microRNA-17 functions as an oncogene by downregulating Smad3 expression in hepatocellular carcinoma

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Abstract
The sekelsky mothers against dpp3 (Smad3) functions as a transcriptional modulator activated by transforming growth factor-β (TGF-β). Accumulated evidences indicated that Smad3 played the important roles in carcinogenesis and progression of hepatocellular carcinoma (HCC). Up to now, the regulatory mechanism of Smad3 in HCC still remains unclear. It has been known that some particular microRNAs (miRNAs) involve in carcinogenesis through the regulation of gene expressions with targeting mRNAs. In our study, the unknown candidates of miRNAs that target Smad3 mRNA were searched by using a newly established in vivo approach, the miRNA in vivo precipitation (miRIP). Using a loss-of-function assay, we demonstrated that miR-17 directly targeted Smad3 in HCC cells and inhibition on miR-17 increased Smad3 expression. Furthermore, we found that downregulation on Smad3 expression was consistent with high level of miR-17 in HCC tissues of patients when compared with around normal liver tissues. The manipulated miR-17 silence in HCC cells suppressed their growth of both in vitro and in vivo. Such suppression on cell growth could be recovered through downregulating Smad3. In addition, miR-17 affected cell proliferation through arresting cell cycle in G1 phase. The negative correlation between levels of miR-17 and protein levels of Smad3 was supported by the results of analysis with HCC tissue chip. In summary, for the first time, we confirmed that miR-17 directly targeted Smad3 mRNA and downregulated Smad3 protein expression in HCC. Our results indicated that the increased expression of miR-17 promoted carcinogenesis of HCC through down-regulations of Smad3, suggesting miR-17 might serve as the potential diagnostic and therapeutic targets for clinical HCC.

Introduction
Hepatocellular carcinoma (HCC) is one of the most common human malignant tumors in the world. Among all types of cancers in clinic, HCC is the second leading cause of cancer-related death in China¹. The improvements of clinical treatments on HCC were promoted with the helps from advancements in cancer researches. However, unpredictable recurrences or metastases still happened often, which led to poor prognosis²,³. Thus, it is necessary to continuously discover the molecular mechanisms of HCC in both tumorigenesis and progression. Particularly, the coexisted inflammation and cirrhosis in the HCC of late stages made the unique characters of HCC, which are represented by their significant differences from other solid malignant tumors. For instance, their early diagnosis and prognostic assessment were more difficult when compared with other tumors⁴. Therefore, identifications on the still unknown biomarkers for HCC diagnosis and key regulators for HCC treatment are studied deeply and extensively in nowadays cancer researches.
A growing body of evidences revealed that the sekelsky mothers against dpp3 (Smad3) might involve in the pathogenesis of fibrosis and carcinogenesis. Results from recent studies indicated that the decreased Smad3 expression was necessary for tumor cell proliferation in the presence of transforming growth factor-β (TGF-β), which suggested that Smad3 could play an important role in tumor suppression."}
by using immunohistochemistry staining or qRT-PCR assays. We found that the protein levels of Smad3 were decreased in HCC tumor tissues when compared with those in surrounding normal tissues (Fig. 3a). In contrast, the expression level of miR-17 was increased in HCC tumor tissues when compared with those in surrounding normal tissues (Fig. 3b). Together, our results suggested that the co-existence of high expression levels of miR-17 and low expression of Smad3 could happen in human HCC tissues.

**Downregulation of miR-17 inhibited the growth of HCC cells**

It has been known that TGF-β functions as a tumor inhibitor for suppressing tumor growth in the early stage of tumor progression\(^\text{21}\). During the process, Smad3 plays an important role in the TGF-β signaling pathway. Here, we found that Smad3 was a direct target of miR-17 and that it regulated Smad3 at the protein translation level in HCC cells. Therefore, we hypothesized that miR-17 could involve into the regulation on the function of TGF-β signaling pathway through targeting Smad3 in HCC cells. In order to examine whether miR-17 potentially involves promoting the proliferation of HCC cells, we detected the expression levels of miR-17 in HepG2 and SMMC-7721 cells. The results showed that miR-17 was highly expressed in HepG2 and SMMC-7721 cells, and then two types of cells were treated with miR-17 inhibitors (Supplementary Fig. S2a, b). Downregulations of miR-17 were successfully obtained in both types of treated cells, which finally resulted in the significant decreases in their rate of cell proliferation (Fig. 4a). When both miR-17 and Smad3...
levels were downregulated simultaneously in either HepG2 or SMMC-7721 cells, however, the decreased rates of cell proliferation could be completely rescued (Fig. 4a). Next, the flow cytometric analysis was conducted to detect whether the cell-cycle arrest was mainly caused by the downregulation of miR-17. Our results revealed that both HepG2 and SMMC-7721 were arrested at G1 phase of cell cycle when their miR-17 were downregulated, which supported that to downregulate miR-17 activation were sufficient to specifically block HCC cells at G1 phase of cell cycle (Fig. 4b).

During the process of study on miR-17 in HCC cells, we also found that the differences in miR-17 levels existed in some particular HCC cells. For instance, the level of miR-17 in MHCC-97H cells was much lower than those in both HepG2 and SMMC-7721 cells (Supplementary Fig. S2a). As the miRNA mimics, the synthesized miR-17 was transfected into MHCC-97H cells for upregulation their basal level of miR-17 (Supplementary Fig. S2c). Results of CCK-8 assays revealed that the proliferation levels of MHCC-97H cells were significantly increased after their miR-17 levels were upregulated (Supplementary Fig. S2d). Results of flow cytometric analysis also revealed that upregulation of miR-17 could promote the synthesized miR-17-treated MHCC-97H cells shifted from G1 phase to S or G2/M phase (Supplementary Fig. S2e, f).

Next, p-Smad3, Smad3, and p21 of cell-cycle inhibitor, as the known regulators for HCC cell proliferation were analyzed for their actual involvements in the miR-17 regulated process. Remarkably, we found that the levels for all of these proteins increased in the HCC cells that were only treated with miR-17 inhibitor, but decreased in other HCC cells that were treated with both miR-17 inhibitor and Smad3 siRNA (Fig. 4c). Together, these results further supported that downregulation of miR-17 inhibited the growth of HCC cells.

Downregulation of miR-17 suppressed tumor growth of HCC-derived xenografts after transplantation

The xenografting mouse model was used to assess the effects of miR-17 on HCC tumorigenesis. Either HepG2 cells transfected with miR-17 inhibitor or HepG2 cells transfected with negative control (NC) were injected subcutaneously into different sites of same Nod/Scid mice. After 4 weeks, our results showed that the tumor growth levels were significantly lower in the group with treatment of miR-17 inhibitor than in NC group. Thus, our results suggested that miR-17 played an oncogenic role during the in vivo HCC tumorigenesis (Fig. 5a–c). All of protein levels for p-Smad3, Smad3, and p21 increased together in the engrafted tumors with reduced growth levels because of the suppression of miR-17 expression.

Fig. 2 Downregulation of miR-17 increases the expression of Smad3. a Schematic representation of the construction of pMIR firefly luciferase reporter plasmid. b Analysis of luciferase activity. HEK-293T cells were co-transfected with pMIR firefly luciferase reporter plasmids, pTK-Renilla luciferase plasmids, together with negative control and miR-17 inhibitor. After 36 h, firefly luciferase activity was measured and normalized by Renilla luciferase activity. c, d Effects of miR-17 inhibitor on the endogenous gene levels were analyzed by qRT-PCR (c) and western blotting (d). The data were subjected to Student’s t-test. Data were shown as mean ± SD (n = 3), **p < 0.01, ns, not significant. NC, negative control.
We also confirmed that the amounts for cells positive to either Ki-67 or PCNA were reduced in the tumors that were derived from the HepG2 cells after treatments of miR-17 inhibitors (Fig. 5e). Taken together, our results suggested that miR-17 could contribute to the inhibition of Smad3 expression, leading to the growth promotion of HCC cells in the xenografted tumors.

(Fig. 5d). We also confirmed that the amounts for cells positive to either Ki-67 or PCNA were reduced in the tumors that were derived from the HepG2 cells after treatments of miR-17 inhibitors (Fig. 5e).
Negative correlation between miR-17 and Smad3 was found in the tumor samples of patients with HCCs

The correlation between miR-17 expression level and Smad3 protein level was further confirmed through the assay of HCC tissue chip. In situ hybridization was conducted to evaluate the expression of miR-17, while immunohistochemical staining was conducted to analyze the protein expression of Smad3 in the patients with HCCs during studies with the array of tissue chip. As shown in Fig. 6a–f, the results indicated that miR-17 expression levels were significantly higher in HCC tissues when compared with their adjacent paratumor tissues. Different from those detected miR-17 expression levels, Smad3 expression levels showed the opposite manners, which were significantly lower in HCC tissues when compared with the control of their adjacent paratumor tissues. Remarkably, the negative correlation between expression levels of miR-17 and protein levels of Smad3 in HCC tissues and adjacent paratumor tissues were further confirmed by the statistical analysis ($R = -0.6216$, $p < 0.0001$), as shown in Fig. 6g.

Furthermore, we also analyzed whether high endogenous miR-17 in HCC tissues were relative to HCC tumor stage. As shown in Table 1, the statistical analysis indicated that the cases of negative expression of miR-17 were mainly found in the HCC patients at the early stage of malignancy ($p = 0.015$).

Collectively, these results suggested that high expression of miR-17 could induce the decrease of Smad3 expression, which showed in HCC by tissue chip. The results also suggested that upregulation of miR-17 could contribute to the tumorigenesis of HCC.

Discussion

Our present study suggests that in-depth investigation of the underlying molecular mechanisms for HCC
tumorigenesis may result in the identification of unknown regulators that can be used as the potential targets for developing novel therapeutic treatment of HCCs or/and the biomarkers valuable for the diagnosis of HCCs. Here, miR-17, as one of such newly identified microRNA regulator, was successfully found for its involvement into the activations in TGF-β signaling pathway.

TGF-β super family regulated many diverse cellular behaviors including cell growth, differentiation, apoptosis, adhesion, and motility. TGF-β signaling pathway was triggered by a series of complicated events that included the association between the TGF-β ligands and membrane receptors type I and type II and the intracellular mediation of Smad proteins. The binding between the TGF-β ligands and its receptors induced the phosphorylation of Smad2 and Smad3. The phosphorylation promoted Smad2 and Smad3 to form a complex with Smad4. The complex could be transported to the nucleus and targeted the binding elements of genes, which induced a class of co-activators to straightly regulate TGF-β-mediated gene expression. Therefore, the tumor cells would gain the resistance to TGF-β-mediated growth suppression and apoptosis while the Smad proteins or TGF-β receptors was inactivated.

Although the inactivation of Smad2 or Smad4 had been found in many cancers due to the deletion or mutation of their genes, there was still little evidence to show that such situations happened for Smad3. Decreases of Smad3 occurred in diverse types of cancers, including B-cell lymphomas, gastric cancers, ovarian adeno-carcinomas, prostate carcinomas, and etc. Smad3 was also shown to suppress liver tumorigenesis by promoting apoptosis in tumor cells. Decrease of Smad3 expression was necessary for the proliferation of tumor cells in the presence of TGF-β, which provided strong evidence to support that Smad3 had an important tumor suppression function. Through our preliminary studies, we found that the level of Smad3 expression was downregulated in liver...
cancer tissues. Therefore, we started to focus on the study to realize the mechanism for downregulation of Smad3 in HCC.

miRNAs were known to play the important roles in the regulation of gene expression in diseases progression including tumorigenesis\textsuperscript{13}. Therefore, we designed our strategy to search for the candidates of miRNAs that can target or regulate the expression of Smad3. Currently, most of the prediction algorithms used to find candidate miRNAs, such as miRanda, TargetScan, and PicTar, were programmed according to the basic combination principle\textsuperscript{32}. The “seed region” (2–8 nt at 5’ end) of miRNA was supplementary with 3′ untranslated region (3′-UTR) of mRNA\textsuperscript{15,19}. Normally, using these prediction algorithms could bring many predicted targets, including some of the predicted targets that were proven to be false positives after the experimental validation\textsuperscript{33}. Thus, it is vital to find a feasible and simple strategy to reveal and study the existing miRNAs that target the special mRNA.

Therefore, in order to explore the roles of TGF-β/Smad3 signaling pathway in HCC, we used a newly established in vivo approach, miRNA in vivo precipitation (miRIP)\textsuperscript{19}, which resulted in the successful identification on the previously unpredictable miRNAs that targeted Smad3 mRNA. With one array analysis of 92 miRNAs, we found that miR-17 showed the strongest evidence to involve into the regulation of Smad3 expression. Therefore, we designed in vitro experiments to illustrate that miR-17 could bind to the 3′-UTR of Smad3 mRNA to stop the protein translation of Smad3. To explore the function of miR-17 in HCC, we conducted both in vivo and in vitro experiments. These experiments proved that miR-17 could promote the growth of HCC tumor cell via inhibiting protein expression of Smad3. In order to
further investigate that whether miR-17 could be used as a potential biomarker for HCC, we collected total of 75 HCC samples of clinical patients, which were paired with the normal nearby tissues. The levels of Smad3 and miR-17 were systematically analyzed in these clinic samples. We finally found that Smad3 expression level had the negative correlation with miR-17 expression level. Our study revealed that the close relationship between miR-17 and Smad3 existed during tumorigenesis of HCC. Therefore, miR-17 might possibly play the oncogene-like role in the early stage of HCC, which was not suggested from previous studies.

However, at the later stages of HCC, increases of TGF-β/Smad3 signaling activities promoted tumor progression inducing and facilitating migration, leading to metastasis. When cancer cells lost TGF-β/Smad3 tumor-suppressive responses, they could use TGF-β/Smad3 signaling to initiate immune evasion, growth factor productions, differentiation into an invasive phenotype and metastatic dissemination. They could also use TGF-β/Smad3 signaling to establish and expand metastatic colonies. For example, it was reported that miRNA-708 downregulated Smad3 expression for inhibiting HCC cell metastasis.

Thus, both miRNA-17 and miRNA-708 could be regarded as the upstream regulators of TGF-β/Smad3 signaling to regulate the expression of Smad3. However, they played the opposite roles. There could be several reasons to explain the difference between miRNA-708 and miRNA-17: (1) the activation of miRNA-17 was mainly during the early stages, but miRNA-708 acted at late stages; (2) The upstream of TGF-β/Smad3 signaling might be involved with a more complicated regulation to balance the final effect of Smad3 expression; and (3) activations of either miRNA-17 or miRNA-708 could also induce some unknown activities. Therefore, the final effects of TGF-β/Smad3 signaling could also have relations with process of HCC carcinogenesis. The more clear understandings of the dual roles of TGF-β/Smad3 signaling through particular regulations are expected from our future studies. Especially, the potential mechanism that could control TGF-β/Smad3 to switch from anti-to pro-tumorigenic role will also be focused in our future study, which is expected to provide us with some improvements on clinical methods for both diagnosis and combinatorial cancer chemotherapies.

In conclusion, our study for the first time suggested that increase of miR-17 expression level promoted HCC development through the downregulation of Smad3, and that miR-17 might be served as a potential biomarker for both diagnosis and a potential target for treatment of HCC patients.

Materials and methods

Cell lines and specimens

HEK-293T (ATCC® CRL-1573™), HepG2 (ATCC® HB-8065™) were purchased from American Type Culture Collection (ATCC) (Rockville, MD). SMMC-7721,

| Characteristics                  | Patients number | miR-17 | p-value |
|----------------------------------|-----------------|--------|---------|
| Gender                           |                 |        |         |
| Male                             | 62              | 10     | 52      | 0.677  |
| Female                           | 13              | 1      | 12      |
| Age                              |                 |        |         |
| < 50                             | 29              | 5      | 24      | 0.741  |
| ≥ 50                             | 46              | 6      | 40      |
| Liver cirrhosis                  |                 |        |         |
| Absent                           | 58              | 8      | 50      | 0.704  |
| Present                          | 17              | 3      | 14      |
| Tumor size                       |                 |        |         |
| ≤ 5 cm                           | 39              | 4      | 35      | 0.335  |
| > 5 cm                           | 36              | 7      | 29      |
| Vascular invasion                |                 |        |         |
| No                               | 52              | 8      | 44      | 1.000  |
| Yes                              | 23              | 3      | 20      |
| Metastasis                       |                 |        |         |
| No                               | 67              | 9      | 58      | 0.333  |
| Yes                              | 8               | 2      | 6       |
| AJCC 7th edition classification  |                 |        |         |
| I                                | 20              | 5      | 15      | 0.015* |
| II                               | 25              | 0      | 25      |
| III                              | 22              | 1      | 21      |
| IV                               | 8               | 1      | 7       |

*p < 0.05
probes were listed in Supplementary Table 2. All patients obtained the required information about the research, and the assent was obtained.

miRNAs in vivo precipitation
The miRIP technology was used to identify the unpredictable miRNAs that targeted Smad3 mRNA in HepG2 cells. All the experiments in this technology were performed according to the previous report19. The used probes were listed in Supplementary Table 2.

RNA isolation and qRT-PCR
Total RNA was extracted from HCC tissues, paired normal tissues and HCC cell lines using TRIzol (15596018, Invitrogen) according to the manufacturer’s protocol. The expression of miRNAs was examined by qRT-PCR assay using All-in-One miRNA qRT-PCR Detection Kit (AOMDQ020, GeneCopoeia). All primers were showed in Supplementary Tables 3 and 4. Expression level of miR-17 was normalized and quantified to U6 small RNA. β-actin was applied as an internal control to normalize and quantify the mRNA expression level of Smad3.

Luciferase assays
Luciferase reporter plasmid was gained by cloning the mRNA sequence of human Smad3 into pMIR-Report construct (AM5795, Invitrogen). The mutant or wild-type mRNA sequence of Smad3 was inserted to the luciferase reporter through SpeI and HindIII sites. The plasmids were constructed. HEK-293T cells were seeded in 96-well plates. Co-transfection was performed with 50 nM miR-17 inhibitors (GenePharma, Shanghai) or NC oligonucleotides (GenePharma, Shanghai), 20 ng luciferase reporter plasmids and 10 ng of pRL-TK (E2231, Promega) by the transfection reagent INTERFERin (89129-130, Polyplus Transfection). The sequences of miR-17 inhibitors or NC oligonucleotides used in this experiment were showed in Supplementary Table 5. Cells were gained at 24 h after transfection and results were detected by Dual-Luciferase Reporter Assay System.

Oligonucleotides and plasmids transfection
RNA oligonucleotides were purchased from GenePharma Co., Ltd (Shanghai, China). The human miR-17 inhibitors sequence was 5′-CUACCUGACUGUAGC ACUUUG-3′. The NC oligonucleotide sequence for miR-17 was 5′-CAGUACUUUUGGUAGUACAA-3′. miR-17 mimics sequence was 5′-CAAAGUGCUCUACAGGUGUAG-3′. The negative control (NC) sequence was 5′-UUCUCCGAAGUGUCAGUTT-3′. INTERFERin reagent (89129-130, Polyplus Transfection) was used to conduct the transfection. The final concentration of miRNA inhibitors or miR-17 mimics or NC was 50 nM, and the final concentration of siRNAs or NC was 20 nM.

Immunological histological chemistry
Expression of Smad3 protein in HCC and paired normal tissues were detected using rabbit anti-Smad3 antibody (ab40854, Abcam), then incubated with secondary antibodies conjugated with HRP (ab205718, Abcam). Ki-67 (ab15580, Abcam) and PCNA (ab92552, Abcam) staining were performed according to similar protocols.

In situ hybridization (ISH) and IHC in tissue chip
The array of tissue chip was gained from commercial company (Shanghai Superchip Biotech, Shanghai, China) containing 75 HCC specimens. The clinicopathologic features of 75 patients were listed in Table 1. miR-17 level in HCC was evaluated by ISH using specific Digoxin labeled miR-17 probe. The sequence of the miR-17 probe was Digoxin-5′-CTACCTGCACTGTAAGCACTTTG-3′-Digoxin. Protein of Smad3 was evaluated by IHC using above antibody (ab40854, Abcam). Staining intensity of every sample was given a modified histochemical score (MH-score) considering both intensity and percentage of cells stained with the intensity. 0–2 was considered as negative, 3–5 was considered as positive. All samples were divided into no/weak staining (−) or strong staining (+) according to the average MH-score.

Cell proliferation assay
CCK-8 assay kit (CK04, Dojindo) was used to examine the ability of cell proliferation according to the manufacturer’s protocol. For cell counting, a total of 2500 were seeded in 96-well plates (n = 6). The miRNA inhibitors or siRNA or miRNA mimics or NC was transfected at 24 h after cell seeding. 24, 48, and 72 h after transfection, cells were added to 10 μl CCK-8 and cultured for 2 h at 37 °C. The growth curve was drawn according to the absorbance at 450 nm. Three independent experiments were conducted.

Western blot
Cell protein lysates were divided by a 12% SDS-PAGE gel, transferred to a 0.22 μm nitrocellulose membrane (N8645, Sigma) and blocked with 5% non-fat milk. After blocked, the membrane was incubated with special antibodies. SuperSignal West Pico Chemiluminescent...
Substrate (34080, Thermo scientific) was used to detect the expression of proteins. anti-Smad3 (1:1000, ab40854, Abcam), anti-p-Smad3 (1:1000, ab52903, Abcam), anti-p21 (1:1000, ab188224, Abcam), anti-beta-actin antibody (1:1000, ab8227, Abcam).

Cell-cycle analysis
HepG2 and SMMC-7721 cells were seeded in 6-well plates 24 h prior to transfection. Then cell was transfected with NC or miR-17 mimics or miR-17 inhibitors or Smad3 siRNA. Cells were collected after 48 h and washed with PBS, then fixed in 75% alcohol for 60 min at 4 °C. After washed with cold PBS for three times, cells were stained with 40 μg of PI (P4170, Sigma) and 100 μg of RNase for 30 min at 37 °C. Then the percentage of cells in G0/G1, S and G2/M phase were analyzed by FACSCalibur.

Tumorigenicity assays
All mice were maintained under specific care according to the guidelines approved by the Animal Care and Use Committees of Shanghai Zhongshan Hospital. miR-17 inhibitors and NC (Genepharma) were transfected into HepG2 cells. Then cells were suspended in PBS at a concentration of 1 × 10⁷ cells/ml, 100 μl cells was injected subcutaneously into a 6-week-old Nod/Scid mice. Each group had five mice and the tumor size was detected every 3 days until 4 weeks. The measured length (L) and width (W) was used to calculate tumor volume, the formula \( V = (L \times W^2) \times 0.5^{1/3} \).

Statistical analysis
Statistical analysis was performed with GraphPad Prism 5 and R version 3.3.2. The expression of miR-17 in distinct tissue types and the correlation between the marker and clinicopathological parameters were calculated by \( \chi^2 \) test and Fisher's exact test. Pearson correlation analysis was applied to evaluate the correlation between the relative expressions of miR-17 and Smad3. The statistical differences between groups were evaluated by Student's t-test or paired t-test. All p values were considered statistically significant when \( p < 0.05 \).

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