MicroRNA-106b regulates the tumor suppressor RUNX3 in laryngeal carcinoma cells

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ABSTRACT

Our study focuses on a set of laryngeal tumors that show reduced RUNX3 expression in the absence of transcriptional silencing of tumor suppressor gene RUNX3 by aberrant methylation of CpG islands. We report that the loss of expression of RUNX3 correlates with up-regulation of miR-106b in human laryngeal carcinoma tissue. The downregulation of RUNX3 is mediated by miR-106b through binding of its 3'UTR. Moreover, miR-106b can promote the proliferation and invasion of laryngeal carcinoma cells by directly targeting RUNX3, and RUNX3 knockdown can abolish this phenotype. These results shed a new insight into the mechanism of miRNA regulation in laryngeal carcinoma.

1. Introduction

Laryngeal carcinoma is a common head and neck malignancy with high incidence because it accounts for approximately 2.4% of new malignancies worldwide every year [1,2]. To further improve its survival and cure rates, the carcinogenic mechanisms of laryngeal carcinoma need to be elucidated.

Various genetic alterations associated with laryngeal carcinoma, such as hTERT, CD24, PTEN, and RUNX1–3, have been described [3–5]. RUNX3, which is located on chromosome 1p36, has been identified as a critical tumour suppressor in many human cancer types [6–11]. The loss of RUNX3 gene expression has been reported to contribute to the tumourigenesis of many human tumour types, including gastric cancers, colorectal cancers, breast cancers, endometrial cancers, and laryngeal carcinomas [12–15]. The downregulation of RUNX3 has been mainly attributed to the hypermethylation of its promoter region [10]. However, the results of a previous study [16] demonstrated that there remains a proportion of laryngeal carcinoma that shows reduced RUNX3 expression in the absence of its methylation regulation mechanism, which suggests that other factors may be involved in the regulation of RUNX3.

MicroRNAs (miRNAs) were recently characterised as endogenous phylogenetically conserved small RNA molecules of approximately 21–25 nucleotides in length that regulate target gene expression by affecting the mRNA translation and stability or by modulating the promoter activity of the target gene [17].

An emerging body of evidence indicates that miRNAs play an important role in a variety of pathogenic conditions, including cancer [18,19]. A differential expression of miRNAs between tumour tissues and normal tissues has been observed in various cancer types [20], which suggests a possible link between miRNA expression and the development of cancer. For example, the members of the let-7 miRNA family have been found to be underexpressed in lung cancer. The let-7 miRNA targets the oncogene RAS, and the loss of let-7 expression results in the overexpression of RAS [21]. In addition, miR-15 and miR-16 are downregulated in chronic lymphocytic leukaemia and cause the overexpression of the tumour suppressor gene BCL2, which protects cells from apoptosis [22]. The aims of this study were to determine whether the dysregulation of miRNAs that target RUNX3 in laryngeal carcinoma is responsible for the observed downregulation of RUNX3 and to evaluate the role of this mechanism in laryngeal carcinoma.
2. Materials and methods

2.1. Clinical samples and cell lines

The 17 paired laryngeal carcinoma tissues used in this study were obtained from The First Hospital of Shanxi Medical University in China. The specimens, which included seven laryngeal carcinomas at stage I and II and seven laryngeal carcinomas at stage III and IV, were snap-frozen in liquid nitrogen. The collection and use of the patient samples were reviewed and approved by the Institutional Ethics Committees, and written informed consent was appropriately obtained from all of the patients. The Hep-2 and TU212 cells were purchased from ATCC.

2.2. Selection of candidate miRNAs

The candidate miRNAs that bind to RUNX3 were identified using three web-based bioinformatics algorithms. TargetScan, DIANA LAB, and microRNA predict miRNA-binding sites based on the complementary nucleotide sequence in the 3'-untranslated region of the RUNX3 mRNA.

2.3. Luciferase reporter assay

The luciferase reporter assay was performed as described in the Supplementary materials.

2.4. In vivo tumour xenograft studies

The tumorigenicity in nude mice was determined as described previously [23,24], and the details of this procedure are described in the Supplementary materials.

2.5. Cell proliferation, colony formation, and invasion assays

The details of these procedures are described in the Supplementary materials.

2.6. Statistical analysis

The data are expressed as the means ± standard deviation (S.D.), and differences that were found to have $P < 0.05$ using the Students–Newman–Keuls test are considered statistically significant. All of the data were analysed with the SPSS 17.0 software to confirm their statistical significance.

3. Results

3.1. The downregulation of RUNX3 in laryngeal carcinoma cells is independent of DNA methylation

Twelve laryngeal carcinoma tissues were analysed by methylation-specific PCR (MSP) to detect the methylation status of the RUNX3 CpG islands. As shown in Fig. 1A, tumours 2–4, 6, 9, 10, and 11 were unmethylated at the RUNX3 CpG islands, whereas tumours 1, 7, 8, and 12 were fully methylated at this locus. We then determined the expression level of RUNX3 protein in these unmethylated and methylated laryngeal carcinoma tissues. The Western blot results showed that the level of RUNX3 protein, which was normalised to that of GAPDH, was always lower in laryngeal carcinoma tissues compared to normal laryngeal epithelium, regardless of the methylation status of the RUNX3 CpG islands (Fig. 1B). This result was confirmed with an immunohistochemistry assay. As shown in Fig. 1C, RUNX3 exhibited a loss of expression in the laryngeal carcinoma tissues compared to the normal laryngeal epithelium.

Fig. 1. (A) Representative samples of MSP analysis. After bisulfite modification, the products that were amplified with primers specific to the unmethylated or methylated alleles of RUNX3 were loaded onto 2.5% agarose gels. (B) Western blot assay was performed to detect the RUNX3 protein levels in these tissues. The RUNX3 protein levels were normalised to that of GAPDH, and representative images are shown. (C) Immunohistochemistry (IHC) was performed using anti-RUNX3 antibody to stain, and representative images from the four groups (normal laryngeal epithelium, adjacent larynx tissues, methylated RUNX3 laryngeal carcinoma, and non-methylated RUNX3 laryngeal carcinoma), are shown.
theilum, irrespective of the methylation status of the CpG islands. These results suggest that RUNX3 is downregulated in laryngeal carcinoma cells and that there is likely another potential mechanism responsible for its downregulation.

3.2. miR-106b targets the RUNX3 transcript and downregulates its expression

The TargetScan, microRNA, and DIANA LAB programs were selected for the miRNA predictions. As shown in Fig. 2A, 11 candidate miRNAs were identified. Of these, miR-130a/b and miR-301a/b have been reported to target RUNX3 and regulate its expression [25]. To determine which miRNA most effectively regulates RUNX3, a luciferase reporter assay was performed. Hep2 cells were transfected with a reporter vector and the 11 miRNA mimics. Obviously, miR-106b significantly downregulated the luciferase intensity of pGL3/luciferase-RUNX3-3’-UTR (Fig. 2B). To further determine whether miR-106b directly regulates RUNX3, another luciferase reporter assay was used to validate the target site in the RUNX3 3’-UTR (Fig. 2C). Hep2 cells were transfected with the reporter vector and miR-106b mimics, control mimics, ASO-miR-106b, or control ASO. Interestingly, the intensity of luciferase in the cells transfected with the miR-106b mimics was significantly decreased compared with the luciferase intensity of the control cells, whereas the intensity of luciferase in the cells transfected with ASO-miR-106b was significantly increased (Fig. 2C). In contrast, the luciferase intensity generated by the reporter vector containing the mutant RUNX3 3’-UTR was not affected by miR-106b mimics or miR-106b ASO (Fig. 2C). These results indicate that RUNX3 is a direct target of miR-106b. Moreover, we examined the effect of miR-106b on the expression of endogenous RUNX3 by Western blot. The RUNX3 protein level was increased approximately three-fold in Hep2 and two-fold in TU-212 cells transfected with ASO-miR-106b compared with the control group (Fig. 2D). In addition, the RUNX3 protein level was decreased approximately 40% in Hep2 and 70% in TU-212 cells transfected with miR-106b mimics compared with the control group (Fig. 2D).

3.3. miR-106b promotes the growth and invasion of human laryngeal carcinoma cells

To determine the role of miR-106b in the proliferation and invasion of laryngeal carcinoma cells, ASO-miR-106b was used to block endogenous miR-106b expression in the Hep2 and TU-212 laryngeal carcinoma cell lines. The efficiency of the ASO-mediated blocking of miR-106b was confirmed by quantitative RT-PCR (Fig. 3A). A colony formation assay and transwell invasion assay were used to further elucidate the effect of miR-106b on the
growth and invasion of laryngeal carcinoma cells. The colony formation rate of Hep2 cells transfected with ASO-miR-106b was decreased approximately 50% compared with the control group (Fig. 3C). The same phenomenon was observed in the TU-212 cells (Fig. 3C). The transwell invasion assay indicated that the blocking of miR-106b expression by ASO affects the growth capacity of Hep2 and TU-212 cells, as determined by a colony formation assay. All of the histograms show the normalised mean percentages of colony formation ± S.D. from three independent experiments. *P < 0.05. (D) The blocking of miR-106b by ASO affects the invasion capacity of Hep2 and TU-212 cells, as determined through a transwell invasion assay. The histograms show the normalised means of invading Hep2 or TU-212 cells ± S.D. from three independent experiments. *P < 0.05. (E) The expression of RUNX3 in the tumours of SCID mice treated with ASO-miR-106b and control ASO was measured by Western blot analysis, and the histograms show the normalised mean of RUNX3 expression from five SCID mice in each group. *P < 0.05.

3.4. The overexpression of RUNX3 represses the cell growth and invasion of Hep2 and TU-212 cells, and the knockdown of RUNX3 abrogates the ASO-miR-106b-induced suppression of Hep2 and TU-212 cell growth and invasion

To determine the influence of RUNX3 on the growth of Hep2 cells, RUNX3 was overexpressed using an RUNX3 expression plasmid (pCMV6/RUNX3). The Western blot assay showed that pCMV6/RUNX3 effectively increased the expression of RUNX3 approximately three-fold in Hep2 cells and two-fold in TU-212 cells compared with the control cells (Fig. 4A). The growth curve assay indicated that the overexpression of RUNX3 resulted in a slower growth than that observed in the control Hep2 and TU-212 cells (Fig. 4B). A colony formation assay was then performed to evaluate the effect of the overexpression of RUNX3 on the growth of Hep2 cells. The colony formation rate of the cells transfected with the pCMV6/RUNX3 vector was markedly lower than that observed in the control group (Fig. 4C). In addition, the transwell invasion assay indicated that the overexpression of RUNX3 by pCMV6/RUNX3 resulted in a lower level of invading cells than that observed in the respective Hep2 and TU-212 control cells (Fig. 4D).
Li et al. had previously reported that the RUNX3-induced growth suppression partially regulated various proteins, including inhibiting cdk2 and cdk4 and increasing p27, Rb, and TIMP-1. Thus, we also determined the effect of RUNX3 overexpression on the expression of its downstream genes in laryngeal carcinoma cell lines, and the Western blot results confirm that the RUNX3 overexpression vector was effective and exhibits a homogeneity function. These results indicate that the knockdown of RUNX3 repressed the growth and invasion of Hep2 and TU-212 cells.

To verify whether miR-106b promotes the proliferation and invasion of Hep2 cells and TU-212 cells by regulating RUNX3, an RUNX3 knockdown vector (RUNX3-siRNA) was designed to reduce the RUNX3 protein level. This vector, which does not affect the expression of miR-106b in transfected laryngeal carcinoma cells (Fig. 5A), was used to rescue the suppressive effect of the knockdown of miR-106b on Hep2 and TU-212 cell growth and invasion. When Hep2 and TU-212 cells were cotransfected with ASO-miR-106b and either RUNX3-siRNA or a control vector, the inhibition of the colony formation rate and cell invasion viability caused by ASO-miR-106b was counteracted by the knockdown of RUNX3 but not by a control vector (Fig. 5C and D). Western blot assays showed that the RUNX3 expression was also rescued (Fig. 5B). These results indicate that RUNX3 is a downstream target gene of miR-106b and is involved in the miR-106b-mediated promotion of cell growth and invasion in the Hep2 and TU-212 laryngeal carcinoma cell lines.

3.5. The RUNX3 expression level is inversely correlated with the miR-106b level in human laryngeal carcinoma tissues

All of above results suggest that miR-106b can promote the growth and invasion in the Hep2 and TU-212 laryngeal carcinoma cell lines by directly targeting RUNX3. To determine whether this regulation axis exists in larynx cancer tissues, we examined the level of miR-106b and RUNX3 in 14 human larynx cancer tissues. miR-106b was detected by real-time PCR, and RUNX3 was detected by Western blot. As shown in Fig. 6A and B, the expression of miR-106b was inversely correlated with the expression of RUNX3 in the examined larynx cancer tissues. The comparison of the larynx cancer tissues and matched normal tissues showed that miR-106b is
significantly inversely correlated with the expression of RUNX3, which indicates that the miR-106/RUNX3 axis may play an important role in the process of larynx cancer tumorigenesis.

4. Discussion

An increasing body of evidence indicates that the miRNA repression of tumour suppressor genes may be a common mechanism involved in the tumourigenesis of laryngeal carcinoma, including the regulation of Rb by miR-106b [26]. In fact, an increasing number of miRNAs have been linked to the development of laryngeal carcinoma. These identified miRNAs target the expression of a diverse set of genes, e.g., miR-21 targets PTEN, miR-203 targets survivin, miR-24 targets S100A8, miR-206 targets VEGF, miR-16 targets Zyxin, miR-1 targets fibronectin1, and miR-34c targets c-Met [5,27–32].

As an important tumour suppressor, the expression of RUNX3 is lost in gastric cancer cell lines and in various types of human cancers, including laryngeal carcinoma, and this loss is mostly accompanied by the aberrant methylation of its promoter [16,33]. Kim et al. reported the RUNX3 methylation rate in 37 larynx tumour tissues was 62%. In contrast, 38% of these laryngeal carcinoma tissues may have a lower expression level of RUNX3 without DNA methylation. Indeed, Lai et al. claimed that there exists a mechanism of miR-130b-induced RUNX3 downregulation in gastric cancer tumorigenesis. In this study, we first determined the RUNX3 methylation status and evaluated its expression level in methylated and non-methylated laryngeal carcinoma tissues. Our results demonstrated that RUNX3 is always downregulated both in the methylated and in the non-methylated laryngeal carcinoma tissues (Fig. 1). To explore whether the mechanism of miRNA-mediated suppression of tumour suppressors contributes to the loss of RUNX3 expression in both methylated and non-methylated laryngeal carcinoma, a bioinformatics approach was used to predict miRNAs that might bind to the RUNX3 3′-UTR. Based on the results...
from three alignment algorithms, 11 miRNAs were selected as the most putative candidates for the study of the miRNA regulation of RUNX3 (Fig. 2A). The subsequent luciferase assay (Fig. 2B) showed that miR-106 significantly decreased the luciferase activity of pGL3/luciferase-RUNX3-3'-UTR. Thus, a functional validation and assessment of miR-106b in laryngeal carcinoma cell lines and clinical samples was then performed (Fig. 2C and D, Fig. 6).

miR-106b, which is located on chromosome 7, has been reported to participate in the development and progression of human tumours, such as hepatocellular cancer, prostate cancer, gastric cancers, and renal cell carcinoma [34–37]. In our study, the repression of miR-106b resulted in the inhibition of laryngeal carcinoma cell proliferation and invasion (Fig. 3). This phenotype of laryngeal carcinoma cells regulated by miR-106b was partly attributed to the miR-106b-induced downregulation of RUNX3 through targeting of the 3'-UTR of RUNX3. The rescue assays performed in this study demonstrated that miR-106b downregulated the expression of RUNX3 directly because the knockdown of
RUNX3 abolished the miR-106b-ASO-mediated inhibition of laryngeal carcinoma cell proliferation and invasion (Fig. 5). A previous study had reported that miR-106b promotes cell proliferation by targeting Rb in laryngeal carcinoma [26]. In this study, we evidenced that RUNX3 is a novel direct and functional target of miR-106b that is involved in the proliferation of laryngeal carcinoma cells.

It has been proposed that a single miRNA can target several genes and that multiple miRNAs can target a single gene in a comprehensive manner [38,39]. Several studies have reported that a single miRNA can regulate the growth, invasion, migration, and cell cycle arrest laryngeal carcinoma cells by directly targeting a single gene [5,27–32]; this type of system is called a one-miRNA-targets-one-gene model. Based on the results reported by Cai et al., we found a new model, which we called a one-miRNA-targets-two-genes model, in laryngeal carcinoma. In this model, miRNA-106b regulates laryngeal carcinoma cell growth and invasion by targeting two tumour suppressors, RUNX3 and Rb (Fig. 7). Additionally, RUNX3 can positive regulate the expression of Rb in renal cell carcinoma (RCC) [40]. Thus, this one-target-two model exhibits positive feedback regulation in laryngeal carcinoma and thus acts as an advanced regulatory mechanism in laryngeal carcinoma cells. This finding may have important implications for the development of future therapies for the treatment of laryngeal carcinoma.

In conclusion, our study has several major findings. (a) RUNX3 is down-regulated in laryngeal carcinoma irrespective of the methylation status of its CpG region. (b) This study provides the first demonstration that miR-106b directly targets RUNX3 and down-regulates the expression of RUNX3 in laryngeal carcinoma cells. (c) The suppression of miR-106b causes a marked inhibition of proliferation and invasion in the laryngeal carcinoma Hep2 and TU-212 cell lines, and this phenotype can be rescued by the knockdown of RUNX3. (d) miR-106b regulates laryngeal carcinoma cell growth and invasion in a one-miRNA-targets-two-genes manner, which involves a positive feedback regulation mechanism. (e) In clinical laryngeal carcinoma tissues, the expression level of miR-106b and RUNX3 exhibit a significant inverse correlation. These results provide strong evidence that RUNX3 can also be inactivated in laryngeal carcinoma cells by miRNA expression in addition to hypermethylation. Further studies are required to determine whether the miR-106b-target-RUNX3 mechanism contributes to the tumourigenesis of other cancers.

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