Original Article
Epigenetic silencing of miR137 in gastric cancer

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Abstract: The small non-coding RNAs termed microRNAs can induce post-transcriptional silencing of genes critical to the pathogenesis of multiple cancers. Such microRNAs are deregulated and play important roles in carcinogenesis. In this study, we explored the regulation and function of miR137 in gastric cancer. We found that miR137 was downregulated in a variety of gastric cancer cell lines and primary gastric carcinoma tissues. Its downregulation was resulted from DNA methylation of its promoter. Pharmaceutical demethylation restored its expression and its promoter was highly methylated in gastric cancer cells. FMNL2 (formin like 2) was found as a new target regulated by miR137. Both miR137 mimics and FMNL2 siRNA inhibited the growth of gastric cancer cells in vitro. Therefore, we concluded that promoter methylation-mediated downregulation of miR137 can stimulate FMNL2 expression to promote gastric carcinogenesis.

Keywords: Gastric cancer, miR137, methylation

Introduction
Carcinogenesis is believed to be a stepwise process with the accumulation of multiple genetic and epigenetic changes that often results in the aberrant activation of oncogenes and/or inactivation of tumor suppressor genes (TSGs). TSGs need to be inactivated to lose their tumor inhibitory functions during carcinogenesis, whereas oncogenes, once activated, can promote the transformation of normal cells into tumor cells. Both TSGs and oncogenes are usually protein coding genes. Recently an unusual class of small RNAs have been found to play an important role in cancer development, although they are not protein-coding RNAs [1]. These small RNAs are termed microRNAs since their mature form are at a length of only 19-25 nucleotides. MicroRNAs can induce post-transcriptional gene silencing through base-pairing with partially complementary sites in the 3'-nontranslated region (3'-UTR) of their target mRNAs. During cancer development, microRNAs can function as either TSGs or oncogenes, depending on the target gene they regulated [2-4]. For example, Let-7 is down-regulated in many cancers and functions as a TSG by suppressing the expression of oncogenes like Ras and HMGA2 [5-8]. In contrast, the miR-17-92 cluster, which is located on chromosome 13q31, are upregulated and functions as oncogenes in multiple cancers including colorectal cancer [9, 10]. Therefore, the importance of microRNAs acting as a new layer of gene regulation in tumorigenesis is emerging.

These cancer-related microRNAs are often deregulated in many cancers including gastro-intestinal cancers [11-14]. Similar to protein-coding cancer-related genes, these deregulated microRNAs in cancer development are subjected to genetic and epigenetic regulations. In this study, we explored the regulation and function of miR137 in gastric cancer. It was downregulated in a variety of gastric cancer cell lines and primary gastric carcinoma tissues due to DNA methylation of its promoter. Moreover, it inhibited the growth of gastric cancer cells by target the expression of FMNL2 (formin like 2).

Materials and methods

Cell culture and patients samples
All cancer cell lines were grown at 37°C in 10% CO₂ atmosphere and maintained routinely in
DMEM or RPMI1640 medium supplemented with 10% fetal bovine serum. All primary gastric carcinoma tissues as well as their adjacent non-tumor tissues were collected from patients who underwent surgical resection of tumors. All patients provided written informed consent for the use of their tissues. All tissues had been pathologically confirmed. Tissue samples were immediately snap-frozen in liquid nitrogen, and stored at -80°C until further analysis.

**Total RNA extraction**

Cell pellets or tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA containing small RNA was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNase treatment was carried out to remove any contaminating DNA. The concentration of all RNA samples were quantified by NanoDrop 1000 (Nanodrop, Wilmington, DE, USA).

**Real-time PCR for microRNA quantification**

For microRNA quantitative PCR (qPCR), reverse transcription was performed using the miScript microRNA quantification system (Qiagen). In brief, total RNA was polyadenylated and reverse transcribed to cDNA using miScript Reverse Transcription kit (Qiagen). Real-time PCR was performed using miScript SYBR Green PCR kit (Qiagen) in ABI PRISM 7900 Real-time PCR system (Applied Biosystems). The expression levels of microRNAs were normalized to U6. The amplification was performed at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for 30 s.

**SiRNA and microRNA mimic transfection**

FMNL2 depletion and microRNA re-expression were achieved by transfection with siRNAs and microRNA mimic (Qiagen, Hilden, Germany), respectively. Cells were seeded in 12-well plates (1x10^5/well) and transfected with siRNA duplexes or miRNA mimic (10 nM) using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cells were harvested for RNA and protein extraction after 72 h.

**Cell growth assay (MTS assay)**

Cell growth was measured by a non-radioactive proliferation assay based on the ability of metabolically active cells to convert 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into formazan using CellTiter 96® AQueous Assay kit (Promega, Madison, WI, USA). Briefly, cells are seeded in 12-well plates and transfected with siRNA or microRNA mimic for 48 h. Cells were then harvested and reseeded into 96-well plates. After another 24 h, the quantity of formazan was measured as 490 nm absorbance after 1 hour incubation with CellTiter 96 AQueous One Solution Reagent following instructions provided.

**Bisulfite treatment of DNA and methylation analysis**

Methylation status of miR137 was determined by BGS (bisulfite genome sequencing) using bisulfite modified genomic DNA as the template. Genomic DNA was bisulphite-treated with Zymo DNA Modification Kit (Zymo Research, Orange, CA, USA) according to the protocol provided by the manufacturer. MSP was carried out for 40 cycles with annealing temperature at 62°C, as previously described [15]. For BGS, PCR products amplified with BGS primers were purified with Illustra GFX™ PCR and gel band purification kit (GE Healthcare life science, Uppsala, Sweden) and cloned into pCR4-TOPO vector for sequencing (Invitrogen). At least 6 colonies were randomly chosen for plasmid extraction and sequencing analysis using ABI PRISM BigDye Terminator Cycle Sequencing Kit in ABI 3100 sequencer (Applied Biosystems).

**Western blot analysis**

Cells were lysed in Lammeli’s lysis buffer, resolved in SDS-PAGE minigel and transferred onto Nitrocellulose membrane (Millipore, Billerica, MA). Membranes were probed overnight at 4°C with primary antibodies against FMNL2 (Abnova, Taipei, Taiwan) or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:10000 dilution, washed extensively with 0.1% Tween-20 in PBS and incubated with secondary antibodies conjugated with horse-radish peroxidase at 1:10000 dilution. The signals were visualized with enhanced chemiluminescence (Amersham Life Science Inc., Buckinghamshire, UK).
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Construction of microRNA reporter vector

The 3'-UTR of FMNL2 mRNA containing intact miR137 binding sequences was inserted immediately downstream of a CMV promoter-driven firefly luciferase cassette in a pMIR-reporter vector according to the protocol provided (Ambion, Austin, TX, USA). Restriction enzymes Spe I and Hind III were used. To make control vectors, the mutant construct contains point mutations of the seed sequence of miR-137-FMNL2 interaction. The sequences of the oligonucleotides used were.

Luciferase activity assay

1x10^5 of cells were co-transfected with pMIR-Luciferase-FMNL2-3'UTR and pRL-CMV-Renilla (Promega) constructs in the presence of 30 pmol of miR-137 mimic or control oligonucleotides. At 48 h post-transfection, activities of firefly luciferase and renilla luciferase were
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measured by using the Dual-Glo™ luciferase assay system (Promega) as described by the manufacturer. Relative luciferase activity was normalized with renilla luciferase activity and then compared with the pMIR-reporter vector control.

Results

MiR137 is downregulated in gastric cancer

The expression of miR137 in in vitro cultured gastric cancer cell lines and primary gastric carcinoma tissues were determined by real-time RT-PCR. MiR137 was significantly downregulated in a variety of gastric cancer cell lines (Figure 1A). In addition, its expression was also significantly reduced in gastric carcinoma tissues (n=58, p<0.05, Wilcoxon matched pairs test) (Figure 1B).

Promoter methylation mediates miR137 downregulation

We then set to understand how miR137 was downregulated in gastric cancer. It has been
reported that miR137 downregulation in human cancers was attributed to promoter hypermethylation [16-19]. Therefore, we asked whether promoter hypermethylation was responsible for miR137 downregulation in gastric cancer cells. Indeed, miR137 promoter was highly methylated in gastric cancer cell lines such as AGS, MKN28 and NCI-N87 but not non-tumor stomach epithelial cell line GES-1 (Figure 2A). In consistent with this, miR137 expressions in gastric cancer cell lines were significantly restored after pharmacological demethylation (Figure 2B).

**FMNL2 is the direct target of miR-137**

We then investigated whether downregulation of miR-137 was relevant to the development of gastric cancer. After restoration of miR137 expression by transfection of miR137 mimic into gastric cancer cell lines, cell growth was significantly inhibited accompanied with the induction of cell apoptosis (Figure 3A and 3B).

**FMNL2 is the direct target of miR137**

Using algorithms such as PicTar (http://pictar.bio.nyu.edu/) and TargetScan (http://www.targetscan.org/), FMNL2 (formin-like 2) was identified as one possible target of miR137. FMNL2 expression was indeed reduced after transfection of miR137 mimic (Figure 4A). Moreover, miR137 can significantly suppress the expression of luciferase controlled by wild-type FMNL2 3'-UTR but not mutated FMNL2 3'-UTR (Figure 4B). Importantly, similar to miR137 mimic transfection, siRNA mediated-FMNL2 knock-down led to significant growth inhibition of gastric cancer cells (Figure 4C).

**Discussion**

Like many other microRNAs with tumor suppressor function, miR137 was found to be downregulated in various type of human cancers such as colorectal cancer, glioblastoma, and oral squamous cell carcinoma [20-22]. Such microRNAs with tumor suppressor function could be downregulated in human cancer either genetically or epigenetically. Indeed, epigenetic silencing of miR137 have been reported in human cancers including lung cancer and squamous cell carcinoma of the head and neck [18, 19]. Interestingly, promoter methylation of miR137 was also reported in human gastric carcinoma tissues [17]. In this study, we found promoter methylation of miR137 was indeed responsible for its epigenetic silencing in gastric cancer cells. Furthermore, we have previously reported that miR137 seemed to be one of microRNAs suppressed by active wnt signaling in colorectal cancer [23]. Therefore, it would be interesting to know whether and how the active wnt signaling contributes to the promoter methylation of miR137.

MicroRNAs function to regulate the expression of target genes mainly in the post-transcriptional manner. More than one target genes could be targeted by one single microRNA, as long as they contain the microRNA-binding sites in the 3'-UTR. MiR137 has been reported to affect the expression of several target genes such as DCLK1, BMP7, cdc42 and MCL-1 [24-27]. In this study, we identified FMNL2 a new target of miR137. Transfection of miR137 mimic reduced protein expression of FMNL2 (Figure 3). Moreover, miR137 significantly inhibited the activity of luciferase driven by wild-type but not miR137-interaction deficient 3'-UTR (Figure 3). Similar to miR137 transfection, knock-down of FMNL2 results in the growth inhibition of gastric cancer cells (Figure 3).

As named, FMNL2 belongs to formin superfamily, encoding a formin-related protein that are a group of proteins involved in the polymerization of actin and associate with the fast-growing endof actin filaments. Bioinformatic analysis revealed it was evolutionally conserved with orthologs in fish, Drosophila, and nematode. The gene contains at least 27 exons and spans around 314 kb in the genome. In human, its expression was high in many fetal and adult organs such as brain, liver, spleen, kindey and ovary [28]. Formin-related proteins have been implicated in morphogenesis, cytokinesis, and cell polarity. However, their relevance to human cancer pathogenesis remain controversial. For example, FMNL2 was found to promote metastasis in colorectal cancer while down-regulation of formin-like 2 predicted poor prognosis in hepatocellular carcinoma [29, 30]. In colorectal cancer, miR137 was down-regulated to promote cell invasion and metastasis by directly targeting FMNL2 [31]. Nevertheless, FMNL2 may have formin-independent functions in the development of cancers. The biological rele-
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vance and underlying mechanisms warrants further investigations.

In summary, we found that miR137 was down-regulated through promoter methylation in human gastric cancer. It can inhibit the in vitro growth of gastric cancer cells probably through direct targeting of FMNL2. Therefore, FMNL2 could be a novel target for the diagnosis and therapy of human gastric cancer.

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Disclosure of conflict of interest

None.

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