patients signed informed consent before the surgery to allow 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

Yanjie Lu and Xiaoru Li contributed equally to this work, who performed the research and wrote the manuscript; Yuhong Li as the corresponding author reviewed the manuscript, made significant revisions and contributed to the reagents; Yanzhen Zuo, Qian Xu, Lei Liu, Haiying Wu and Long Chen contributed to discussions about the manuscript; Ying Zhang, Wenjing Fan and Ying Liu carried out experiments and analyzed data.

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References

1. Lurain JR: Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole. AM J

OBSTET GYNECOL 2010, 203(6):531-539.

2. Mehlen P, Puisieux A: Metastasis: a question of life or death. NAT REV CANCER 2006, 6(6):449-458.
3. Berkowitz RS, Goldstein DP: Current advances in the management of gestational trophoblastic disease. GYNECOL ONCOL 2013, 128(1):3-5.
4. Shukla V, Adiga D, Jishnu PV, Varghese VK, Satyamoorthy K, Kabekkodu SP: Role of miRNA clusters in epithelial to mesenchymal transition in cancer. Front Biosci (Elite Ed) 2020, 12:48-78.
5. Shin VY, Chu KM: MiRNA as potential biomarkers and therapeutic targets for gastric cancer. World J Gastroenterol 2014, 20(30):10432-10439.
6. Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, Cha KY, Chung HM, Yoon HS, Moon SY et al: Human embryonic stem cells express a unique set of microRNAs. DEV BIOL 2004, 270(2):488-498.
7. Keklikoglou I, Koerner C, Schmidt C, Zhang JD, Heckmann D, Shavinskaya A, Allgayer H, Guckel B, Fehm T, Schneeweiss A et al: MicroRNA-520/373 family functions as a tumour suppressor in estrogen receptor negative breast cancer by targeting NF-kappaB and TGF-beta signaling pathways. ONCOGENE 2012, 31(37):4150-4163.
8. Qiu X, Zhu J, Sun Y, Fan K, Yang DR, Li G, Yang G, Chang C: TR4 nuclear receptor increases prostate cancer invasion via decreasing the miR-373-3p expression to alter TGFbetaR2/p-Smad3 signals. Oncotarget 2015, 6(17):15397-15409.
9. Das V, Bhattacharya S, Chikkaputtaiah C, Hazra S, Pal M: The basics of epithelial-mesenchymal transition (EMT): A study from a structure, dynamics, and functional perspective. J CELL PHYSIOL 2019.
10. E DJ, Pollheimer J, Yong HE, Kokkinos MI, Kalionis B, Knofler M, Murthi P: Epithelial-mesenchymal transition during extravillous trophoblast differentiation. Cell Adh Migr 2016, 10(3):310-321.
11. Turini S, Bergandi L, Gazzano E, Prato M, Aldieri E: Epithelial to Mesenchymal Transition in Human Mesothelial Cells Exposed to Asbestos Fibers: Role of TGF-beta as Mediator of Malignant Mesothelioma Development or Metastasis via EMT Event. INT J MOL SCI 2019, 20(1).
12. Howley BV, Howe PH: TGF-beta signaling in cancer: post-transcriptional regulation of EMT via hnRNP E1. CYTOKINE 2019, 118:19-26.
13. Park SH, Cho HJ, Jeong YJ, Shin JM, Kang JH, Park KK, Choe JY, Park YY, Bae YS, Han SM et al: Melittin inhibits TGF-beta-induced pro-fibrotic gene expression through the suppression of the TGFbetaRII-Smad, ERK1/2 and JNK-mediated signaling pathway. Am J Chin Med 2014, 42(5):1139-1152.
14. Tan Y, Xu Q, Li Y, Mao X, Zhang K: Crosstalk between the p38 and TGF-beta signaling pathways through TbetaRI, TbetaRII and Smad3 expression in placental choriocarcinoma JEG-3 cells. ONCOL LETT 2014, 8(3):1307-1311.
15. Li Y, Xu Q, Zhang Z, Liu S, Shi C, Tan Y: The impact of TGF-beta1 on the mRNA expression of TbetaR I, TbetaR II, Smad4 and the invasiveness of the JEG-3 placental choriocarcinoma cell line. ONCOL LETT 2012, 4(6):1344-1348.

16. Huang Y, Zhang Z, Huang Y, Mao Z, Yang X, Nakamura Y, Sawada N, Mitsui T, Takeda M, Yao J: Induction of inactive TGF-beta1 monomer formation by hydrogen sulfide contributes to its suppressive effects on Ang II- and TGF-beta1-induced EMT in renal tubular epithelial cells. *Biochem Biophys Res Commun* 2018, 501(2):534-540.
17. Hatta M, Miyake Y, Uchida K, Yamazaki J: Keratin 13 gene is epigenetically suppressed during transforming growth factor-beta1-induced epithelial-mesenchymal transition in a human keratinocyte cell line. *Biochem Biophys Res Commun* 2018, 496(2):381-386.
18. E DJ, Pollheimer J, Yong HE, Kokkinos MI, Kalionis B, Knofler M, Murthi P: Epithelial-mesenchymal transition during extravillous trophoblast differentiation. *Cell Adh Migr* 2016, 10(3):310-321.
19. Kokkinos MI, Murthi P, Wafai R, Thompson EW, Newgreen DF: Cadherins in the human placenta–epithelial-mesenchymal transition (EMT) and placental development. *PLACENTA* 2010, 31(9):747-755.
20. Nakaya Y, Sheng G: EMT in developmental morphogenesis. *CANCER LETT* 2013, 341(1):9-15.
21. Dubruc E, Allias F, Morel AP, Golfier F, Puisieux A, Devouassoux-Shisheboran M: Gestational trophoblastic neoplasms (GTNs) do not display epithelial-to-mesenchymal transition (EMT) features. *VIRCHOWS ARCH* 2019, 475(1):121-125.
22. Wong O, Cheung C, Ip P, Ngan H, Cheung A: Amyloid Precursor Protein Overexpression in Down Syndrome Trophoblast Reduces Cell Invasiveness and Interferes with Syncytialization. *AM J PATHOL* 2018, 188(10):2307-2317.
23. Xu G, Guimond MJ, Chakraborty C, Lala PK: Control of proliferation, migration, and invasiveness of human extravillous trophoblast by decorin, a decidual product. *BIOL REPROD* 2002, 67(2):681-689.
24. Khaled N, Bidet Y: New Insights into the Implication of Epigenetic Alterations in the EMT of Triple Negative Breast Cancer. *Cancers (Basel)* 2019, 11(4).
25. Shi Y, Shi H, Zhang B, Yan Y, Han X, Jiang W, Qian H, Xu W: miR-373 suppresses gastric cancer metastasis by downregulating vimentin. *MOL MED REP* 2018, 17(3):4027-4034.
26. Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ et al: The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. *NAT CELL BIOL* 2008, 10(2):202-210.
27. Bordinon P, Bottoni G, Xu X, Popescu AS, Truan Z, Guenova E, Kofler L, Jafari P, Ostano P, Rocken M et al: Dualism of FGF and TGF-beta Signaling in Heterogeneous Cancer-Associated Fibroblast Activation with ETV1 as a Critical Determinant. *CELL REP* 2019, 28(9):2358-2372.
28. Mu Y, Li N, Cui YL: The lncRNA CCAT1 upregulates TGFbetaR1 via sponging miR-490-3p to promote TGFbeta1-induced EMT of ovarian cancer cells. *CANCER CELL INT* 2018, 18:145.
29. Ibrahim MG, Elghonaimy EA, Schafer S, Vennemann M, Kliesch S, Kiesel L, Gotte M, Schuring AN: Seminal plasma (SP) induces a rapid transforming growth factor beta 1 (TGFbeta1)-independent up-regulation of epithelial-mesenchymal transdifferentiation (EMT) and myofibroblastic metaplasia-markers in endometriotic (EM) and endometrial cells. *ARCH GYNECOL OBSTET* 2019, 299(1):173-183.

Table

Table1. Primers and RNA sequence used in this study

Transcript	Primers sequence
GAPDH	-TGAAGGTCGGAGTCAACGGAT (Sense); -CTGGAAGATGGTGATGGGATT (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 ACCCCAAAAUCGAAGCACUUCUU
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-PCR analysis of EMT marker expression in HTR-8 trophoblast cells, JAR and JEG-3 choriocarcinoma 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. (C) The relative expression levels of plexin-A1 were shown. β -actin was used as a loading control. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).

Figure 2

Trophoblasts have strong invasion and migration characteristics (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. (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. (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). (G-H) The relative expression levels of plexin-A1 were shown. β -actin was used as a loading control. 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 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 and JAR 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. TGF-β treatment decreased the mRNA levels of the epithelial marker E-cadherin and increased the levels of the mesenchymal markers N-cadherin, ZEB1 and vimentin in a dose-dependent (C) and time-dependent manner (D) in JAR cells. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).

Figure 6

Figure 6

TGF- β signalling induces EMT in choriocarcinoma. 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 (A) and JAR (C) in a dose-dependent manner. The corresponding protein quantitative result is shown of JEG-3 (B) and JAR (D). TGF- β treatment decreased the protein levels of epithelial markers and enhanced the levels of

mesenchymal markers in JEG-3 (E) and JAR (G) in a time-dependent manner. The corresponding protein quantitative result is shown of JEG-3 (F) and JAR (H). 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 7

Figure 7

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 and JAR cells were pretreated for 12 h with TGF β before transfection with the miR 373 3p mimic for 6h. The protein levels and quantitative results of JEG-3 (E-G) and JAR (F-H) are shown. β -actin was used as a loading control. Data are presented as the mean $\pm$ SD (n=3 for each group, *p<0.05, **p<0.01).