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miRNA-1 targets fibronectin1 and suppresses the migration and invasion of the HEp2 laryngeal squamous carcinoma cell line

Feifei Wang [1], Ge Song [1], Min Liu, Xin Li, Hua Tang *

Tianjin Life Science Research Center and Basic Medical School, Tianjin Medical University, 22 Qi-Xiang-Tai Road, Tianjin 300070, China

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ABSTRACT

MicroRNAs (miRNAs) are an evolutionarily conserved class of endogenous, non-coding RNAs that modulate gene expression at the post-transcriptional level and are involved in tumorigenesis. In this study, we demonstrate that miR-1 suppresses the potential for growth, migration and invasion in the HEp2 cell line. Furthermore, we validate that FN1 is a direct target gene for miR-1 via fluorescent reporter assay and is negatively regulated by miR-1. Moreover, the knockdown of FN1 has the same phenotypic effects as the overexpression of miR-1. Taken together, our results provide evidence that miR-1 may play a role as a tumor suppressor gene in laryngeal carcinoma.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate mRNA translation or stability [1] by interacting with the 3′ UTRs of their target gene mRNAs. The 3′ UTRs of mRNAs contain key stability elements that are subject to various regulatory proteins and are also miRNA binding sites [2]. The miRNAs play critical roles in myriad processes, including cell fate, cellular differentiation, cancer, apoptosis, metabolism, immunity and development stress responses [3–5].

Aberrations in the expression of miRNA are associated with diseases, such as neuronal disorders [6], inflammation [7] and cancer [8]. Tumor-related miRNA expression has been identified by profiling, and distinct miRNA signatures for many types of cancers, including laryngeal carcinoma, have been reported [9–11]. Furthermore, depending upon the cell type, several miRNAs that are deregulated in human malignancies exhibit oncogenic or tumor suppressor properties [8]. For example, let-7a and miR-34c are downregulated in laryngeal carcinoma and function as tumor suppressors [11,12], whereas miR-21 functions as an oncogene [13]. Thus, miRNAs play critical roles in tumorigenesis and may potentially serve as biomarkers and targets for anticancer therapy.

One of the most evolutionarily conserved miRNAs is miR-1, which is highly enriched in heart and muscle where it plays important roles in the proliferation of progenitor cells and myogenesis [14,15]. In addition, it has been reported that miR-1 was released into the blood of Duchenne muscular dystrophy (DMD) patients as a consequence of muscle degeneration, and the amount of miR-1 paralleled the severity of the disease [16]. Several other observations showed that miR-1 is downregulated in the liver cancer and functions as a tumor suppressor [17]. These findings indicate that miR-1 is a molecule with multiple roles. Our previous study found that miR-1 was downregulated in laryngeal carcinoma tissues compared to adjacent normal tissues [13]. However, the function and molecular mechanism of miR-1 in laryngeal carcinoma cells remains unknown.

In this study, we addressed miR-1 as a negative regulator of migration and invasion of HEp2 laryngeal carcinoma cells and identified fibronectin 1 (FN1) as a miR-1 target gene that mediated its effect on the cellular phenotype.

Abbreviations: ASO, antisense oligonucleotide; cDNA, complementary DNA; EGFP, enhanced green fluorescent protein; FN1, fibronectin1; GAPDH, glyceraldehyde phosphate dehydrogenase; HRP, horseradish peroxidase; miR-1, microRNA-1; miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RFP, red fluorescent protein; UTR, untranslated region

* Corresponding author. Fax: +86 22 23542503.
E-mail address: htang2002@yahoo.com (H. Tang).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Cell culture, transfection and isolation of RNAs

The human laryngeal carcinoma cell line HEp2 was maintained in RPMI1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The cell line was incubated at 37 °C in a humidified chamber supplemented with 5% CO_2. Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Total RNA was extracted from HEp2 cells using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.2. Bioinformatic method

The miRNA targets were predicted using the algorithms TargetScan, PicTar, and MiRBase Targets. We used MatchMiner to identify the genes commonly predicted by the three different algorithms.

2.3. Immunohistochemistry

Five pairs of primary laryngeal squamous carcinoma tissues and adjacent normal tissues were obtained from the patients in Tianjin People's Hospital (Tianjin, China). The detail information of specimens was in the suppl. table S1. The study was approved by the Institutional Review Board of Tianjin People's Hospital. Immunohistochemistry (IHC) was performed according to the methods described previously [18]. Being pretreated with microwave, the sections were blocked and incubated using monoclonal mouse anti-human FN1 (Tianjin Saierbio, China). Staining intensity was assessed.

2.4. Statistical analysis

The statistical significance of differences between groups was analyzed by the unpaired Student's t test, and $P \leqslant 0.05$ was considered to be statistically significant. One representative experiment is shown in triplicates in the statistical analysis.

The other materials and methods are in the Supplementary data.

3. Results

3.1. miR-1 suppresses the growth of HEp2 cells

In a previous study, we found that miR-1 was significantly downregulated in laryngeal carcinoma tissues [13]. In order to investigate the effect of miR-1 on cellular phenotypes, we first made a miR-1 expression construct, pcDNA3/pri-miR-1, and confirmed its efficiency. Real-time PCR showed that miR-1 level was increased approximately 3.8-fold in HEp2 cells transfected with pcDNA3/pri-miR-1 compared to the control vector (Fig. 1A). However, miR-1 level decreased by approximately 70% in HEp2 cells transfected with miR-1 ASO compared to the control oligomer (Fig. 1B). The MTT assay was used to evaluate cell viability. The assay indicated that either ectopic expression of miR-1 or blocking miR-1 by ASO did not obviously alter HEp2 cell viability at 24 h, 48 h and 72 h post-transfection (Fig. 1C and D). To further determine the effect of miR-1 on the growth of HEp2 cells, a colony formation assay was performed. The colony formation rate of HEp2 cells transfected with pcDNA3/pri-miR-1 declined by approximately 75% but increased nearly 3-fold in HEp2 cells transfected with miR-1 ASO compared to their respective control groups (Fig. 1F and G). To confirm the effect of miR-1 on HEp2 cell growth, we synthesized a mutant miR-1 in which six bases were mutated. The mutant miR-1 did not affect HEp2 cell viability and abolished the influence of miR-1 in the colony formation assay on HEp2 cells (Fig. 1E and H). These results indicate that miR-1 can promote colony formation in HEp2 cells but does not affect cell viability.

3.2. miR-1 suppresses the properties of migration and invasion of HEp2 cells

Next, to identify the potentials for migration and invasion of tumor cells that might be relevant to malignancy, in vitro migration and invasion assays were employed. First, we performed a migration transwell assay to determine the migratory ability of laryngeal carcinoma cells. As shown in Fig. 2A, 38% of cells overexpressing miR-1 migrated to the basal side of the membrane compared to control cells. In contrast, 40% more of HEp2 cells transiently transfected with miR-1 ASO migrated than the negative control group. Subsequently, we determined invasive properties by the invasion transwell assay. We found that the average number of HEp2 cells transfected with pri-miR-1, which invaded the basal side of the membrane, was 36% fewer than that of the control group. Alternatively, invasion increased 1.9-fold in cells with downregulated miR-1 (using miR-1 ASO), as compared to the control group (Fig. 2B). To confirm this specific effect of miR-1 on the migration and invasion of HEp2 cells, migration and invasion transwell assays were performed with cells transfected with miR-1-mut and NC-mut. The data from the two groups showed no differences (Fig. 2C and D). These data show that miR-1 is an important participant in the reduction of migration and invasion potential of laryngeal carcinoma cells.

3.3. miR-1 directly targets FN1 and down-regulates its expression

The identification of miR-1-regulated targets is a necessary step in understanding how miR-1 functions. Target genes of miR-1 were predicted using three programs, PicTar, TargetScan and miRBase Targets. Although there were a variety of candidate target genes, FN1, ANP32B, CCND2, DDX5, E2F5, EIF4E, GAS2L, MAP4K3, MMD2 and SOX6 etc, we chose FN1 as a target gene of miR-1 due to its tumor migration and invasion associated functions, which were consistent with the effects of miR-1 on the phenotypes in HEp2 cells. The alignment of miR-1 with the FN1 mRNA 3′ UTR, which carries a putative miR-1 binding site, is illustrated in Fig. 3A. To determine whether miR-1 directly regulates FN1, an enhanced green fluorescent protein (EGFP) reporter assay was used to validate the target site in the FN1 3′ UTR. The 334-bp 3′ UTR region containing the predicted miR-1 binding site was inserted downstream of the EGFP gene following a stop codon (pcDNA3/EGFP-FN1-3′ UTR). First, we transfected either pcDAN3/EGFP or pcDNA3/EGFP-FN1-3′UTR into HEp2 cells. We found that cells transfected with pcDNA3/EGFP-FN1-3′UTR had lower EGFP expression, indicating that endogenous miR-1 had an effect on the FN1 3′UTR (Fig. 3B). HEp2 cells were transfected with the reporter vector, as well as pcDNA3/pri-miR-1 or a control vector and miR-1 ASO or a control sequence, respectively. Interestingly, we found that EGFP expression declined by 48% compared to the control group when miR-1 was overexpressed by transfecting cells with pri-miR-1. Alternatively, suppression of miR-1 with miR-1 ASO increased EGFP expression 1.35-fold (Fig. 3C). To further determine the function of the miR-1 binding site, we constructed another EGFP reporter vector containing the FN1 3′ UTR with four mutated miR-1 binding sites (Fig. 3A). As a result, the observed alteration of EGFP intensity was abolished after transfection with this vector (Fig. 3B and C). We thus conclude that FN1 is a direct target gene of miR-1.

To assess whether miR-1 functions in the downregulation of endogenous FN1 expression, HEp2 cells were transfected with either pcDNA3/pri-miR-1 or miR-1 ASO to overexpress or block miR-1, respectively. Expression of FN1 mRNA was then measured

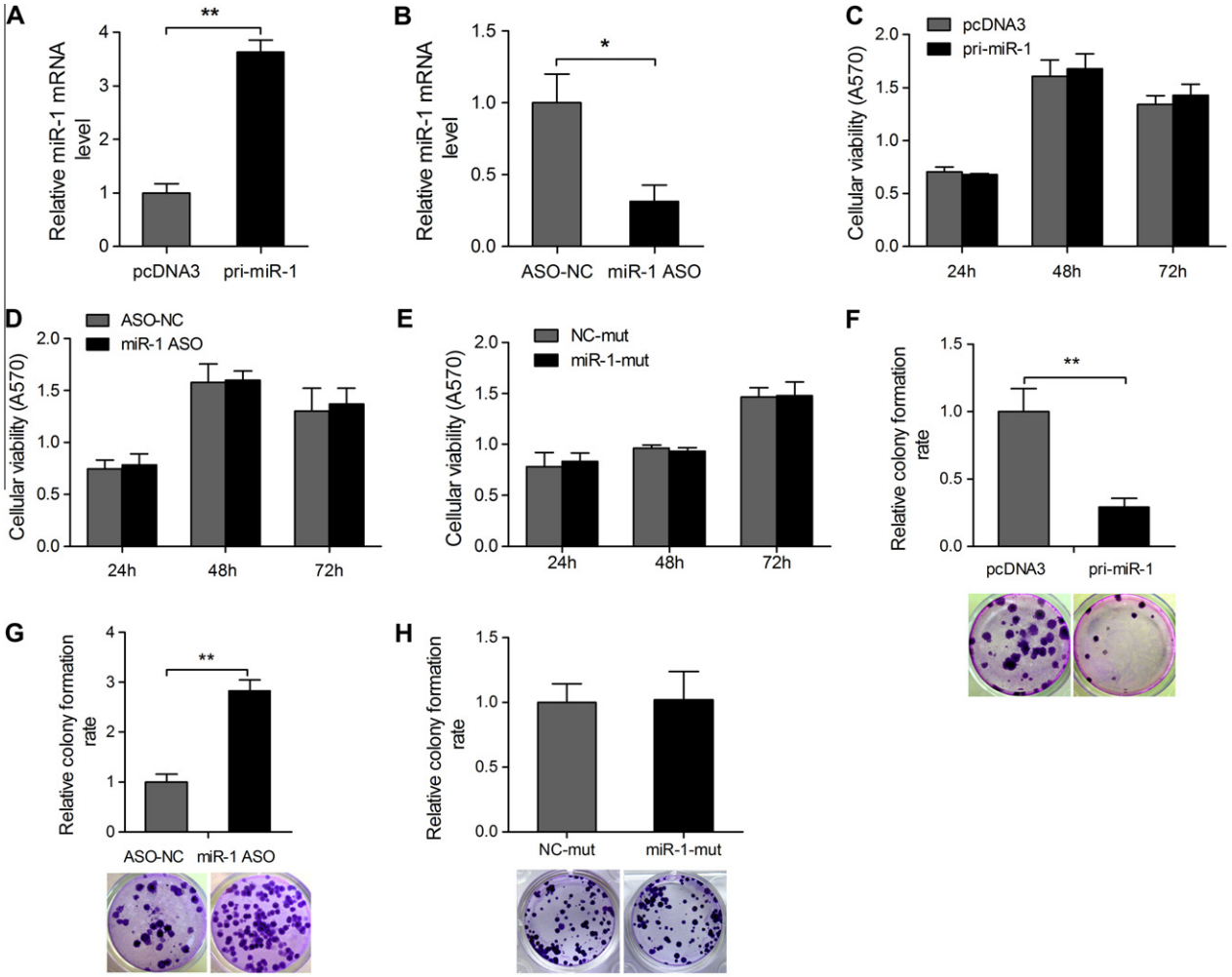

Fig. 1. The growth of HEp2 cells is suppressed in vitro by miR-1. (A, B) The miR-1 expression level in HEp2 cells was effectively altered by transfection of pri-miR-1 or miR-1 ASO, as detected by real-time RT-PCR. U6 snRNA was used for normalization. (C–E) MTT assays were performed to determine the effect of miR-1 on cell vitality. (F–H) Colony formation assays were employed to detect the effect of miR-1 on the cell growth activity of HEp2 cells. Experiments were performed in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$).

by qRT-PCR. When miR-1 was overexpressed or blocked, FN1 mRNA was diminished by 79% or elevated 5.24-fold, respectively, compared to the control groups (Fig. 4A and B). In addition, to determine whether miR-1 effects the expression of the FN1 protein, a western blot was performed. The FN1 protein level was reduced by approximately 77% in HEp2 cells transfected with pri-miR-1 (Fig. 4C) and was elevated 3.56-fold after transfection with miR-1 ASO (Fig. 4D) compared to the respective controls. Moreover, immunofluorescence assays showed that FN1 expression was weaker in HEp2 cells treated with pri-miR-1 following incubation with anti-FN1 compared to controls. However, FN1 expression was stronger in HEp2 cells transfected with miR-1 ASO than in control cells (Fig. 4E). These data show that FN1 is regulated by miR-1 at the mRNA and protein levels.

3.4. Knockdown of FN1 suppresses cellular growth, migration and invasion in vitro

Sequence-specific small interfering RNA (siRNA) can effectively suppress gene expression. To determine the influence of FN1 on the growth, migration and invasion of HEp2 cells, FN1 expression was knocked down by a FN1 siRNA expression plasmid, pRNAT U6.2/si-FN1 (pU6.2/si-FN1). Western blot and qRT-PCR analyses showed that transfection of HEp2 cells effectively suppressed approximately 79% of FN1 expression on the mRNA level and 52% on the protein level when compared to control groups (Fig. 5A and B). Next, MTT assays showed that FN1 did not affect cell vitality. We also explored the capability of cell growth and mobility (Fig. 5C). Subsequently, colony formation assays were employed to evaluate the effect of silencing FN1 on the growth of HEp2 cells. The colony formation rate was lower for cells where FN1 was knocked down with siRNA than in the control group (Fig. 5D). Consequently, transwell assays examining migration and invasion were performed. The average numbers of HEp2 cells transfected with si-FN1 that were seen to migrate and invade were fewer than seen with control cells (Fig. 5E and F). These results indicate that the knockdown of FN1 suppresses cell growth and mobility in the laryngeal carcinoma cell line HEp2.

3.5. Expression of FN1 in laryngeal squamous carcinoma tissues

To detect the level of FN1 in laryngeal squamous carcinoma tissues, we collected 5 pair of laryngeal squamous carcinoma tissues and adjacent normal tissues, and performed immunohistochemistry staining. As shown in Fig. 6, the expression levels of FN1 were higher than that in adjacent normal tissues. The result indicates that miR-1 and FN1 may be negative correlations in laryngeal squamous carcinoma tissues.

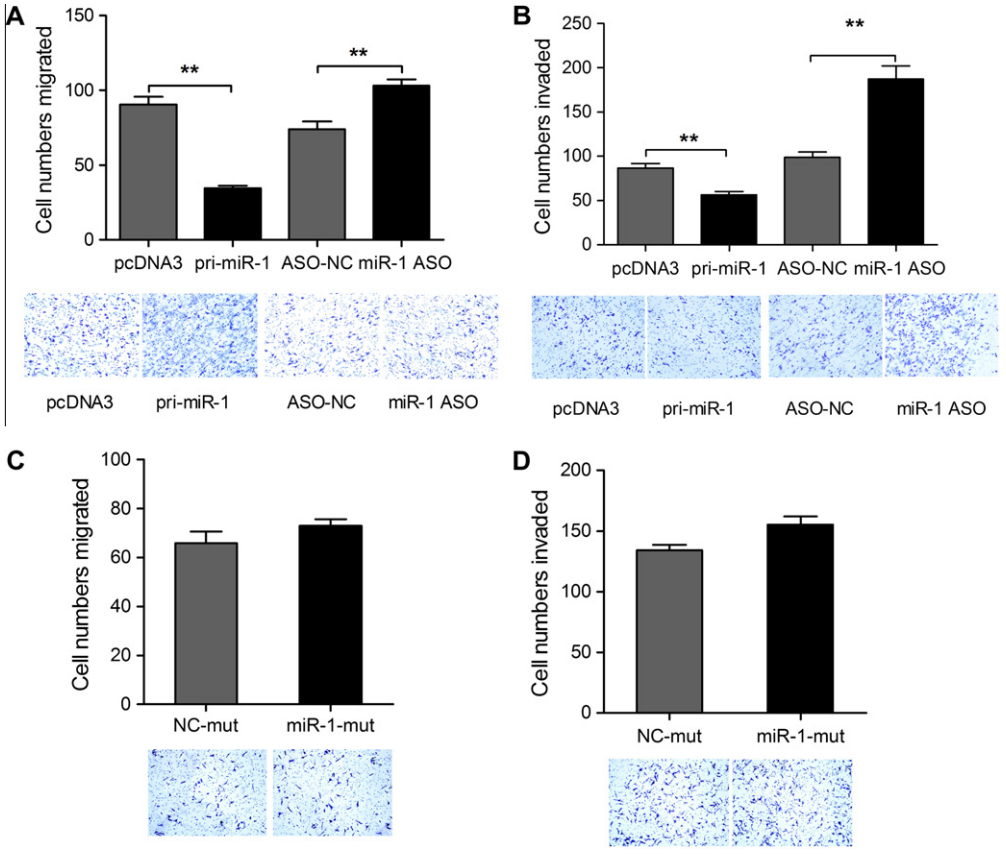

Fig. 2. Effects of miR-1 on the migration and invasion of HEp2 cells. (A, C) Transwell assays without Matrigel were performed to detect the migration activity of HEp2 cells transfected with pcDNA3/pri-miR-1, miR-1 ASO or miR-1-mut and their respective control sequences. (B, D) Transwell assays with matrigel were used to detect the invasion activity of HEp2 cells after transfection. Experiments were performed in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$).

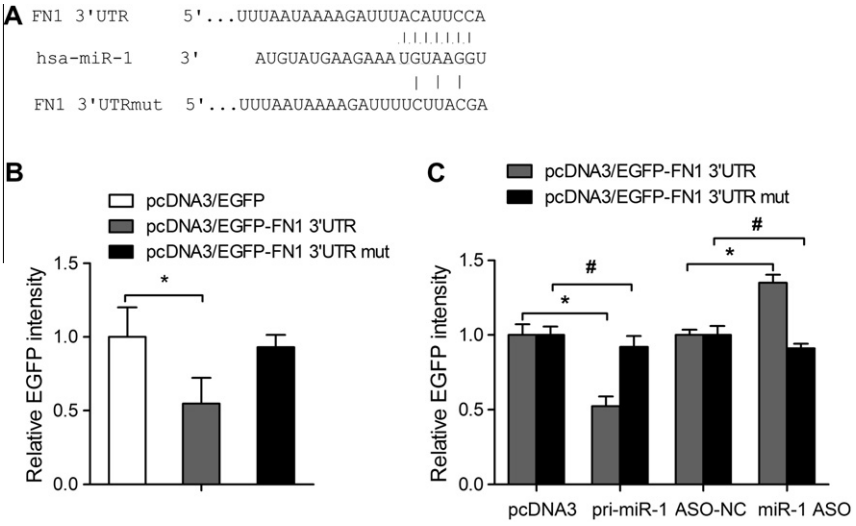

Fig. 3. The 3′ UTR of FN1 mRNA is directly targeted by miR-1. (A) The wild type (wt) and mutant complementary sequences of the FN1 mRNA 3′UTR are shown with the miR-1 sequence. (B, C) EGFP reporter assays were performed to confirm the direct interaction of miR-1 with the FN1 3′ UTR. HEp2 cells were transfected with EGFP reporter vector, as well as the pri-miR-1 or miR-1 ASO, and the relative EGFP intensity was measured. The experiment was performed in triplicate ($^{*}P < 0.05$, $^{\#}$no statistical significance).

4. Discussion

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer, and laryngeal squamous cell carcinoma (LSCC) is the most common HNSCC [19,20]. Although considerable advantages are seen with multimodality therapy, the overall five-year survival rate for patients with this type of cancer is among the lowest of all major cancer types and has not improved in recent years [21]. Understanding the carcinogenic mechanisms of LSCC may help to find a marker and further develop treatment approaches to improve the cure and survival rates of this cancer. Recently, an increasing number of miRNAs are being discovered that are involved in the

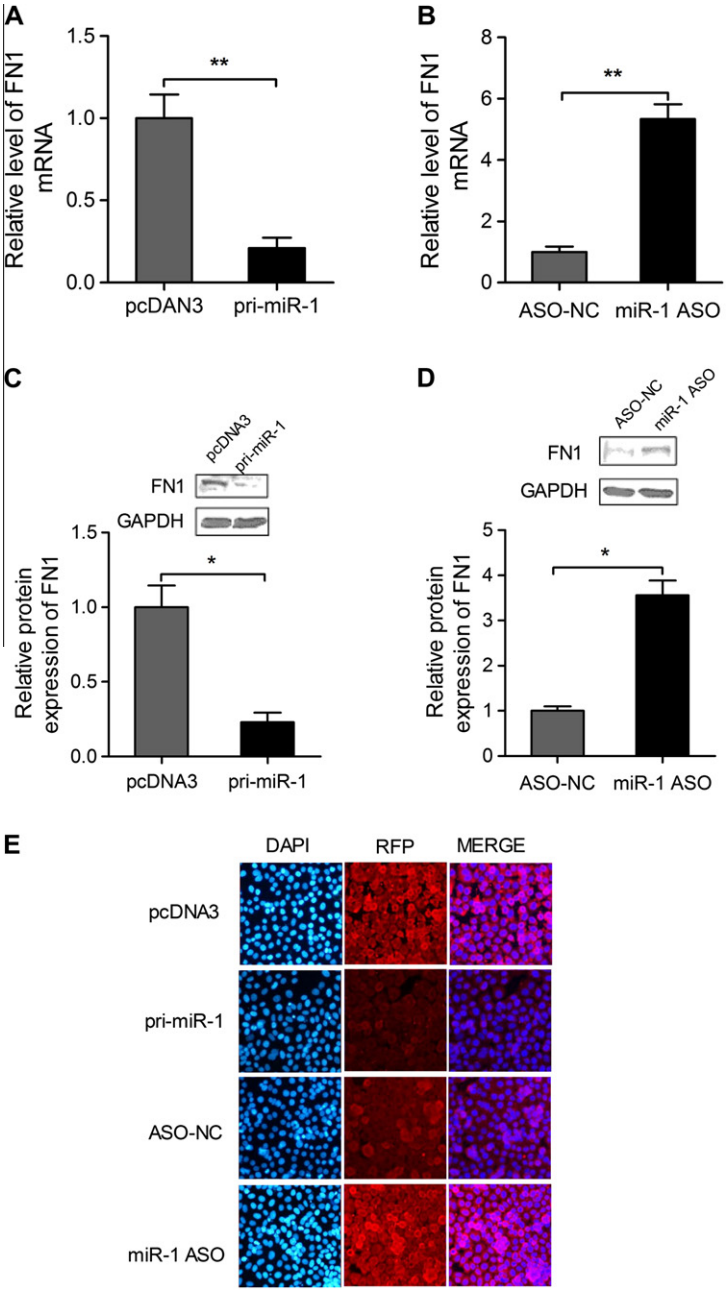

Fig. 4. Fibronectin1 (FN1) is negatively regulated by miR-1. A and B: When miR-1 was overexpressed or blocked, the level of FN1 mRNA was subsequently diminished or elevated, respectively, as compared to the control group. C and D: When miR-1 was overexpressed or blocked, the level of FN1 protein was subsequently diminished or elevated, respectively, as compared to the level in the control group. E: HEp2 cells were transfected with pri-miR-1 or miR-1 ASO, and FN1 protein expression was reduced or enhanced, respectively. Experiments were performed in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$).

regulation of gene expression at the post-transcriptional level. Accumulating evidence suggests that miRNAs play important roles in both normal cellular identity and in the pathological state, especially in tumorigenesis.

Previously, we performed miRNA microarray assays to find 13 miRNAs that were differentially expressed between laryngeal carcinoma samples and normal laryngeal tissues. We showed that miR-1 was notably downregulated in cancer tissues compared with normal tissues [13]. We presumed that the lower expression level of miR-1 was associated with the malignancy of laryngeal carcinoma. Therefore, we performed a further investigation of the effect of miR-1 on the growth and invasion of the human laryngeal carcinoma cell line HEp2. Interestingly, though miR-1 had no significant effect on the viability of HEp2 cells (Fig. 1), the impact of miR-1 on cell growth, migration and invasion is remarkable (Fig. 2). Mutating miR-1 abolished these effects. We just used HEp2 cell in this study because no other laryngeal carcinoma cell line was available. Even though, we detected the effects of miR-1 on the cellular phenotypes in HeLa human cervical cancer cell line (Suppl. Fig. S1) and QGY-7703 human hepatocellular carcinoma cell line (Suppl. Fig. S2) (data shown in the Supplementary data) and got similar results with those in HEp2 cells. Besides the two tumors, it was reported that miR-1 was downregulated and had suppressive effects on cell proliferation and mobility in lung cancer

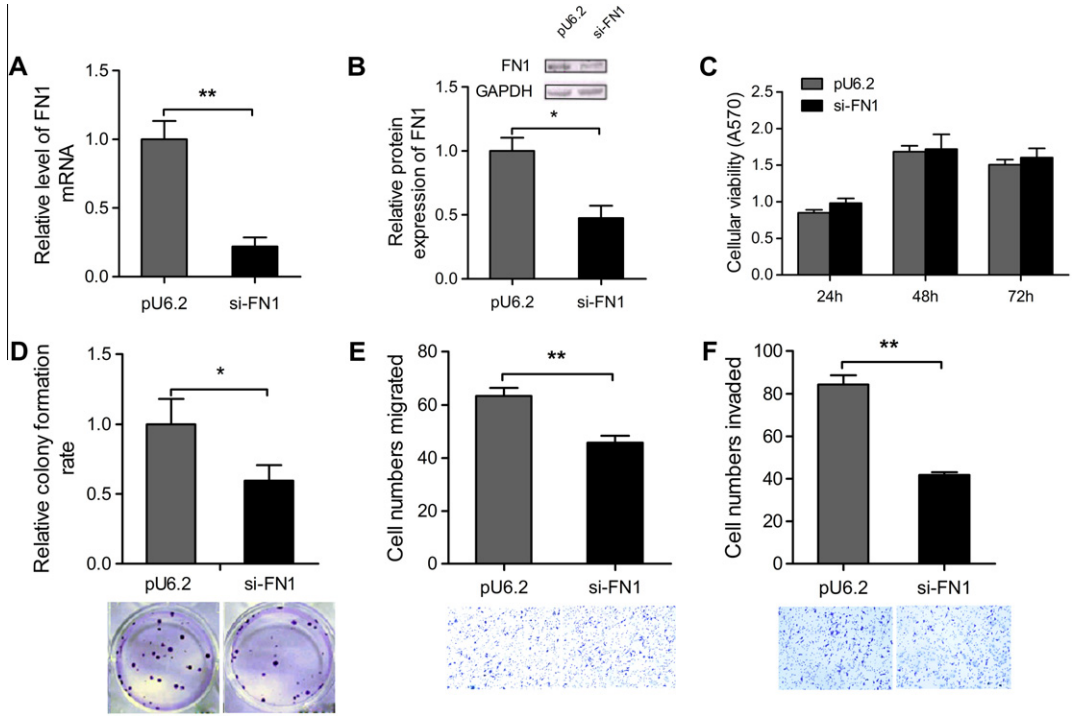

Fig. 5. FN1 promotes cell growth and mobility in HEp2 cells. (A, B) HEp2 cells were transfected with a siRNA targeting FN1, causing the suppression of the mRNA and protein levels of FN1. (C, D) FN1 expression was knocked down in HEp2 cells, and cell growth activity was detected by MTT and colony formation assays. (E, F) Knockdown of FN1 suppresses the migration and invasion potential of HEp2 cells. Experiments were performed in triplicate ($^{*}P < 0.05$, $^{**}P < 0.01$).

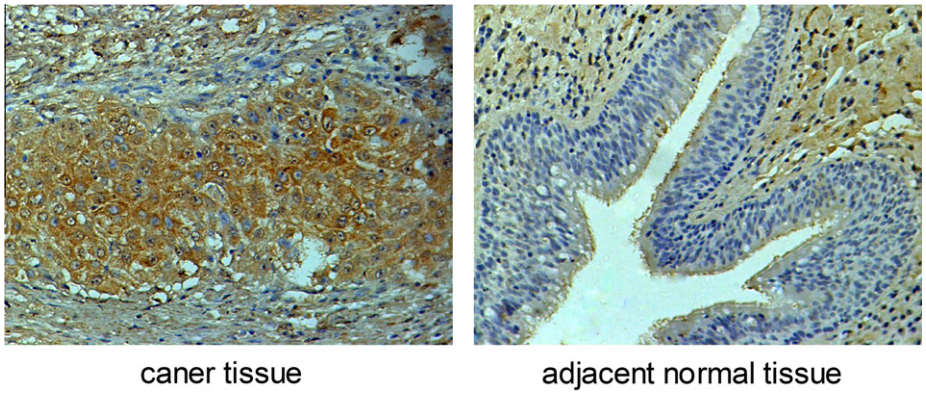

Fig. 6. The expression level of FN1 in laryngeal squamous carcinoma tissues. The expression of FN1 was detected in laryngeal squamous carcinoma tissues and adjacent normal tissues by IHC ($n = 5$).

[22] and thyroid carcinoma [23]. These results indicate that miR-1 may play a tumor suppressor role in several kinds of tumor cells, including the human laryngeal carcinoma cells.

It has been reported that the aberrant expression of miR-1 contributed to the malignant phenotype through the inhibition of cell cycle progression and the induction of apoptosis in hepatocellular carcinoma [17]. Moreover, a reported study implicates miR-1 in the initial dampening of stress response genes and in governing sets of genes used during periods of pressure overload. Serum response factor (SRF) is a direct upstream regulator of both c-Fos and miR-1, suggesting that SRF activates the transcriptional mediators of the immediate early gene response and, in a negative feedback loop, limits the extent of the stress response by activating miR-1 expression [24]. An additional study demonstrates a functional relationship between EVI1 and miR-1-2/miR-133a-1 cluster expression in acute myeloid leukemia (AMP) [25]. These findings are consistent with our results, showing that miR-1 functions as a tumor suppressor.

As is commonly known, miRNAs are required to bind to their target genes in order to regulate gene expression [26]. We used bioinformatics to predict that FN1 is a candidate target of miR-1 and employed an EGFP reporter system, real-time PCR, western blot analysis and immunofluorescence to confirm that FN1 is a direct target gene of miR-1. Moreover, miR-1-mediated suppression of FN1 is dependent on the 3′ UTR of the FN1 mRNA. Furthermore, immunostaning indicates the FN1 is overexpressed in laryngeal squamous carcinoma tissues compared with adjacent normal tissues. Although no sufficient samples were available, FN1 was detected in the laryngeal squamous carcinoma tissues and it had a negative relationship with miR-1. Because we did not collect enough samples, it is difficult to tell the relationship between the clinical stage of laryngeal carcinoma and the expression of miR-1

and FN1. Finally, knockdown of FN1 with siRNA represses cell growth, migration and invasion activity, which is consistent with ectopic expression of miR-1. Therefore, these results highlight the significance of miR-1 as a tumor suppressor in the malignancy of laryngeal carcinoma by targeting FN1.

FN1 is an extracellular matrix glycoprotein that plays major roles in cell differentiation, growth and migration. It is involved in such processes as wound healing and embryonic development, as well as oncogenic transformation [27,28]. For instance, fibronectin (FN) and TG2 facilitate the metastatic activity of A431 tumor cells, and this mediation may be partly attributed to the enhancement of the association of FN and β integrin by TG2 [29]. Also, FN is a key mediator of glioma progression through a mechanism that involves the maintenance of integrin β1 fibronectin receptors in glioma cells. These receptors are required by fibronectin to induce a Src kinase-dependent survivin activity, which promotes brain tumor proliferation [30]. In addition, the expression levels of fibronectin and the fibronectin type-III domain containing 3A (FNDC3A) protein are reduced by miR-17 in vitro and in transgenic mice. Also, miR-17 causes cellular defects through its repression of fibronectin expression [31]. In this study, we found that miR-1 can negatively regulate FN1 by targeting its 3′ UTR and suppresses the growth and invasion of HEp2 human laryngeal carcinoma cells.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.08.052.

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