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Original Article

MicroRNA-23b regulates esophageal squamous cell carcinoma proliferation and metastasis by targeting IMP2

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Abstract: Background: Esophageal squamous cell carcinoma (ESCC) dominates in esophageal carcinomas and remains a serious threat to public health in China with an extremely poor prognosis. Thus, it is still necessary to deeply explore the underlying mechanisms and comprehensive management of ESCC. Materials and methods: 45 ESCC patients and equal amount of healthy volunteers were recruited in our study. QPCR was used to test the expression of miRNAs in the serums. *In vitro*, after transfection of miR-23b mimics into EC109 cells, the biological function of miR-143 was examined using CCK8 assay, caspase 3 activity assay, wound healing, transwell chamber and Western blot. The correlation between miR-23b and IMP2 was validated using luciferase reporter and Western blot. Results: (1) Serum levels of miR-23b were significantly increased in ESCC patients compared with healthy controls ($P<0.001$) and upregulation level of miR-23b tended to be associated with clinicopathological grade of ESCC patients. (2) Over-expression of miR-23b inhibited proliferation, caspase 3 activity migration and invasion ability of EC109 cells *in vitro*. (3) Luciferase reporter identified 3'-UTR of IMP2 mRNA harbored the target sequence of miR-23b in EC109 cells, which was also validated by Western blot result. (4) Restoration of IMP2 significantly reversed miR-23b induced anti-proliferation and anti-metastasis of EC109 cells. Conclusion: These findings pointed out that miR-23b might function through targeting IMP2 to suppress ESCC proliferation and metastasis. Our results provide new insights into the function of miR-23b in the development of ESCC and suggest it might represent a potential therapeutic target for ESCC.

Keywords: Esophageal squamous cell carcinoma, microRNA-23b, IMP2, proliferation, metastasis

Introduction

Esophageal carcinoma represents the eighth most common malignancy and the sixth most common worldwide cause of cancer-related death. The fact that about 500000 new cases of esophageal carcinoma were diagnosed annually makes it a global health problem [1, 2]. Esophageal squamous cell carcinoma (ESCC) accounts for approximately 90% of esophageal carcinomas with regard to histological classification and dominates in Asian countries [3]. As one of the most aggressive carcinomas of the gastrointestinal tract, ESCC remains a serious threat to public health in China with an extremely poor prognosis [4]. Despite the progress in perioperative, chemo surgical techniques and/or radiotherapy regimens, facilitating appropriate therapeutic strategies for ESCC patients and comprehensive management of advanced disease are needed [5].

MicroRNAs (miRNAs) are a family of endogenous, small noncoding RNAs. They regulate the translation or induce degradation of specific protein coding genes through binding to the 3'-untranslatd regions of the mRNA [6]. Bioinformatic analysis predicted that more than 60% of human genes are targets of miRNAs [7]. Although the exact origins and biological functions of circulating miRNAs are still unclear, studies revealed that altered miRNA expression were involved in tumorigenesis and the development of various cancers [8-10]. Thus, miRNAs are thought to be markers of cancers pro-

gression, prognosis and diagnosis [11]. Several human miRNAs have been shown to be dysregulated in ESCC, including miR-23b, miR-23a, miR-103a and miR-223 [12-15]. Although miR-23b has been reported to act as tumor suppressor in many diseases [13, 15-17], the functions of miR-23b in ESCC were rare unexplored previously. We, therefore, investigated the role of miR-23b in ESCC prognosis and therapeutic monitoring.

IMP2/p62 (insulin-like growth factor 2 mRNA binding protein, IMP) was originally identified as an autoantigen in a hepatocellular carcinoma patient [18]. And the autoantibodies against the IMP2/p62 was reported to be elevated in patients with esophageal squamous carcinoma [19]. Here we hypothesize that miR-23b may be involved in esophageal carcinoma through regulating IMP2 expression in ESCC.

In this study, the serum miR-23b concentration was compared between patients with ESCC and healthy people. The relationship between miR-23b and IMP2, as well as their biological activity, were identified in ESCC cells.

Materials and methods

Participants and samples

26 males and 19 females ESCC patients (aged 45-75 years) and a control group of 45 healthy volunteers were recruited in our study. Serum samples were obtained from patients and volunteers. All 45 patients did not receive surgical, preoperative chemotherapy or radiotherapy. The 45 ESCC serum samples consisted of 19 cases of well differentiated, 15 cases of moderately differentiated, 11 cases of poorly differentiated. All of these patients agreed to participate in this study gave written informed consent. The study authorized by the Xiangyang Hospital, Hubei University of Medicine Ethics Committee in China. Serum samples obtained as follows: venous blood was extracted, 4000 rpm, 10-minutes centrifugation. The supernatant was stored at -80°C.

Cell lines and cell culture

ESCC cell lines EC109 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (10% FBS) (Bioind, Israel) and 1% of 100 U/mL penicillin and streptomycin sulfates. All the cell lines were incubated in a humidified incubator at 37°C with 5% CO_2.

Cell transfection

Hsa-miR-23b mimics (pGCMV/EGFP) was synthesized by GenePharma, and Human IMP2 gene was constructed into pcDNA3.1+HA-empty vector by Life Technologies, while the empty vector (NC) served as negative control (Both, Shanghai, China). 1×10^5 per well of EC109 cells were added to the 24 well-plates. After 24 h, Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen-Life Technologies, Shanghai, China) (DNA/Lipofectamine 2000=1/2.5) according to the manufacturer's instructions. Incubated in RPMI-1640 medium with blasticidin (12 μg/mL) or G418 (500 mg/ml) (Both, Sigma, Shanghai, China) for 15 days, Stable transfection expression of cells were verified by Western blot and real-time quantitative polymerase chain reaction (RT-PCR). Successful clones were pooled and frozen in liquid nitrogen for further experiments.

Cell proliferation assay

Cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. EC109 cells with established stable expression (NC, miR-23b mimics, miR-23b mimics+pcDNA3.1+HA-empty vector, miR-23b mimics+pcDNA3.1+HA-IMP2) were seeded at a density of 8×10^3 cells per well in 96-well plates and allowed to grow for 24 h, 48 h, and 72 h. After incubation, the absorbance at 450 nm was measured using a microtiter plate reader (Thermo Fisher Scientific, Waltham, MA) and a growth curve was drawn.

Caspase-3 activity analysis

Caspase-3 colorimetric activity assay kit (Beyotime, Shanghai, China) was used to determine Caspase-3 activity according to the manufacturer's instructions. EC109 cells with established stable expression were collected and lysed with the cell lysis buffer included in the kit. Concentration of extracted proteins was determined by the Bicinchoninic acid (BCA) method. Equal amounts of protein were then

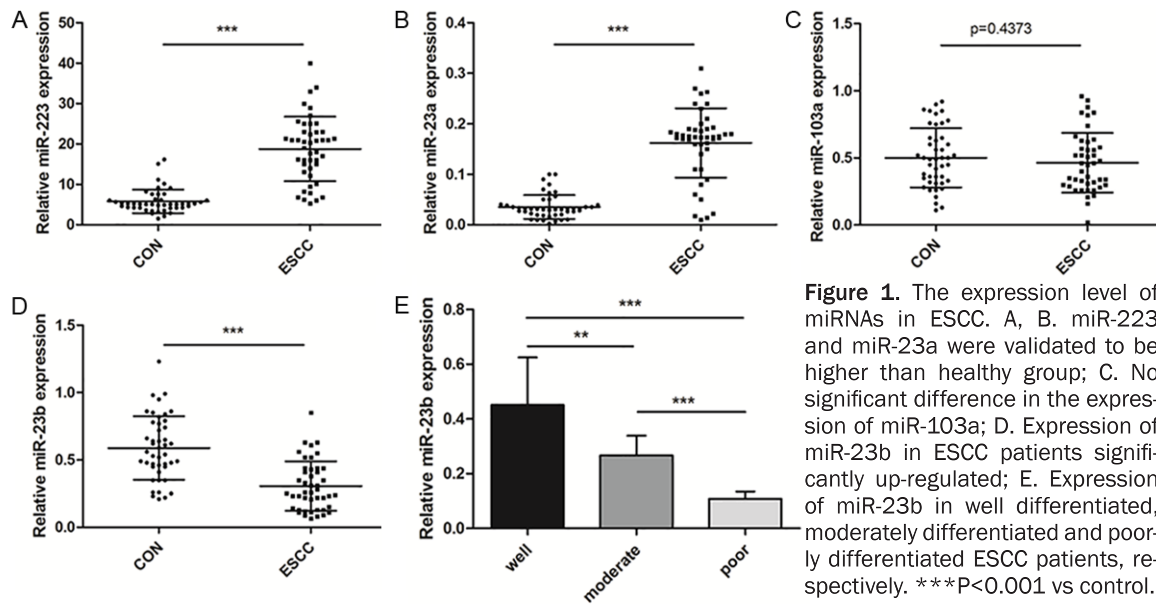

Figure 1. The expression level of miRNAs in ESCC. A, B. miR-223 and miR-23a were validated to be higher than healthy group; C. No significant difference in the expression of miR-103a; D. Expression of miR-23b in ESCC patients significantly up-regulated; E. Expression of miR-23b in well differentiated, moderately differentiated and poorly differentiated ESCC patients, respectively. ***P<0.001 vs control.

incubated with Ac-DEVD-AMC (a caspase-3 substrate) at 37°C for 2 h. After incubation, the absorbance at 405 nm was measured using an electroluminescence immunosorbent assay reader.

Cell migration assay

Cell migration ability was determined by wound-healing assay. EC109 cells with established stable expression were plated into 12-well plates, after grew approximately 90% confluence, the cells layer was scratched gently. After wash twice with cold PBS to remove float cells, plates were continue to be incubated in RPMI-1640 medium containing 2% FBS for additional 48 h. Digital camera system (Olympus Corp., Tokyo, Japan) was used to acquire five random images of the scratches of each group at a magnification of 200×.

Cell invasion assay

Transwell assay was used to determine cell invasion. EC109 cells with established stable expression were incubated in RPMI-1640 medium supplemented with 1% FBS, which added in the upper chamber of 24-well plates precoated with diluted Matrigel. The lower chamber was filled with 20% FBS as a chemoattractant. After 48 h at 37°C, invading cells migrating to the lower surface were fixed and stained, while non-invading cells in the upper chamber were removed by cotton swab. Five random fields in each chamber were photographed and counted at ×100 magnification.

Isolation of RNA and quantitative polymerase chain reaction analysis

Total RNA from serum and ESCC cells were extracted using TRIzol (Invitrogen, USA) following the manufacturer's protocols. MiRNA-specific RT primers (RiboBio, Guangzhou, China) for miR23b and random primer (TaKaRa, Dalian, China) for IMP2 were synthesized. Quantitative polymerase chain reaction (qPCR) was used to measure Reverse-transcribed cDNA with SYBR Green PCR Kit (QIAGEN, Shanghai, China) under the following conditions: predenaturation at 95°C for 5 min, denaturation at 95°C for 10 sec, annealing and extension at 60°C for 30 sec, the followed steps were running for 40 cycles. The relative miRNA and mRNA expression levels were normalized by U6 and GAPDH, respectively.

The reverse transcription primer miR-23b: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATCA-3'; *The qPCR primers* miR-23b: 5'-GAGGGTTCCTGGCATGC-3' (forward), 5'-GTGCAGGGTCCGAGGT-3' (reverse).

Western blot

Proteins, extracted from serum and ESCC cells, were determined by the bicinchoninic acid (BCA) method (Beyotime, Hangzhou, China). An aliquot of 25 μg of denatured protein from each sample were loaded onto a 10% SDS polyacrylamide gel. Samples were transferred to nitrocellulose membrane. The membranes were probed with antibodies against IMP2 (1:1000,

Table 1. Association between serum mir-23b levels and clinicopathological factors

Variables	Patients (n=45)	MiR-23b median[b]	*P*-value[b]
Age			
>60	23 (51%)	0.4016	0.1423
≤60	22 (49%)	0.3754	
Sex			
Male	26 (58%)	0.2610	0.1650
Female	19 (42%)	0.3431	
Venous invasion[a]			
v0	27 (60%)	0.1267	0.0486*
v1-3	18 (40%)	0.3911	
Lymphatic invasion[a]			
Ly0	21 (47%)	0.1465	0.0712
Ly1-3	24 (53%)	0.3022	
*P*t-stage[a]			
T0-1	12 (26%)	0.1003	0.0398
T2	9 (20%)	0.1352	
T3	21 (47%)	0.2513	
T4	3 (7%)	0.4611	
*P*N-stage[a]			
N0	17 (38%)	0.2155	0.1068
N1	19 (42%)	0.1620	
N2	4 (9%)	0.3623	
N3	5 (11%)	0.4011	
*P*stage[a]			
I	5 (11%)	0.1121	0.0361*
II	17 (38%)	0.1835	
III	20 (44%)	0.2674	
IV	3 (7%)	0.5360	

[a]TNM classification. [b]The Mann-Whitney *U*-test and Kruskal Wallis *H*-test were performed to compare serum miRNA concentrations. *P*-value was considered significant at 0.05.

Abcam, England), PCNA (1:1000, Abcam), vimentin (1:500, Abcam), and GAPDH (1:1000, Abcam) overnight at 4°C followed by incubation with secondary antibody (1:2000 dilution; both, Cell Signaling Technology, Boston, MA) for 1 h at room temperature. The specific proteins were visualized with Odyssey™ Infrared Imaging System (Gene Company, Lincoln, NE, USA).

Luminescent reporter gene transfection and luciferase assays

The potential miR-23b binding sites of IMP2 were predicted by using the TargetScan and miRanda database. In the luciferase reporter assay, ESCC cells were added to 96-well plates at 1×10^4 cells per well, after 48 h post-transfection, the dual-luciferase reporter assay system with the luminometer (Promega, Madison, WI, USA) was used to measure the luciferase activity. IMP2 mRNA 3'-UTR contained sequences with mutations (MUT) in the putative binding sites of miR-23b. The firefly luciferase activities were used as an internal control for transfection efficiency.

Statistical analyses

All experiments were performed at least three independent times. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) were used for the statistical analyses. Statistical graphs were drawn using GraphPad 6.0 software. Comparisons between groups were analyzed by analysis of variance or 2-tailed Student t-test. Data were presented as the mean value ± SD. A *p*-value <0.05 was considered statistically significant.

Results

Expression level of miRNAs in ESCC

The expressions of miRNAs from serum were investigated by qRT-PCR methodologies in our study. We selected four miRNAs (namely miR-23a, miR-223, miR-23b and miR-103a), which were reported to be aberrant expressed in serum of ESCC patient [12-15]. As shown in **Figure 1A**, **1B**, the plasma level of miR-223 and miR-23a were validated to be higher than healthy group (all panels, P<0.001). In addition, the results revealed no significant difference in the expression of miR-103a (P=0.4373) (**Figure 1C**). However, the expression of miR-23b in ESCC patients showed significant up-regulation compared with healthy control (P<0.001) (**Figure 1D**). Furthermore, miR-23b (P<0.01 well differentiated vs moderately differentiated; P<0.001 moderately differentiated vs poorly differentiated, well differentiated vs poorly differentiated) tended to be higher in the serum of ESCC patients with well differentiated than in those with moderately differentiated or poorly differentiated (**Figure 1E**). We also analyzed whether miR-23b was correlated with some clinicopathological factors in ESCC patients. Result showed that serum miR-23b level tended to be high in the presence of venous invasion (P=0.0486) and advanced P stage (P=0.0361; **Table 1**).

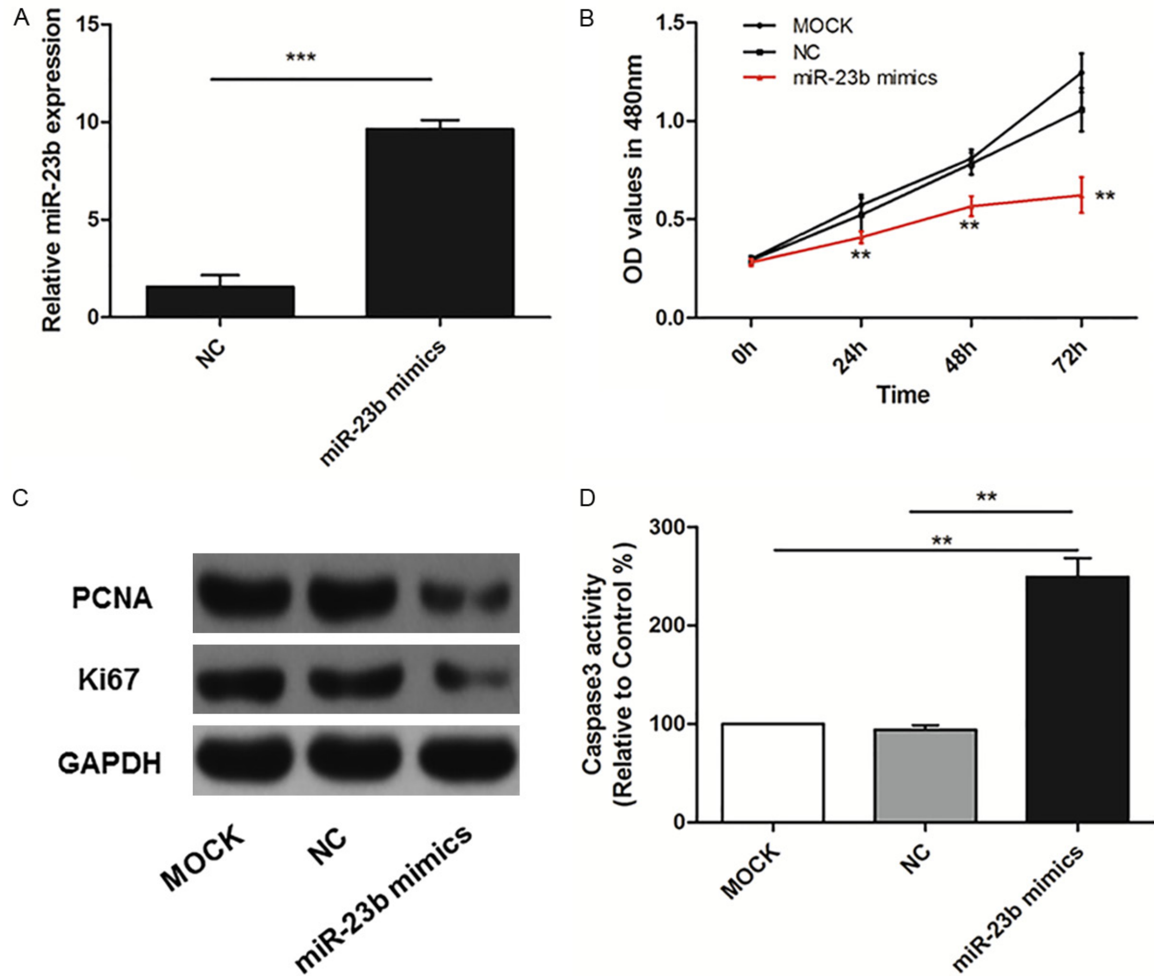

Figure 2. MiR-23b overexpression inhibits the proliferation of EC109 ESCC cells. A. Expression of miR-23b was significantly increased in EC109 cells after treatment with miR-23b mimics; B. Over-expression of miR-23b dramatically inhibited proliferation ability of EC109 cells at 24 h, 48 h and 72 h, no significant difference was found between MOCK group and NC group; C. miR-23b decreased the protein expression levels of Ki67 and PCNA; D. Caspase-3 activity was increased significantly after transfected with miR-23b mimics. **P<0.01 ***P<0.001 vs control.

MiR-23b overexpression inhibits the proliferation of EC109 ESCC cells

As shown in the **Figure 2A**, the expression of miR-23b was significantly increased in EC109 cells after treatment with miR-23b mimics (P<0.001). Ectopic over-expression of miR-23b could dramatically inhibit proliferation ability of EC109 cells at 24 h, 48 h and 72 h (all panels, P<0.01), no significant difference was found between MOCK group and NC group (P>0.05) (**Figure 2B**). In addition, we also found that restoration of miR-23b decreased the protein expression levels of Ki67 and PCNA (**Figure 2C**). Meanwhile, Caspase-3 activity was increased significantly after transfected with miR-23b mimics (all panels, P<0.01) (**Figure 2D**).

MiR-23b overexpression inhibits EC109 cells migration

In the wound healing assay, transfected with miR-23b mimics dramatically inhibited the migration ability of EC109 cells compared to the control (P<0.01, **Figure 3A**, **3B**).

MiR-23b overexpression inhibits EC109 cells invasion

In accordance with migration, over-expression of miR-23b resulted in strong invasion inhibitory of EC109 cells (all panels, P<0.01) (**Figure 4A**, **4B**). Moreover, the protein expression levels of MMP2 in EC109 cells transfected with miR-23b mimics were also decreased significantly compared with control (**Figure 4C**).

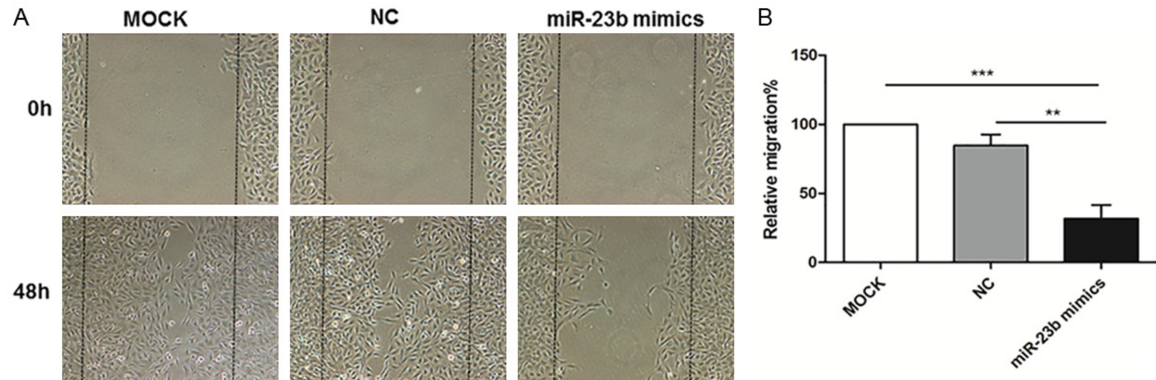

Figure 3. MiR-23b overexpression inhibited EC109 cells migration. A, B. miR-23b mimics dramatically inhibited the migration ability of EC109 cells compared to the control. **P<0.01 ***P<0.001.

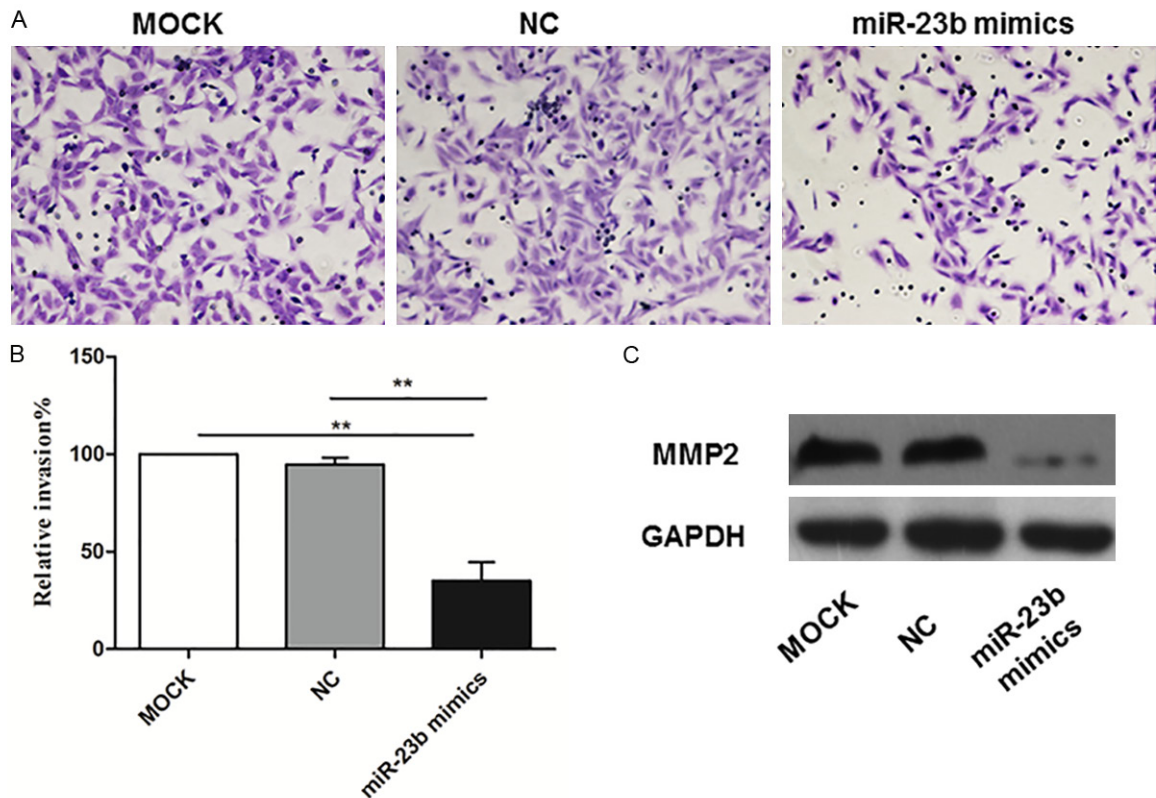

Figure 4. MiR-23b overexpression inhibits EC109 cells invasion. A, B. miR-23b mimics dramatically inhibited the invasion ability of EC109 cells; C. miR-23b mimics decreased the protein expression levels of MMP2 in EC109 cells. **P<0.01 vs control.

IMP2 is a direct target of miR-23b

The potential miR-23b binding sites of IMP2 were predicted by three computer-aided algorithms including TargetScan, miRanda and PicTar. **Figure 5A** showed the potential target sequence of miR-23b harbored in 3'-UTR of IMP2 mRNA. Luciferase reporter assay was then performed and result demonstrated that miR-23b mimics in the wild type (WT) significantly inhibited the luciferase activity, while the inhibitory effect of miR-23b mimics was vanished in the MUT vector (all panels, P<0.05) (**Figure 5B**). Moreover, qPCR and Western blot analyses revealed that ectopic over-expression of miR-23b dramatically decreased the expres-

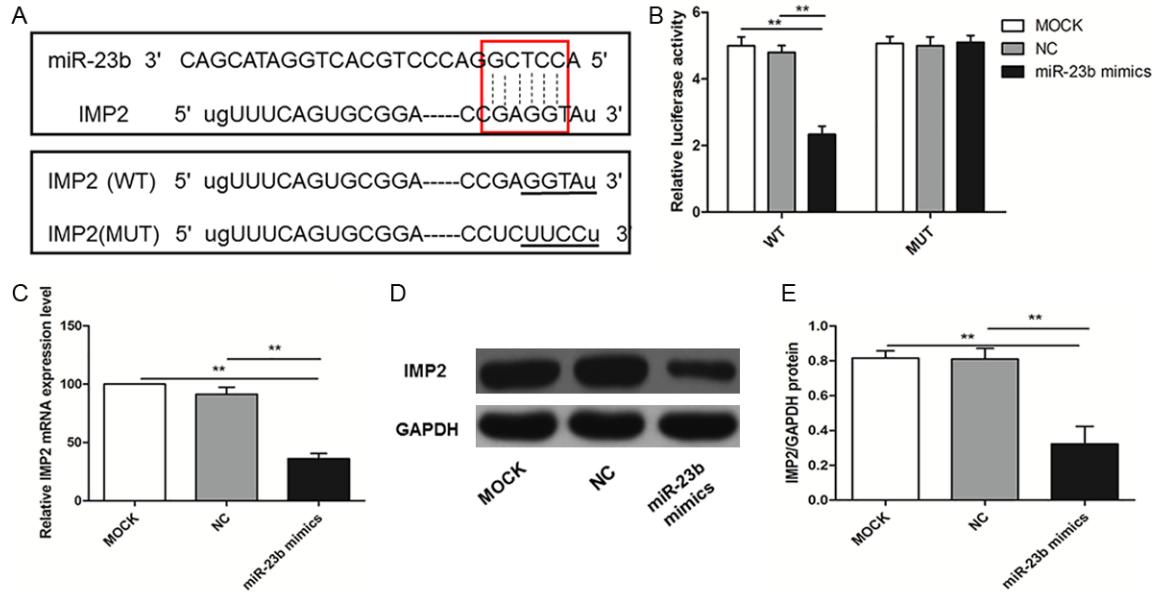

Figure 5. IMP2 is a direct target of miR-23b. A. Target sequence of miR-23b harbored in 3'-UTR of IMP2 mRNA; B. miR-23b mimics in the wild type (WT) significantly inhibited the luciferase activity, inhibitory effect of miR-23b mimics was vanished in the MUT vector; C-E. Over-expression of miR-23b dramatically decreased the expression of IMP2 in EC109 cells. **P<0.01 vs control.

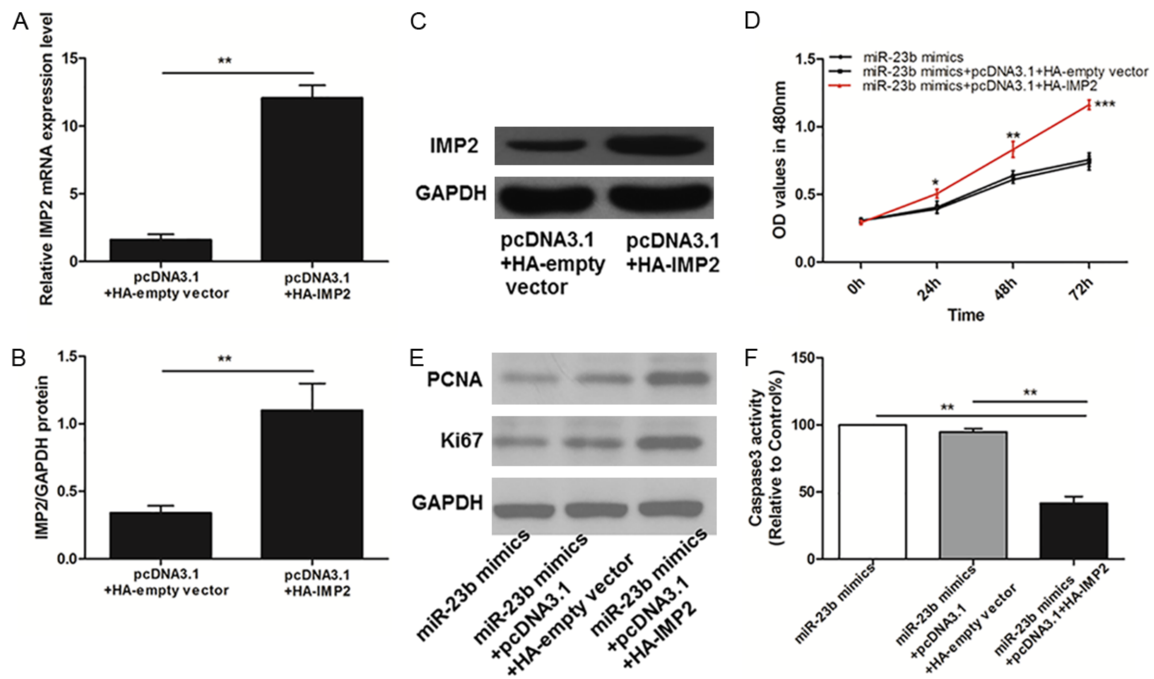

Figure 6. IMP2 contributes to miR-23b increased proliferation of EC109 cells. A-C. IMP2 was increased in the cells transfected with pcDNA3.1+HA-IMP2 compared with the pcDNA3.1+HA-empty plasmid; D. IMP2 re-introduction reversed the anti-proliferation role of miR-23b; E. Expression levels of PCNA and ki-67 in EC109 cells transfected with miR-423-5p mimics were no longer decreased after overexpression of IMP2; F. Overexpression IMP2 also decreased caspase-3 activity of cells transfected with miR-423-5p mimics significantly. *P<0.05 **P<0.01 ***P<0.001 vs control.

sion of IMP2 in EC109 cells (P<0.01) (**Figure 5C-E**). In summary, our results suggest that IMP2 might be a target of miR-23b in EC109 cells.

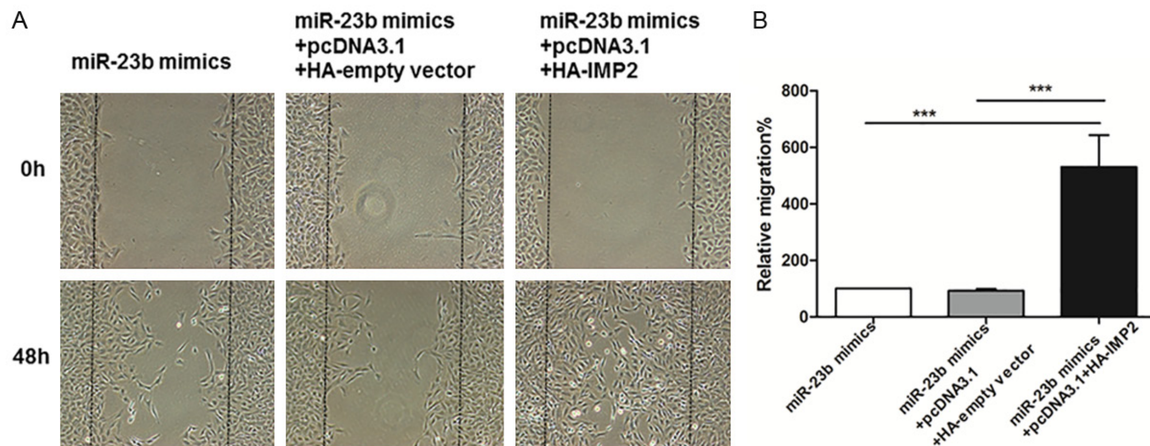

Figure 7. IMP2 contributes to miR-23b induced migration of EC109 cells. A, B. IMP2 re-introduction reversed the inhibitory role of miR-23b in cell migration. ***P<0.001 vs control.

IMP2 contributes to miR-23b increased proliferation of EC109 cells

PcDNA3.1+HA-IMP2 were transfected in over-expression of miR-23b mimics cells to increase the expression of IMP2 in EC109 cells. qPCR and western blot validated that the IMP2 was increased in the cells transfected with pcDNA3.1+HA-IMP2 compared with the pcDNA3.1+HA-empty plasmid (P<0.01) (**Figure 6A-C**).

As shown in **Figure 6D**, miR-23b contributed to a decrease of EC109 cell proliferation, whereas IMP2 re-introduction reversed the anti-proliferation role of miR-23b. Additionally, Western blot examined that protein expression levels of PCNA and ki-67 in EC109 cells transfected with miR-423-5p mimics were no longer decreased after overexpression of IMP2 (**Figure 6E**). As for Caspase-3 activity, overexpression IMP2 also decreased caspase-3 activity of cells transfected with miR-423-5p mimics significantly (all panels, P<0.01) (**Figure 6F**).

IMP2 contributes to miR-23b induced migration of EC109 cells

IMP2 re-introduction reversed the inhibitory role of miR-23b in cell migration (all panels, P<0.001) (**Figure 7A**, **7B**).

IMP2 contributes to miR-23b induced invasion of EC109 cells

As with migration, over-expression of IMP2 resulted in invasion increasing of EC109 cells (all panels, P<0.001) (**Figure 8A**, **8B**). Moreover, IMP2 also increased the protein expression levels of MMP2 in EC109 cells transfected with miR-23b mimics significantly compared with control (**Figure 8C**).

Discussion

In the present study, we showed that the serum levels of miR-23b were significantly increased in ESCC patients compared with healthy controls. Further research revealed that the expression of miR-23b tended to be higher in the serum of ESCC patients with well differentiated than in those with moderately differentiated or poorly differentiated. Over-expression of miR-23b inhibited proliferation, migration and invasion ability of EC109 cells *in vitro*. More importantly, we identified IMP2 as a direct target gene of miR-23b in EC109 cells. Restoration of IMP2 significantly reversed miR-23b induced anti-proliferation and anti-metastasis of EC109 cells.

ESCC dominates in esophageal carcinomas and remains a serious threat to public health in China with an extremely poor prognosis [4, 20]. Despite of the recent rapid promotion in the diagnosis and therapeutic strategies for ESCC patients, prognosis improvement and comprehensive management of such disease are needed [5]. MiRNAs have been shown to aberrantly express in a variety of cancers including ESCC [12, 21-23]. Since Tanaka *et al.* first investigated serum miRNAs and proved the high expression of *miR-27a/b* in serum to be correlated to poor prognosis [24], Komatsu etc

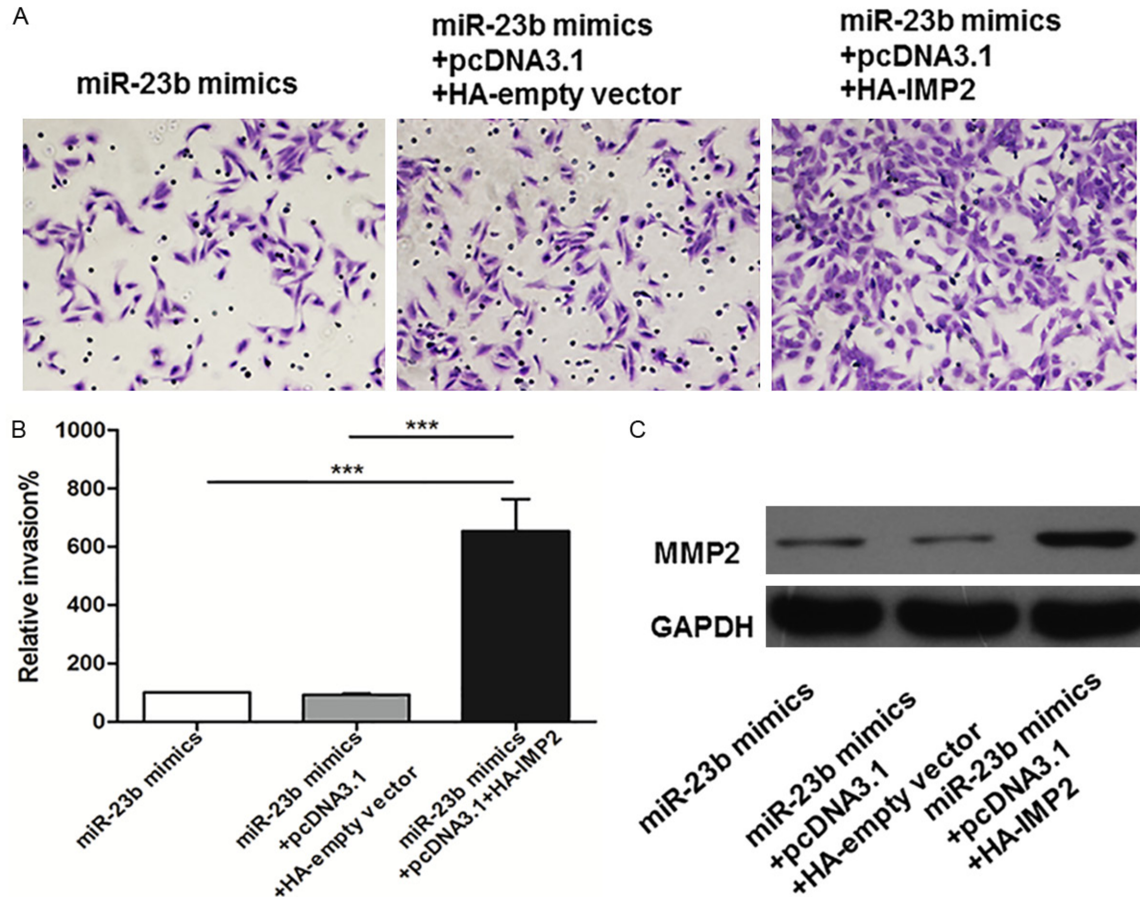

Figure 8. IMP2 contributes to miR-23b induced invasion of EC109 cells. A, B. Over-expression of IMP2 resulted in invasion increasing of EC109 cells pretransfected with miR-23b mimics; C. IMP2 increased the protein expression levels of MMP2 in EC109 cells transfected with miR-23b mimics. ***$P<0.001$ vs control.

also identified the plasma miRNA miR-23b as a predictive biomarker for chemoresistance in ESCC [12]. Decreased miR-23b has been reported in ovarian cancer, bladder cancer and Human Gliomas [13, 15, 25]. Among these cancers, miR-23b might be regarded as an tumor suppressive miRNA and miR-23b overexpression was correlated to restrain cell proliferation, migration and invasion. Which are consistent with our results that miR-23b was significantly downregulated in ESCC patients and elevated miR-23b expression was oppositely associated with capability of ESCC cell proliferation, caspase 3 activity, migration and invasion.

To further understand the mechanism of miR-23b in ESCC, predicting potential binding sites of miR-23b were conducted. We found that 3'-UTR of IMP2 mRNA harbored the target sequence of miR-23b. Moreover, ectopic overexpression of IMP2 in EC109 cells transfected with miR-23b mimics led to anticancer capability silence of miR23b. Previous studies showed that *IMP2* expression was increased with tumor size and clinical tumor stage, similar to the results in our study.

These findings pointed out that miR-23b might function through targeting IMP2 to suppress ESCC proliferation and metastasis.

In conclusion, our current study demonstrates that serum levels of miR-23b were significantly increased in ESCC patients compared with healthy controls and upregulation level of miR-23a tended to be associated with clinicopathological grade of ESCC patients. Moreover, findings validated that miR-23b might function through targeting IMP2 to suppress ESCC proliferation and metastasis. Our results provide new insights into the function of miR-23b in the

development of ESCC and suggest it might represent a potential therapeutic target for ESCC.

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Disclosure of conflict of interest

None.

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