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**Abbreviations:** GBC, Gallbladder carcinoma; TGF-β1, Transforming Growth Factor-β1; Smad7, Mothers Against Decapentaplegic homolog 7; EMT, epithelial-mesenchymal transition.

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Abstract

Background & Aims: The dysfunction of miRNAs has been demonstrated participating in the regulation of various tumor developments. However, whether miRNAs are involved in metastasis and progression of GBC remains obscure.

Methods: A new designed gain-of-function miRNAs screening technology was applied to filter out pro-metastatic miRNAs in GBC. Their expression in GBC tissues was validated by real-time PCR. The biological functions of miRNAs were intensively studied by transwell, immunoblot, immunohistochemical and in situ hybridization assays. Tumorigenicity and liver metastasis were further examined in nude mice.

Results: Of 880 miRNAs, 17 were filtered out as the prominent metastatic inducers for GBCs. Among them, the up-regulation of pro-metastatic miR-20a was closely associated with the local invasion, distant metastasis and poor prognosis of 67 followed-up GBC patients clinically. Patients with higher miR-20a expression exhibited worse overall survival (OS, median OS time were 5 and 20 months, respectively) than the lower expression group. The dramatically increased TGF-β1 level was found in GBC patients, which was responsible for the elevation of miR-20a. The ectopic expression of miR-20a could induce Epithelial-mesenchymal transition and enhance the metastasis of GBC cells in vitro and in vivo, through directly targeting the 3’UTR of Smad7 and promoting nucleus translocation of β-catenin subsequently. Conversely, the blockage of miR-20a by specific antagomir effectively restored the expression of Smad7 and attenuated TGF-β-induced cell metastasis.
Conclusions: TGF-β1-mediated activation of miR-20a/Smad7/β-catenin axis plays a pivotal role in the pathogenesis and worse prognosis of GBCs, which may serve as a potential therapeutic target in the future.
Introduction

Gallbladder carcinoma (GBC) is the seventh gastrointestinal cancer [1] worldwide, with an annual incidence of over 10,000 and mortality of about 3,300 [2]. GBC has an extremely poor prognosis, with a median survival time for suspected carcinomas of 9.2 months, and for incidental carcinomas of 26.5 months [3]. Up until now, surgical resection is the only potentially curative therapy for GBC. However, even after complete resection, loco-regional recurrence rates are extremely high. And all other treatments, including chemotherapy and radiotherapy are palliative, which also have no remarkable improvement for its dismal prognosis and survival. Local tumor growth, hepatic invasion and lymph node metastasis are the main prognostic factors in patients with GBC. Although several genes have been identified involving the progression of GBC, such as C-erbB2, iNOS, COX-2 [5] in the past few decades, the detailed molecule mechanisms are still under evaluated.

Many miRNAs have been demonstrated to actively participate in the regulation of tumor development as tumor suppressor genes or oncogenes. Their emerging roles in the development and progression of human cancers may present novel diagnostic and therapeutic opportunities, also supply the prognostic information of tumor development. However, the potential roles of miRNAs in GBC have not yet been documented.

MiR-20a is located in the 13q31 chromosomal region on which DNA amplification is correlated with malignant phenotype and poor prognosis in several cancers [6]. Recently, it was shown that miR-20a encoded by the miR-17-92 cluster
enhances the proliferation and metastatic potential of ovarian cancer cells and osteosarcoma cells [7,8], and the over-expression of miR-20a also contributes to chemotherapeutic resistance of colorectal adenocarcinoma [9]. On the contrast, miR-20a overexpression inhibited proliferation and metastasis in breast cancer and pancreatic cancer [10,11]. In the miR-17-92 cluster, there are many miRNAs which were shown as oncogenes. However, in this study only miR-20a was filtered out to be pro-metastasis in GBC by high content screening (HCS).

Here, we first showed that miR-20a is up-regulated in GBC samples, which in turns promotes the invasion and proliferation of GBC cells, accompanied with the dysregulation of several EMT-related genes in vitro and in vivo. Smad7 (mothers against decapentaplegic homolog 7), a potential inhibitor of TGF-β1 signaling pathway, was identified as the direct target of miR-20a. Exogenous expression of Smad7 in GBC cells significantly reversed miR-20a-induced β-catenin translocation and cell migration/invasion. Patients with abnormal enrichment of miR-20a and lower expression of Smad7 in GBC tissues showed very poor prognosis. Moreover, we also found that the elevation of miR-20a in response to TGF-β1 treatment is necessary for TGF-β1 induced cell migration and invasion, implying such TGF-β1/miR-20a/Smad7 axis plays an important role in the progression of GBC, and may be benefit for the development of potential therapeutics against GBC.
Materials and methods

Tissue samples and tumor cell lines

A total of 85 GBC specimens were randomly retrieved from GBC patients who underwent curative resection, and 11 normal gallbladder mucous membrane tissues were obtained from liver transplantation donators, following informed consent according to an established protocol approved by the Ethic Committee of Eastern Hepatobiliary Surgery Hospital. All GBC patients were retrospectively followed up until September 2012, none of whom received chemotherapy or radiotherapy after surgery. Overall survival(OS) was defined as the interval between the dates of surgery and death. OS exceeding 30 months was considered the censored value. Supporting Table.S2 shows patient clinicopathological features. The GBC cell lines GBC-SD and SGC-996(Chinese Academy of Science) were used in this study, which were cultured in Dulbecco’s modified Eagle medium(Gibco, Carlsbad, CA, USA) with 15% and 10% fetal bovine serum(PAA Laboratories, Pasching, Austria) separately, supplemented with 100U/mL penicillin and 100 μg/mL streptomycin.

Pro-metastatic miRNAs screening

880 mature miRNA mimics(100nM)(Ribobio, Guangzhou, China) were transfected into GBC-SD cells(1500 cells per well) in the 96 microplates(BD). 60h after transfection, GBC-SD cells were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with Triton X-100 for 5 minutes. Then F-actin was stained with Phalloidin(Invitrogen, USA) in PBS(1:50) for 20 minutes at room temperature(RT). And cell nuclei were stained by DAPI for 3 minutes at RT. Finally, the fluorescent images of F-actin were acquired using ImageXpress Micro XL.
high-content screening system (Molecular Devices, USA), and the microfilament total microfilament length per cell in each well was analyzed by MeteXpress 4.0 software (Molecular Devices, USA).

For detailed materials and methods, please refer to the Supporting information.
Results

Identification of metastasis-related miRNAs via high content miRNA library screening

To identify novel miRNAs that manipulate GBC cell metastasis, a HCS-based gain-of-function method was developed by monitoring the formation of F-actin microfilament with Cy3-labeled phalloidin (Invitrogen, UK). F-actin is one type of stress fiber that regulated cell motility and polarization, which is often documented in several kinds of cancer cells with highly metastatic property [12]. The simplified flowchart was shown in Fig.1A, detailed procedure, filtration criteria and original data can be referred in Supplementary information and Fig.S1A.

As shown in Fig.1B, of 880 miRNAs, only 24 miRNAs were filtered out as the pro-metastasis factors with the cutoff value of more than AVG(average)+2SD (standard deviation). After verification with microscopic examination (Wells with cell confluence >80% or <30%), 17 of 24 were identified as listed in Fig.1C. Interestingly, several miRNAs have been recently reported as the key factors for tumor metastasis in vitro and in vivo, which provides strong evidence that our developed method is a powerful tool for large-scale screening motility or metastasis phenotype(Table.S1).

To further study the potential roles of such miRNAs in GBC cells, we examined the expression of top 10 miRNAs and two mature miRNAs, miR-20a and miR-1470, can be detected in both GBC tumor and normal gallbladder tissues, (CT values of other miRNAs were above 35 or undetected)(Fig.1D, S1B). Moreover, only miR-20a was found highly expressed in tumor tissues by real-time PCR and LNA in situ assay.
(Fig.1D and 1E). In comparison with TGF-β1, a well-known inducer for fiber polymerization, miR-20a significantly enhanced the length and thickness of F-actin microfilaments, whereas the control miR-27b didn’t show any changes (Fig.1F). In addition, other miRNAs in miR-17-92 cluster, miR-17-5p, miR-17-3p, miR-18a, miR-19a and miR-19b, didn’t show significant F-actin increase (Fig.S1B).

MiR-20a plays a key role in GBC metastasis and growth

To explore the potential role of miR-20a on GBC cells, we first applied mature miR-20a mimics and its specific inhibitor miR-20a antagomir. As shown in Fig.2A, GBC-SD cells with exogenous expression of miR-20a exhibited the reduction of cell-cell contacts and a more elongated morphological shape. Meanwhile, the treatment of miR-20a antagomir resulted in the formation of clear fusiform and multi-angle cell-cell adhesion in comparison with control cells. We also applied lentivirus system expressing mir-20a-GFP or mir-NC-GFP to generate four stable cell lines with GBC cell lines, GBC-SD and SGC-996. As expected, the morphology of GBC-SD-20a cells showed similar morphology as mimic miR-20a transient transfection group. Furthermore, both “wound healing” and Boyden chamber assays showed that enhanced migration and invasion activity of GBC-SD, SGC-996 and GBC-SD-20a cells in the presence of miR-20a (Fig.S1C, Fig.2B-2D), but those were remarkably reduced upon miR-20a antagomir treatment, which suggested that miR-20a can significantly promote the metastasis of GBC cells in vitro. Correspondingly, western blot and real-time PCR analysis showed that miR-20a
significantly increased the expression of EMT-related genes N-cadherin, Vimentin and Snail, whereas decreased that of E-cadherin (Fig. 2E, S1D). Additionally, the proliferation rate of GBC-SD cells was enhanced after miR-20a mimic transfection in comparison with their counterpart cells treated with miR-20a antagomir during the indicated time (Fig. S1E).

We then compared the growth and metastasis activity of GBC-SD cells in vivo. After incubated with miR-20a antagomir or the scramble miRNA antagomir, about 2500,000 GBC-SD cells were injected into left dorsal subcutaneous tissues of nude mice. Three weeks after inoculation, miR-20a antagomir was injected into the tumor tissues twice a week (5nmol/mouse). As shown in Fig. 2F, the tumor growth was significantly inhibited in the presence of miR-20a antagomir. IHC staining showed the membrane localization of E-cadherin, lower expression of Vimentin and less Ki-67 positive cells in miR-20a antagomir inoculated tumor tissues (Fig. 2G). The mRNA expression of E-cadherin, N-cadherin and Vimentin also showed similar trends (Fig. S1F-1G).

Next, we employed liver tumor metastasis model via spleen injection [13] to monitor the metastasis ability of GBC-SD cells in vivo. As compared with miR-20a antagomir-treated mice, the total metastasis occurrence number was extremely higher in control mice (7 for control mice vs. 1 for antagomir treated ones) (Fig. 2H, S2A). IHC with duct-specific antibody CA19-9 was shown to confirm the live metastases (Fig. 2H).

In addition, the stable transfected cell line GBC-SD-20a and GBC-SD-GFP were
also used to perform the opposite experiments in both subcutaneous xenograft and liver tumor metastasis model via spleen injection. The results in Fig.S2B and S2C showed that GBC will be more aggressive and invasive in response to the over-expression of miR-20a. Collectively, our data demonstrated that miR-20a plays the potential role in promoting both cell proliferation and metastasis \textit{in vitro} and \textit{in vivo}.

\textit{Smad7 is the direct target of miR-20a}

To address the mechanisms by which miR-20a induces proliferation and migration/invasion in GBC, we employed three steps to identify the potential target genes of miR-20a: First, we picked up the shared genes whose 3'UTR sequences were predicted with miR-20a binding sites via different programs (miRanda and TargetScan) simultaneously; Second, we aligned the target regions of shared genes of different organisms to further filter out the conversed target regions and genes for miR-20a; Third, the conversed genes whose expression levels were reduced in response to miR-20a treatment were nominated as the potential target genes for further experiments.

Basing on above criteria, Smad7, which contains a conserved 3'UTR element complementary to miR-20a across over eight vertebrate organisms (Fig.3A), was selected for further experiments. To verify the regulation of miR-20a on Smad7, we constructed the 3'UTR reporter plasmids coupled with full-length wild-type (pGLO-Smad7-WT) or mutant miR-20a sites 3'UTR (pGLO-Smad7-MT) of
Smad7. Luciferase analysis showed that miR-20a could repress the reporter activities of pGLO-Smad7-WT plasmid but not that of pGLO-Smad7-MT(Fig.3A). Fig.3B(left two panels) showed that the mRNA level of Smad7 was dramatically enhanced or decreased in response to the incubation of miR-20a antagomir or miR-20a mimics in GBC-SD and SGC-996 cells as compared with control cells, separately. Analogously, the expression level of Smad7 in GBC-SD-20a cells also displayed the similar change trend(Fig.3B right two panels). To further evaluate the potential relationship between Smad7 and miR-20a in clinical GBC specimens, expression of Smad7 and miR-20a were analyzed. As illustrated in Fig.3C, the enrichment of Smad7 protein was found chiefly in normal gallbladder cells as compared with the tumor tissues. Reciprocally, the negative correlation between miR-20a and Smad7 was observed in 18 GBC tissues(Fig.3D). Analogously, IHC staining with xenograft subcutaneous tumors also showed the enhanced expression of Smad7 protein in response to miR-20a antagomir treatment(Fig.3E). These data provided strong supports for our hypothesis that the reduction of Smad7 is likely the result of enhanced expression of miR-20a.

**Negative modulation of Smad7 expression by miR-20a is essential for miR-20a-promoted GBC cells metastasis and epithelial-to-mesenchymal transition**

Respecting the distinct roles of Smad7 in various cancers [14-16], we applied synthesized short interfering RNA(siRNA) specifically against Smad7(Table.S3) expression to investigate its functions in GBC cells. As shown in Fig.S2D, the migration and invasion of GBC-SD cells were significantly enhanced after the
inhibition of Smad7 expression. Moreover, the inhibition of Smad7 by siRNA significantly induced EMT of GBC-SD cells (Fig. S2E-2F). In addition, we also observed that Smad7 could retard the proliferation of GBC-SD cells (Fig. S2G). Correspondingly, the metastasis and proliferation of GBC-SD cells were further attenuated upon the exogenous expression of Smad7 (Fig. S2H).

Given that miR-20a could down-regulate the expression of Smad7, we wondered whether Smad7 is responsible for miR-20a-mediated malignant phenotypes of GBC-SD cells. Hence, we examined the effect of miR-20a on the cell proliferation, migration and invasion in the presence or absence of Smad7 expression. As shown in Fig. 3F&S2F, exogenous expression of Smad7 blocked the majority of miR-20a-induced migration/invasion and proliferation. Expectedly, western blot demonstrated that Smad7 could reverse miR-20a contributed E-cadherin down-regulation and Vimentin up-regulation (Fig. 3G). Taken together, these data suggested that Smad7 could negatively modulate GBC cells metastasis and EMT phenotypes, which is indispensable for the pro-metastasis capability of miR-20a during GBC pathogenesis.

The reduction of Smad7 upon miR-20a results in nuclear translocation and transactivation of β-catenin signaling

It has been documented that Smad7 promotes cell-cell adhesion by stabilizing β-catenin and consequently increases the β-catenin-E-cadherin complex level at the plasma membrane, we thus wondered whether Smad7 inhibits the activity of miR-20a
by sequestrating the nuclear translocation of $\beta$-catenin. After co-transfecting $\beta$-catenin reporter gene together with miR-20a or Smad7 siRNA, we observed that the activity of $\beta$-catenin signaling was increased significantly (Fig. 3H left two panels, S3A). But exogenous expression of Smad7 effectively abrogated miR-20a-enhanced $\beta$-catenin reporter gene activity (Fig. 3H right panel).

Further, immunofluorescent assay showed more positive nuclei staining with anti-$\beta$-catenin antibody in GBC-SD-20a cell line, in accordance with the subcellular fraction analysis by western blot after mimic miR-20a transfection (Fig. 3I). The nuclei location of $\beta$-catenin was significantly enhanced after siRNA-Smad7 (Fig. S3B) or miR-20a incubation, but over-expression of Smad7 could eliminate miR-20a-induced accumulation of $\beta$-catenin into nucleus (Fig. 3I right panel). Similarly, we observed less positive nuclei staining of $\beta$-catenin (Fig. S3C, left panel) and the reduction of c-myc or cyclinD1, the potential mediators of EMT, promoter activities (Fig. S3C, right two panels) in miR-20a antagonim treated tumor tissues. Moreover, we confirmed the metastasis-promoting effects of translocation of $\beta$-catenin by administration of LiCl (Lithium chloride), the selective GSK-3$\beta$ inhibitor, to GBC-SD cells (Fig. S3D-3F). These data indicated that the cytoplasm-nucleus translocation of $\beta$-catenin is responsible for miR-20a/Smad-7 axis manipulated GBC cells metastasis.

**Elevated expression of miR-20a in an inverse relationship with Smad7 is associated with unfavorable prognosis of GBC**

To further determine the pathogenic implication of miR-20a in the development
and progression in GBC, we first analyzed the potential relationship between miR-20a expression and clinical-pathological characteristics of GBC patients. LNA-based in situ assay was applied to examine the level of miR-20a for 67 primary GBC patients (Table S2). Basing on the overall expression level of miR-20a, we divided GBC specimens into two groups (relative high miR-20a expression level group and low group). Elevated miR-20a expression was observed in patients with larger tumor size \( (P = 0.0465) \), more local invasion \( (P = 0.0392) \), distance metastasis \( (P = 0.0291) \) and TNM III-IV \( (P = 0.0146) \) (Table 1). More importantly, patients with higher miR-20a expression exhibited worse OS (median OS time were 5 and 20 months, respectively, difference = 15 months, \( P = 0.0014 \)) than the lower expression group (Fig. 4A). The reduction of Smad7 in GBCs also predicted a poor prognosis of patients (Fig. 4B). Moreover, those patients with both elevated miR-20a and decreased Smad7 level exhibited even worse prognosis, suggesting that the combination of two factors provides more prognostic accuracy in comparison with each alone (Fig. 4C). In line with our previous results obtained via cell line and animal model experiments, IHC staining with anti-Smad7, β-catenin, Vimentin and E-cadherin antibodies showed similar changes in miR-20a high or low expression tumor tissues (Fig. 4D). Furthermore, univariable (Table S4) and multivariable (Table 2) Cox regression analysis confirmed that miR-20a and Smad7, including local tumor invasion, to be independent indicators of overall survival.

In addition, weak membrane staining of E-cadherin, the enrichment of Vimentin and the nuclear location of β-catenin in tumors also showed close relation with poor
overall survival rate for GBC patients (Fig. S4A). Taken together, these results further suggested that the miR-20a/Smad7/β-catenin axis plays the pivotal role in the progression of GBC, whose activities could be served as the valuable predicting markers for recurrence and poor survival of GBC clinically.

The elevated expression of miR-20a is necessary for TGF-β1-induced cell metastasis

Since TGF-β1 is one of the most potent cytokines linked to inflammation and metastasis in various types of cancers, we wondered whether TGF-β1 involves in pathogenesis of GBC and miR-20a-induced tumor metastasis. We determined the serum level of TGF-β1 protein in healthy or GBC patients by applying ELISA assay, and found that TGF-β1 was significantly increased in serum isolated from GBC patients in comparison with the healthy group (Fig. 4E, left panel). And in accordance with the previous report [17], IHC staining with anti-TGF-β1 antibody also showed that TGF-β1 was dramatically up-regulated in about 70% of GBC tumor tissues. In addition, we also found the positive staining of TGF-β1 in stroma tissues immediately surrounded the tumor cells, infiltrated with more lymphoid and myofibroblast cells, whereas, no such staining was observed in the stroma close to normal gallbladder epithelial cells (Fig. 4E, right panel). These data indicated that the high serum level of TGF-β1 results at least partly from the abnormal enrichment of TGF-β1 in tumor tissues.

Interestingly, the expression of TGF-β1 showed positive correlation with that of
miR-20a in the majority of tumor tissues (Fig. 4E right panel), suggesting that TGF-β1 may play a potential role in regulating miR-20a expression. Hence, we examined the change of miR-20a level in response to TGF-β1 challenge. As shown in Fig. 4F, average 3-4 fold increase of miR-20a mRNA was obtained two days after TGF-β1 treatment in GBC-SD and SGC-996 cells. It has been reported that TGF-β1 could induce EMT of GBC cells [18], and Smad7 is the negative regulator of the TGF-β1 pathway. We wondered whether miR-20a antagomir could inhibit TGF-β1-induced GBC EMT through enhancing the protein level of Smad7 in GBC. As expected, the treatment of miR-20a antagomir in the presence of TGF-β1 significantly blocked the TGF-β1-induced elongated morphology (Fig. S4B). Similarly, TGF-β1-enhanced cell migration and invasion were attenuated after the incubation of miR-20a antagomir (Fig. S4C), which were further verified by western blot assay with several EMT-related genes (Fig. 4G).

Together, these results indicated that miR-20a/Smad7/β-catenin axis plays a pivotal role in TGF-β1-induced metastasis, which provides both potential prognostic markers and useful therapeutic candidates to gallbladder carcinoma.
Discussion

As one of the most aggressive and lethal tumors, gallbladder cancer has a propensity for early lymph node metastasis and for direct invasion into the liver, as well as a remarkable tendency to seed the peritoneal cavity, biopsy tracts, and laparoscopic-port sites. However, the molecular mechanisms manipulating the metastasis and invasion of GBC has not been elucidated clearly yet.

Previous studies identified metastasis-related miRNAs mainly depending on the data obtained from the differential expression profiles of miRNAs in tumor against para-cancer tissues/cells. Whereas due to the complexity of tumor tissues and relative lower rank in differential expression lists, some potential pivotal metastatic inducers may be neglected. High content screening method is a powerful tool for filtering the candidate miRNAs or siRNAs in a large-scale [19]. We here, for the first time, attempted to identify the metastatic-related miRNAs from a large-scale miRNAs library by applying novel designed HCS process. Since EMT is the key process for cell metastasis, we used anti-F-actin phalloidin to monitor the formation of fibers, which is necessary for mesenchymal transition, and applied TGF-β1 as the positive control. As delineated in Fig.1, several pro-metastasis miRNAs were filtered out of 880 miRNAs with the high ratio of fiber length per cell, some of which showed prominent activities similar as TGF-β1 did. Besides further transwell and invasion experiments with individual candidate miRNAs, literature searching also found that several candidate miRNAs identified in our study have been documented with the indicated capabilities in various cancers previously, such as miR-506 [20], miR-135a
[21], miR-556-3p and miR-524-3p [22](Table.S1), illustrating the reliability of HCS platform for metastatic phenotype analysis and the potential important roles of miRNAs during GBC metastasis. The clinicopathological analysis revealed the tight relationship between high expression of miR-20a and local invasion together with distant metastasis of GBCs. Combination with other experiments in vitro and in vivo, we draw the convinced conclusions that miR-20a is a key factor for GBC metastasis.

Our present data further demonstrated that Smad7 is a direct target of miR-20a and that miR-20a-mediated inhibition of Smad7 is dependent on a conserved motif in 3’UTR of Smad7(Fig.3). Smad7 has been proved as a pivotal factor in embryonic development and adult homoeostasis. Altered expression of Smad7 is often associated with human diseases, such as tissue fibrosis and inflammatory diseases. However, the role of Smad7 in cancer is controversial. Smad7 has been shown to play a pro-metastatic function in colorectal cancer and melanoma metastasis to bone [14,15], while overexpression of Smad7 in colon adenocarcinoma cells could inhibit tumorigenicity [16]. Nevertheless, the potential physiologic significance of Smad7 underlying tumorigenicity and progression of GBC remains elusive. We here demonstrated that the decreased expression of Smad7 is correlated with the worse clinical characteristics and poor survival rate of GBC patients. Conversely, the ecotopic expression of Smad7 could definitely attenuate the invasion/migration and proliferation in vitro and in vivo via sequestrating the nucleus translocation of β-catenin, which strongly indicated the key role of Smad7/β-catenin pathway for the prognosis of GBC for the first time.
Inflammation microenvironment has long been known as a localized promoter to cancer development. Cytokine TGF-β1 is highly expressed in various cancer cells, and acts as one of the most potent metastatic inducers. There are few reports on animal experiments or clinical specimens with regard to the high expression of TGF-β1 in gallbladder cancer. In our present studies, we observed that the increased level of TGF-β1 in serum and tumor tissues is responsible for the induction of miR-20a followed with the activation of β-catenin signaling, which may further reinforce TGF-β1-induced cell EMT and form the positive feedback loop for cell metastasis(Fig.4I). Interestingly, TGF-β1-induced phosphorylation of Smad3 was weakened upon miR-20a antagonir treatment, further emphasizing the multiple roles of Smad7 involving the cell transition and metastasis.

In summary, TGF-β1-induced expression of miR-20a plays a pivotal role in GBC metastasis by EMT induction via directly targeting the transcription of Smad7 and enhancing the activity of β-catenin signaling pathway, which sheds a new light on the understanding of TGF-β1-involved GBC metastasis and provides the potential therapeutic targets for its prevention.

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References


### Tables

#### Table 1: Relationship Between MiR-20a Expression and Clinicopathologic Features of GBC Patients

<table>
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<th>Features</th>
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**Note:** TNM, tumor-nodes-metastasis, based on the American Joint Committee on Cancer/International Union Against Cancer staging manual (7th edition, 2009). Differences among variables were assessed by χ² test.

#### Table 2: Multivariable Analysis of Clinical variables Contributing to Overall Survival

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male or Female</td>
<td>1.395</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>≤59 or &gt;59</td>
<td>1.120</td>
</tr>
<tr>
<td></td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>Tumor size (cm)</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 3 or &gt;3</td>
<td>1.039</td>
</tr>
<tr>
<td><strong>Local invasion</strong></td>
<td></td>
</tr>
<tr>
<td>Yes or No</td>
<td>2.697</td>
</tr>
<tr>
<td><strong>Lymph-node metastasis</strong></td>
<td></td>
</tr>
<tr>
<td>Yes or No</td>
<td>1.347</td>
</tr>
<tr>
<td><strong>Distant metastasis</strong></td>
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</tr>
<tr>
<td>Yes or No</td>
<td>1.550</td>
</tr>
<tr>
<td><strong>TNM stage</strong></td>
<td></td>
</tr>
<tr>
<td>I-II or III-IV</td>
<td>1.851</td>
</tr>
<tr>
<td><strong>miR-20a</strong></td>
<td></td>
</tr>
<tr>
<td>Low or High</td>
<td>2.566</td>
</tr>
<tr>
<td><strong>Smad7</strong></td>
<td></td>
</tr>
<tr>
<td>Low or High</td>
<td>2.329</td>
</tr>
</tbody>
</table>

P-values for clinical variables are from multivariate Cox regression after adjusting for each other.
Figure legends

**Fig. 1 miR-20a was identified as candidate pro-metastatic miRNA by HCS.**

(A) Flowchart of high content miRNA library screening via F-actin assay.

(B) More than 20 miRNAs were filtered out basing on the AVG+2SD value. The value of TGF-β1 treated cells was designated as a positive control. More detailed information can be found in supplemented materials and methods.

(C) Several miRNAs were considered as false positive miRNAs by microscopic examination. Finally, only 17 miRNAs were picked out as candidate miRNAs.

(D) Relative expression of top 10 miRNAs in GBC samples (n=18) and normal gallbladder tissues (n=11). Only miR-20a was over-expressed in GBC tumor tissues, **P<0.01. Other 8 miRNAs were undetected or with CT value above 35.

(E) The expression of miR-20a shown by ISH with anti-miR-20a LNA probe. 400×.

(F) The typical Cy3-labeled phalloidin staining (Red) and texture analyzed (green) cellular F-actin microfilament in the presence of TGF-β1, miR-20a or miR-27b. The nucleus was stained by DAPI (Blue). 400×.

**Fig. 2 miR-20a promotes both migration and invasion in GBC-SD and SGC-996 cell lines.**

(A) GBC-SD cells were transiently transfected with miR-20a mimics (100nM), NC mimics (100nM), NC antagonir (100nM), miR-20a antagonir (100nM) for 72h, or stable infected with lentivirus expressing GFP-tagged miR-20a or miRNA-NC (miRNA negative control). The morphology changes were examined by microscope.
Transwell migration and invasion assays were performed after transiently transfected with miR-20a, NC mimics, NC antagonor or miR-20a antagonor, and stably infected with lentivirus expressing miR-20a or NC (miRNA negative control) in GBC-SD and SGC-996 cell lines, respectively. The migration and invasion cells number were shown in histograms.

After transfected with miR-20a mimics or infection by miR-20a-expressing lentivirus, whole cell lysates of GBC-SD and SGC-996 cells were harvested and subjected to western blot assay at the indicated times.

Mice bearing GBC-SD subcutaneous xenografts were killed after 2 weeks of treatment with miR-20a antagonor (5nmol per mouse each time). The right panel showed the volume of tumors, **P<0.01.

The paraffin sections were subjected to hematoxylin & eosin or IHC staining with anti-CA19-9, Ki-67, E-cadherin and Vimentin antibodies respectively.

Left panel: the liver metastasis tumor model via spleen injection was employed to evaluate the pro-metastatic role of miR-20a (n=10 for each group). The total metastases number of each group was shown in histograms. Right panel: CA19-9 assay of liver metastatic nodule via IHC staining. 400×.

**Fig. 3 Smad7/β-catenin axis was indispensable for miR-20a-induced GBC cells metastasis.**

Left panel: diagram of miR-20a putative seed sequences in the 3'UTR of Smad7
mRNA across eight vertebrate organisms. Hs=Homo sapiens, Pt=Pan troglodytes, Mm=Mus musculus, Rn=Rattus norvegicus, Cf=Canis familiaris, Gg=Gorilla gorilla, Fr=Fugu rubripes, Dr=Danio rerio. Right panel: Relative activity of luciferase reporters with wild-type or mutant Smad7 3’UTR after cotransfected with miR-20a mimics (100nM) or NC mimics (100nM) in GBC-SD cells. *P<0.05.

(B) mRNA or protein levels of Smad7 in GBC-SD and SGC-996 cells were monitored 60h after miR-20a mimics/antagomir transfection (100nM) or miR-20a lentivirus infection. *P<0.05, **P<0.01.

(C) mRNA levels of Smad7 in 18 fresh GBC specimens and 11 normal gallbladder specimens. **P<0.01. Right panel showed the representative expression of Smad7 in GBC tumor tissues and normal gallbladder tissues.

(D) The correlation between the expression levels of miR-20a and Smad7 was determined by linear regression analysis with the same samples used in Fig.3C (P=0.0028, r=-0.6606. Pearson’s correlation).

(E) Smad7 immunostaining of subcutaneous tumors.

(F) GBC-SD cells were transfected with pcDNA3.1 empty vector, Smad7 plasmid, miR-20a mimics, or miR-20a plus Smad7 plasmid respectively. Transwell assays were applied to monitor cell metastasis *P<0.05, **P<0.01.

(G) Western blot assay was performed to examine the expression of E-cadherin and Vimentin 60h after miR-20a mimics alone or miR-20a plus Smad7 plasmid transfection.

(H) β-catenin transactivity was monitored using dual-reporter system (pGL-OT and
pRL-TK plasmids), *P<0.05, **P<0.01.

(I) Left panel: Immunofluorescence staining of β-catenin in miR-20a or NC-miRNA stable GBC-SD cells. Right panel: western blot assay was applied to examine the cytoplasm and nucleus distribution of β-catenin.

Fig. 4 Correlations of miR-20a and pathological characteristics of GBC patients.

(A-B) The overall survival rates of 67 GBC patients were compared among different groups.

(C) The overall survival rates of 67 GBC patients were compared between high miR-20a/low Smad7 expression group and low miR-20a/high Smad7 expression group by using Kaplan-Meier analysis. P value was generated from all groups together.

(D) Representative Smad7, β-catenin, E-cadherin and Vimentin expression in high miR-20a or low miR-20a level human GBC tissues (400×).

(E) TGF-β1 level in serum of healthy persons (n=11) and GBC patients (n=18). *P<0.05. Right panel showed the representative TGF-β1 IHC staining in normal gallbladder and GBC (high or low miR-20a expression tissues) (400×).

(F) Relative miR-20a expression changes in GBC-SD and SGC-996 cells after TGF-β1 (10ng/ml) stimuli.

(G) The expression of E-cadherin, Vimentin, Smad7 were examined by western blot assay.

(H) Schematic depiction for the function and potential mechanism of miR-20a in GBC.
**Fig. 1**

**A**
- microRNA library
- Transfection
- GBC-SD cells
- F-actin assays by HCS

**B**
- Total fiber length per cell (µm)
- MicroRNAs

**C**

<table>
<thead>
<tr>
<th>miRNAs increasing the amount of F-actin</th>
<th>Fold Change of F-actin (miRNA vs average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>has-miR-506</td>
<td>1.84</td>
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<tr>
<td>has-miR-20a</td>
<td>1.77</td>
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<tr>
<td>has-miR-556-3p</td>
<td>1.72</td>
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<tr>
<td>has-miR-524-3p</td>
<td>1.72</td>
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<tr>
<td>has-miR-141</td>
<td>1.68</td>
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<td>has-miR-1290</td>
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<td>has-miR-1471</td>
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<td>hsa-miR-1180</td>
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<tr>
<td>TGF-β1 (5ng/ml)</td>
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</table>

**D**
- Human tissue
- Level of miR-20a (ΔCT)

**E**
- Human tissue
- Normal gallbladder
- GBC tumor
- H&E
- miR-20a
Fig. 1 continued

<table>
<thead>
<tr>
<th>GBC-SD</th>
<th>Blank</th>
<th>NC</th>
<th>Tgf-β1</th>
<th>miR-20a</th>
<th>miR-27b</th>
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<tr>
<td>F-actin</td>
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<tr>
<td>Analyzed</td>
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</table>

Quantitation: 1.00  1.06  1.96  1.77  0.95
Fig. 2

A

<table>
<thead>
<tr>
<th>GBC-SD</th>
<th>Blank con</th>
<th>NC mimic</th>
<th>miR-20a</th>
<th>NC antagonir</th>
<th>miR-20a antagonir</th>
<th>LV-miR-NC</th>
<th>LV-miR-20a</th>
</tr>
</thead>
</table>

B

- Migration
  - Blank con
  - NC mimic
  - miR-20a
  - NC antagonir
  - miR-20a antagonir

- Invasion
  - Blank con
  - NC mimic
  - miR-20a
  - NC antagonir
  - miR-20a antagonir

C

D

E

<table>
<thead>
<tr>
<th>GBC-SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
</tr>
<tr>
<td>Vimentin</td>
</tr>
<tr>
<td>GAPDH</td>
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<table>
<thead>
<tr>
<th>SGC-996</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
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<tr>
<td>Vimentin</td>
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<tr>
<td>GAPDH</td>
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<table>
<thead>
<tr>
<th>GBC-SD</th>
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<tbody>
<tr>
<td>LV-miR-NC</td>
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<td>LV-miR-20a</td>
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</tr>
<tr>
<td>LV-miR-20a</td>
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<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>LV-miR-NC</td>
</tr>
<tr>
<td>LV-miR-20a</td>
</tr>
</tbody>
</table>
Fig. 2 continued

F. Subcutaneous xenograft tumor model

G. Tumor tissue

H. Spleen-Liver metastasis model
Fig. 3

A

Hs  aaataagaaaaAGATGCACTTT gtt  3'SMAD7
Pt  aaataagaaaaAGATGCACTTT gtt
Mm  aaataagaaaaAGATGCACTTT gtt
Rn  -----taaagaaaaAGATGCACTTT gtt
Cf  aaataagaaaaAGATGCACTTT gtt
Gg  -------aaaaaaaAGATGCACTTT gtt
Fs  aaagaaaa-acaAGATGCACTTT gtt
Dr  gagaaaaaaagAGATGCACTTT gtt

3' gauggacgugaUAUUCGUGAAu  5' hsa-miR-20a

hsa-miR-20a/SMAD7 Alignment

B

GBC-SD  SGC-996
NC control  NC control
miR-20a stimuli  miR-20a stimuli
NC antagonim  NC antagonim
miR-20a antagonim  miR-20a antagonim

C

Human tissue  GBC tumor
Tumor tissue

Smad7

Human tissue

miR-20a level (ΔCT) vs. Smad7 level (ΔCT)

D

E

Mouse tumor tissue
NC antagonim  miR-20a antagonim

Smad7
Fig. 3 continued

F

GBC-SD

H

GBC-SD

I

GBC-SD

β-catenin DAPI Merge

GBC-SD
cytoplasm nucleus

nc 20a 20a+Smad7 nc 20a 20a+Smad7

β-catenin

Smad7

GAPDH

Histone (H3)
Fig. 4

A

Overall Survival

Time after surgery (Months)

P = .0014

B

Overall Survival

Time after surgery (Months)

P = .0059

C

Overall Survival

Time after surgery (Months)

P = .0007

D Human tissue

Low miR-20a  High miR-20a

H&E

miR-20a

Smad7

β-catenin

Vimentin E-cadherin

100μm
Fig. 4 continued

E

Human tissue

GBC/miR-20a high

Normal gallbladder

Case 1

Case 2

GBC/miR-20a low

Concentration of TGF-β (ng/ml)

N=11  T=18

100X

400X

F

Relative level of miRNA-20a

GBC-SD after TGF-β 1 stimuli

SGC-996 after TGF-β 1 stimuli

G

GBC-SD

Con  TGF-β1  TGF-β1 + NC antagonist  TGF-β1 + miR-20a antagonist

E-cadherin

Vimentin

Smad7

t-Smad3

p-Smad3

GAPDH

H

Inflammation

TGF-β1

Cell membrane

Smad2/3

Snail

E-cadherin

miR-20a

Smad7

Nucleus translocation

β-catenin

C-Myc, cyclin D1, ?

Vimentin, α-sma, F-actin, MMPs,...