Abstract. Accumulating evidence suggests that microRNAs (miRNAs) can function as oncogenes or as tumor suppressor genes depending on the tissue type or target. Therefore, clarification of the specific roles of miRNAs is vital for the diagnosis and treatment of cancer. In the present study, miR-451 was found to be downregulated in hepatocellular carcinoma (HCC) tissues when compared to that in adjacent tissues. Functional analysis showed that, in vitro, miR-451 inhibited the migration of hepatoma cell lines HepG2 and SK-Hep-1. Further investigation of the molecular mechanisms identified activating transcription factor 2 (ATF2) as a target of miR-451. miR-451 inhibited ATF2 expression by binding to the 3'UTR. An in vivo assay revealed a significant negative correlation between miR-451 and ATF2 in liver cancer tissues. According to previous findings reported in the literature, the opposing functions of ATF2 are related to its subcellular localization. In the nucleus, ATF2 displays oncogenic activities in melanoma. In the present study, ATF2 exhibited a higher expression level in the nucleus in tumoral tissues of HCC as detected by immunohistochemistry. In conclusion, in this study, we identified a potential target of miR-451, ATF2, and revealed a novel role of miR-451 in the inhibition of the migratory ability of hepatoma cell lines.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer types in both genders. Due to its high mortality, HCC is the third most common cause of cancer-related death (1). Because of a high incidence of recurrence after initial treatment and high migratory capacity, patients with HCC have a poor prognosis with only a 5-year survival rate of 10% from initial diagnosis (2). The majority of HCC cases occur in developing countries resulting in a serious economic and social burden that cannot be ignored.

Viral infections, abnormal metabolic alteration, epigenetic or genetic changes in liver tissues may finally lead to HCC. During tumor genesis or progression, the expression levels of a series of genes are dysregulated such as oncogenes or tumor suppressors. In addition, the location of specific proteins is also related to the behavior of cancer cells. Notably, the expression levels of almost 60% of genes in mammalian cells are regulated by microRNAs (miRNAs). An increased understanding concerning the molecular mechanisms involved in HCC will facilitate diagnosis and effective treatment.

miRNAs are small conserved noncoding RNAs of approximately 22 nucleotides that function to fine-tune gene expression by targeting preferential sequences in the 3' untranslated region (UTR) of miRNAs (3). The roles of miRNAs in cancer initiation or progression depend on their targets or the specific tissue origin (4). Several miRNAs have been shown to be abnormally expressed and to be related to the prognosis (5), metastasis (6) and therapeutic response of HCC (7) since the beginning of studies on the roles of miRNAs in HCC. Moreover, as an important application that is gradually being explored, various circulating miRNAs can be prognostic biomarkers for HCC (8,9).

In our previous study, an miRNA expression profile was carried out in tumor tissues and adjacent non-tumoral liver tissues from patients with HCC. miR-451 was found to have lower expression in the tumor tissues. Yet, the function of miR-451 remains unknown. In the present study, the result from the microassay was further confirmed by qRT-PCR that miR-451 was downregulated in 24 of the 26 HCC tumor tissues. ATF2, a key component of activator protein 1 (AP1), was found to be a target of miR-451 by sequence analysis and
luciferase reporter assay. In contrast to miR-451, ATF2 was increased in tumor tissues where it was mainly localized in the nucleus. Further study showed that miR-451 inhibited the migration of HepG2 and SK-Hep-1 cells. These data revealed the potential function of miR-451 and a new pathway for the regulation of ATF2 in HCC.

Materials and methods

Clinical tissue specimens. Twenty-six patients with HCCs at the General Hospital of PLA (Beijing, China) were enrolled. No patient in the present study received preoperative adjuvant therapy prior to surgery. The fresh tumoral and peritumoral tissue specimens for RNA extraction were frozen in liquid nitrogen immediately after surgery. The clinical features of the patients are characterized in Table I. Informed consent was obtained from the patients, and this study was approved by the Medical Ethics Committee of the General Hospital of PLA.

Cell lines and cultures. Human liver cancer cell lines HepG2 and SK-Hep-1 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Rockford, IL, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO₂ atmosphere. Types HepG2 and SK-Hep-1 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Hyclone, Thermo Scientific, Rockford, IL, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin in a 5% CO₂ atmosphere. HepG2 cells in 24-well plate and starved cells were trypsinized and resuspended in DMEM and 1X protease inhibitor cocktail (Complete Mini, Roche, Switzerland). Equal amounts of proteins were separated by 10% SDS-PAGE and were electrotransferred onto Immobilon P (Millipore, Billerica, MA, USA). Protein extracts were prepared in a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.25% (v/v) Na deoxycholate, 0.5% (v/w) SDS and 1X protease inhibitor cocktail (Complete Mini, Roche, Switzerland)]. Equal amounts of proteins were separated by 10% SDS-PAGE and were electrotransferred onto Immobilon Hybond-C membranes (Amersham Biosciences, Piscataway, NJ, USA). Primary antibodies against ATF2 (1489; Epitomics) and β-actin (sc-77778; Santa Cruz) were used and detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Western blot analysis. Protein extracts were prepared in a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.25% (v/v) Na deoxycholate, 0.5% (v/w) SDS and 1X protease inhibitor cocktail (Complete Mini, Roche, Switzerland)]. Equal amounts of proteins were separated by 10% SDS-PAGE and were electrotransferred onto Immobilon Hybond-C membranes (Amersham Biosciences, Piscataway, NJ, USA). Primary antibodies against ATF2 (1489; Epitomics) and β-actin (sc-77778; Santa Cruz) were used and detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Luciferase reporter assay. HepG2 cells in 24-well plates were transfected with mixtures containing 100 ng firefly luciferase reporter plasmid with the ATF2 3’UTR (named pGL3-ATF2-3’UTR) or ATF2 3’UTR mutant (named pGL3-ATF2-3’UTRmut) sequence, miR-451 mimics (20 nM), and 1 ng pRL-TK (a normalization control). Luciferase activities were measured using a dual-luciferase reporter assay system (Promega) at 48 h after transfection.

Cell migration assay. HepG2 and SK-Hep-1 cells cultured in 6-well plates were transfected using miR-451 mimics or negative control (NC). Cells were serum-starved for 18 h in DMEM containing 0.1% FBS from 30 h after transfection. Then the starved cells were trypsinized and reseeded in DMEM containing 0.1% FBS, and 2x10⁵ cells were added to the upper chamber with a 6.5-mm diameter, 8-µm pore size membrane (Corning), while the DMEM containing 5% FBS was placed in the lower compartment of the chamber. After incubation at 37°C for 12 h, cells on the supine surface of the membrane...
were removed carefully with a cotton swab, and then the filters were fixed with 95% ethanol for 30 min, stained with 0.2% crystal violet solution for 30 min, and finally observed under a microscope.

**Immunohistochemical analysis.** For detection of ATF2 expression in tissues, the sections (4-µm) were prepared and subjected to deparaffinized in xylol and rehydrated in a graded alcohol series. Antigen retrieval was performed by boiling tissue sections in EDTA buffer (pH 8.0) for 5 min at 100°C in a pressure cooker. Endogenous peroxidase activity was blocked with hydrogen peroxidase (0.3%), and nonspecific proteins were blocked with 5% BSA. Incubation with the primary antibody against ATF2 (1:200) was carried out overnight at 4°C, followed by the biotinylated secondary antibody and detection with diaminobenzidine (Dako) and counterstained with Mayer's hematoxylin. For each tissue specimen, ATF2 expression was scored on a scale of 0-3 according to the extent (0-100%) and intensity (strong, 3; moderate, 2; and weak, 1) which was evaluated by an observer in a blinded manner, and the average was calculated.

**Statistical analysis.** All values are reported as means ± SD. Differences were estimated by the two-tailed Student's t-test using Excel software. Spearman's rank correlation was carried out to evaluate the relationship between miR-451 and ATF2 expression. P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-451 is underexpressed in primary HCC lesions. The clinical features of the HCC patients in this study are described in Table I. Twenty-six pairs of fresh tumor and peritumoral tissues were employed for detecting the expression of miR-451 by real-time RT-PCR. As shown in Fig. 1, miR-451 was underexpressed (1.3- to 315-fold) in 92% of the tumors (24 of 26 patients) compared to the matching peritumoral tissues. The results suggest that miR-451 could be involved in most processes of HCC.

miR-451 interacts with the 3'UTR of ATF2 mRNA. miRNAs usually carry out their function by binding to the 3'UTR and

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F, female; M, male; P, positive; N, negative; HbsAg, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; AFP, α-fetoprotein.
inhibiting the expression of target mRNAs. By bioinformatic analysis using TargetScan (www.targetscan.org), we found that the 3'UTR of ATF2 contained a putative binding site for miR-451 (Fig. 2A). To investigate whether ATF2 is a direct target of miR-451, the human ATF2 wild-type 3'UTR and mutant-type 3'UTR containing the mutant sequence at the miR-451 binding site were subcloned into the pGL3 vector separately after the firefly luciferase opening reading frame (ORF). These vectors were cotransfected with miR-451 mimics or the negative control (NC) RNA duplex into the HepG2 cells. Luciferase activity was normalized to another co-transfected Renilla expression plasmid. Results are shown as the mean relative luciferase activity ± SD for three independent experiments. *P<0.05.

miR-451 suppresses the expression of ATF2. Inhibition of expression by miRNAs may be mediated by degrading mRNAs (12,13) or impeding translation without affecting the stability of mRNAs (14). To ascertain whether miR-451 inhibits endogenous ATF2 expression, miR-451 mimics were transfected into HepG2 and SK-Hep-1 cells, respectively. Total-RNA and protein were extracted at 48 h and 72 h after transfection. Results from RT-PCR and western blot analysis showed that ATF2 mRNA (Fig. 3A and C) and protein (Fig. 3B and D) were significantly reduced after transfection of miR-451 mimics as compared to the negative control. The results revealed that miR-451 suppresses ATF2 expression at the post-transcriptional level by degrading its mRNA.

A significant negative correlation between miR-451 and ATF2 expression is observed in human HCC. To determine the relationship between the expression of miR-451 and...
ATF2, miR-451 and ATF2 expression in the 26 pairs of tissues (including tumoral and peritumoral tissues from the 26 samples) was detected, which was represented by $2^{-\Delta Ct}$ and ATF2 IHC score. Based on Spearman's rank analysis, an inverse correlation between miR-451 and ATF2 ($r=-0.5028$, $P=0.0002$) was shown (Fig. 4A). Research has revealed that the...
miR-451 INHIBITS THE MIGRATION OF LIVER CANCER CELLS AND ATF2 EXPRESSION

LV et al: miR-451 inhibits the migration of liver cancer cells and ATF2 expression. Further observation in this study showed that the upregulated ATF2 was mainly located in the nucleus of cells in the HCC tissues (Fig. 4B), which provides evidence that ATF2 has oncogenic activity in liver cancer. These results suggest that downregulation of miR-451 could contribute to the upregulation of ATF2 and the tumorigenesis of HCC.

**Discussion**

In the present study, we have confirmed that miR-451 expression is downregulated in human HCCs and it is a direct negative regulator of ATF2 by targeting its 3’UTR, which contributes to an increase in ATF2 in the nucleus of neoplastic tumor tissues inversely related to that of miR-451. Functional analysis revealed that miR-451 suppressed the migratory ability of hepatoma cell lines, HepG2 and SK-Hep-1. Thus, miR-451, as a suppressor, has an important role in the progression of hepatic carcinoma.

Dysregulated expression of miRNAs, that function as oncogenes or tumor suppressors, occurs in various types of cancers in a tissue-specific manner. However, widespread deregulation of miRNAs usually occurs in the same type of cancer. For example, in human HCC, miR-22 (17), let-7g (18), miR-29 (19), miR-124 and miR-199b-5p were found to be expressed at a lower level, indicating a poor survival or prognosis for patients with HCC. miR-451 has been reported to be highly conserved across vertebrates, and its biogenesis is independent of Dicer (20), mainly functioning as a tumor suppressor (16,21), or acting to change the response to stress and start conditional control of cell proliferation and migration (22,23). Suppression of miR-451 expression by tamoxifen was found to promote breast cancer cell survival and endocrine resistance (24), while transfection with the mature miR-451 was found to disperse neurospheres, and inhibit glioblastoma cell growth (25). In gastric cancer, miR-451 was also found to be decreased when compared to normal gastric mucosa (26). In the present study, we confirmed that miR-451 was markedly decreased in HCC and suppressed the migration of hepatoma...
cells; thus, it is considered as a potential tumor suppressor in HCC similar to its function in other tumors.

ATF2 may form homodimeric or heterodimeric activator protein 1 (AP1) which is involved in a series of eukaryotic cellular functions from cell proliferation and development to stress response and apoptosis (26,27). ATF2 can elicit oncogene activities or tumor-suppressor activities depending on the cell or tissue type (28). For example, in melanoma, interfering with ATF2 transcriptional activity can inhibit the proliferation of melanoma cells in culture and the formation of tumors and metastasis (29,30). Contrarily, expression of transcriptionally inactive ATF2 in the presence of oncogene activation (such as Ras mutations) in non-melanoma skin cancers increases papilloma formation owing to the deregulated expression of genes that promote proliferation, such as CTNNB1 (31). Consistent with its cell cycle regulatory role, enhanced expression of ATF2 increased cell proliferation in mouse cancer models (32,33).

However, how ATF2 elicits oncogenes or suppressors is not fully known. Increasing research suggests that subcellular localization of ATF2 is a crucial factor for its contrary activities. In melanomas, nuclear ATF2 is associated with metastasis and poor prognosis while cytoplasmic ATF2 is associated with nonmalignant skin cancers and more favorable prognosis (34). Its subcellular localization could be regulated by PKCε that promotes oncogenic functions of ATF2 in the nucleus while blocking its apoptotic function in the mitochondria (15). In this research, we found that ATF2 was significantly increased in tumor tissue where it was localized in the nucleus in HCC compared to adjacent normal tissues. The detailed role of ATF2 in HCC requires further investigation. Choi et al noted that ATF2 can bind directly to HIF-1α thereby competing with p53 leading to inhibition of the degradation of HIF-1α, and is essential for the full induction and maintaining the stability of HIF-1α (35). A previous study confirmed that ATF2 is essential for hepatocyte survival in embryo development (27). Collectively, we speculate that ATF2 plays an oncogenic role in HCC.

Notably, ATF2 usually plays an important role as a transcriptional factor during response to stress. For example, transient elevation of ATF2 was observed in the MAPK pathway by hypoxia or low glucose. In contrast, to maintain survival and migration of tumor cells such as glioma, miR-451 downregulation is necessary at low energy (23). Thereby whether regulation by miR-451 of ATF2 is involved in cellular adaptation in response to cellular stress is a new topic which warrants further study.

Tumor invasion and metastasis are often associated with enhanced synthesis of matrix metalloproteinases (MMPs), and among these, MMP2 and MMP9 are of central importance. As an oncogene, ATF2 mediates MMP2 transcriptional activation induced by p38 in breast epithelial cells (36). If miR-451 could deregulate ATF2 expression, the migration of hepatoma cells could be inhibited. In the present study, miR-451 was found to inhibit the migration of hepatoma cell lines, HepG2 and SK-Hep-1.

In conclusion, miR-451 is a critical regulator of ATF2 in HCC and suppresses the migration of liver cancer cells. These findings suggest that miR-451 is a potential target for liver cancer therapy, and could be developed as a diagnostic or prognostic factor.

Acknowledgements

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References