

Regulation of the cell cycle gene, *BTG2*, by *miR-21* in human laryngeal carcinoma

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MicroRNAs are short regulatory RNAs that negatively modulate gene expression at the post-transcriptional level, and are deeply involved in the pathogenesis of several types of cancers. To investigate whether specific miRNAs and their target genes participate in the molecular pathogenesis of laryngeal carcinoma, oligonucleotide microarrays were used to assess the differential expression profiles of microRNAs and mRNAs in laryngeal carcinoma tissues compared with normal tissues. The oncogenic miRNA, *microRNA-21* (*miR-21*), was found to be upregulated in laryngeal carcinoma tissues. Knockdown of *miR-21* by specific antisense oligonucleotides inhibited the proliferation potential of HEP-2 cells, whereas overexpression of *miR-21* elevated growth activity of the cells, as detected by the colony formation assay. The cell number reduction caused by *miR-21* inhibition was due to the loss of control of the G1-S phase transition, instead of a noticeable increase in apoptosis. Subsequently, a new target gene of *miR-21*, *BTG2*, was found to be downregulated in laryngeal carcinoma tissues. *BTG2* is known to act as a pan-cell cycle regulator and tumor suppressor. These findings indicate that aberrant expression of *miR-21* may contribute to the malignant phenotype of laryngeal carcinoma by maintaining a low level of *BTG2*. The identification of the oncogenic *miR-21* and its target gene, *BTG2*, in laryngeal carcinoma is potentially valuable for cancer diagnosis and therapy.

Keywords: microRNA, cell cycle, *BTG2*, laryngeal carcinoma, *microRNA-21*

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Introduction

MicroRNAs (miRNAs) are a group of non-coding, single-stranded RNAs that are approximately 22 nt in length. They regulate gene expression at the post-transcriptional level, and are involved in the regulation of cellular differentiation, development and metabolic processes. Hundreds of miRNAs have been identified in various mammalian genomes. To date, there have been 706 miRNA genes described in the human genome. It is becoming apparent that miRNAs have the potential to regulate at least 20%-30% of all human genes [1], but their biological function remains largely unknown. Recently, researchers have uncovered both the tumor-suppressive

and oncogenic potential of a number of miRNAs, underscoring their importance in human cancers [2-4]. In fact, either a single miRNA or small sets of miRNAs have been demonstrated to be dysregulated in diverse cancer subtypes. Aberrant expression of miRNAs can contribute to tumor development and progression by targeting certain tumor suppressor genes. Tumor suppressors maintain cell cycle checkpoint integrity and regulate apoptotic responses, and the deregulation of tumor suppressor genes by miRNAs can enhance tumorigenesis and contribute to poor clinical outcomes. Mertens-Talcott *et al.* [5] have reported that *miR-27a* facilitates cancer cell proliferation through the suppression of *Myt-1*, which blocks the cell cycle transition at G2-M. Moreover, *miR-221* and *miR-222* have also been found to be oncogenes that control cell cycle progression by inhibiting *p27^{kip1}* [6, 7].

Laryngeal carcinoma is one of the most common head and neck cancers. Although early-stage laryngeal cancer is often curable with surgery or radiotherapy, for the majority of patients with the advanced disease, the outcome has not improved dramatically in the last two decades despite therapeutic advances. In order to further improve

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Abbreviations: ASO (antisense oligonucleotide); *BTG2* (B-cell translocation gene 2); *miR-21* (*microRNA-21*); UTR (untranslated region)

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survival rates and treatments, it is important to better understand the mechanisms of carcinogenesis. Despite the fact that considerable efforts have been made in recent years, the molecular mechanism involved in the initiation and progression of laryngeal carcinoma remains largely unknown.

This study utilized an oligonucleotide microarray to examine the differential expression profiles of microRNAs and mRNAs in laryngeal carcinoma tissues compared to their adjacent normal tissues. *MicroRNA-21* (*miR-21*), a known oncogenic miRNA, was found to be overexpressed in laryngeal carcinoma tissues, and suppression of *miR-21* inhibited the proliferation of the cells of laryngeal carcinoma cell line, HEP-2, by suppressing cell cycle progression without affecting cell apoptosis. Deregulation of *BTG2*, which was identified to be a *miR-21* target gene, may be responsible for the *miR-21*-mediated abnormal cellular phenotype.

Results

Differential miRNA expression between laryngeal carcinoma and normal laryngeal tissues

Using the miRNA microarray containing 210 human miRNA probe sets, we first assessed the miRNA profiles in three paired laryngeal carcinoma and normal laryngeal tissues. A miRNA was considered to be significantly upregulated only if the fold-change was more than 1.5 in the cancer tissues compared with the normal tissues. Likewise, only if a fold-change was less than 0.66 was the miRNA considered to be significantly downregulated. Statistical analysis identified 13 miRNAs that were differentially expressed in all three tumor tissues compared with the matched non-neoplastic controls. Among these 13 miRNAs, 6 miRNAs (*let-7a-1*, *miR-203*, *miR-205*, *miR-21*, *miR-98* and *miR-16-1*) were upregulated and 7 miRNAs (*miR-100*, *miR-1-2*, *miR-122a*, *miR-143*, *miR-145*, *miR-26a-1* and *miR-34c*) were downregulated in laryngeal carcinoma tissues (Table 1). Among them, *miR-21* is reported to be overexpressed in many types of solid tumors, including glioblastoma and breast cancer [8-11]. Thus, we selected *miR-21* for further studies.

The effect of *miR-21* on the growth of HEP-2 cells

To study the biological significance of elevated *miR-21* levels in laryngeal carcinoma cells, we first used a loss-of-function approach by transfecting the laryngeal carcinoma cell line, HEP-2, with chemically synthesized oligonucleotides complementary to *miR-21*. Transient transfection of HEP-2 cells with *miR-21* ASO (specific antisense oligonucleotide of *miR-21*) caused suppression of cellular proliferation at 48-h and 72-h post-trans-

fection in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay (Figure 1A). Meanwhile, the soft agar colony formation assay indicated that the number of colonies from HEP-2 cells transfected with *miR-21* ASO was significantly lower than that of the negative control group (Figure 1B) and that overexpression of *miR-21* using a *miR-21*-expressing construct (pSilencer/sh-*miR-21*) led to elevated colony formation in tumor cells (Figure 1C). In addition, we also explored the effect of *miR-21* on laryngeal tumorigenesis *in vivo*. Compared with the control group, knockdown of *miR-21* led to suppression of overall tumor growth in nude mice (Figure 1D). Furthermore, the validity of the alterations of *miR-21* expression levels was confirmed using real-time PCR assays (Figure 1E). These data indicate that the aberrant expression of *miR-21* in laryngeal carcinoma may contribute to the malignant phenotype.

Suppression of *miR-21* impairs the HEP-2 cell cycle without affecting apoptosis

To explore how the suppression of *miR-21* inhibits HEP-2 cell growth, we studied the effects of *miR-21* on cell cycle and cell apoptosis. The cell cycle distribution of HEP-2 cells was found to be affected by *miR-21*.

Table 1 Differentially expressed miRNAs in laryngeal carcinoma tissues compared with normal tissues

miRNA name	Chromosome	Ratio (cancer/normal tissue)*	P value
<i>let-7a-1</i>	9	2.465±0.831	1.215E-04
<i>miR-203</i>	14	8.150±3.816	4.350E-04
<i>miR-205</i>	1	7.660±3.665	2.604E-07
<i>miR-21</i>	17	5.268±1.756	6.208E-06
<i>miR-98</i>	X	4.485±2.431	4.252E-07
<i>miR-16-1</i>	13	11.745±8.842	1.607E-03
<i>miR-100</i>	11	0.119±0.038	8.139E-06
<i>miR-1-2</i>	18	0.126±0.053	7.727E-04
<i>miR-122a</i>	18	0.183±0.027	1.968E-06
<i>miR-143</i>	5	0.331±0.173	1.342E-03
<i>miR-145</i>	5	0.186±0.042	9.411E-06
<i>miR-26a-1</i>	3	0.390±0.024	2.718E-06
<i>miR-34c</i>	11	0.201±0.113	6.211E-07

The miRNA microarray analysis was performed to detect the differentially expressed miRNAs in laryngeal carcinoma tissues and matched normal tissues. The prominently changed miRNAs are shown in the figure. The ratio indicates the fold change (mean±SD) of cancer tissues compared with the normal tissues and the P value is also provided.

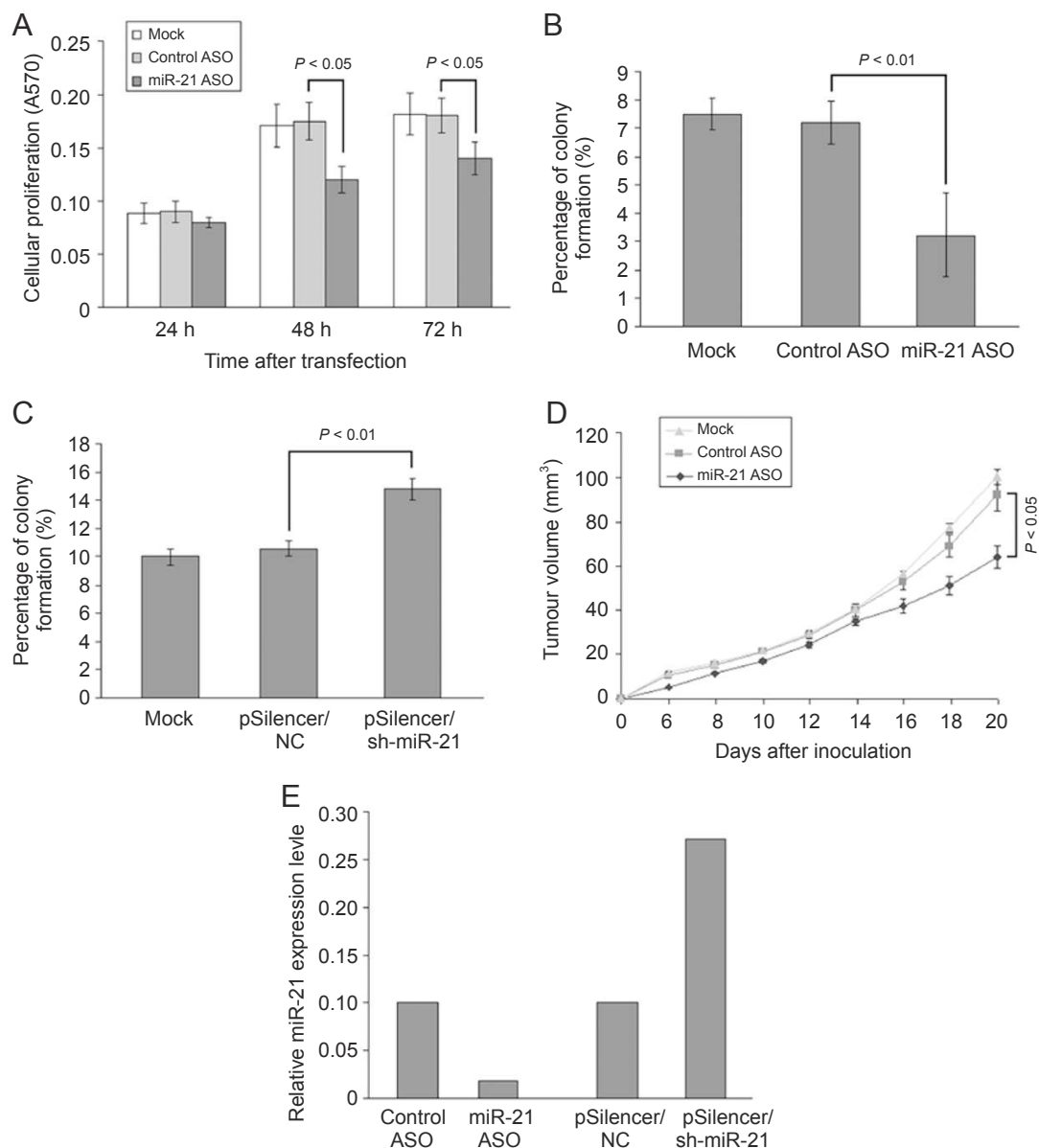


Figure 1 Regulation of tumor cell proliferation by *miR-21*. **(A)** HEP-2 cells were either mock transfected, transfected with *miR-21* ASO or transfected with control ASO. The MTT assay was used to determine relative cellular proliferation at 24 h, 48 h and 96 h. A_{570} is the optical density of MTT measured at 570 nm. The figure shows mean values \pm SD of A_{570} from three independent experiments. After transfection with *miR-21* ASO, the 48 h and 72 h data points showed a statistically significant difference. **(B)** HEP-2 cells were either mock transfected, transfected with *miR-21* ASO, or transfected with control ASO. At 48-h post-transfection, tumor cell proliferation was analyzed by a soft agar colony formation assay. **(C)** pSilencer/sh-*miR-21* or control vector (pSilencer/NC) was transfected into HEP-2 cells. At 48-h post-transfection, tumor cell proliferation was analyzed by a soft agar colony formation assay. All the histograms show normalized mean value \pm SD of percentage of colony formation from three independent experiments. **(D)** Growth curves of primary subcutaneous tumors in nude mice formed by HEP-2 cells either non-transfected or transfected with *miR-21* ASO or control ASO. Each data point represents the mean \pm SD of six mice. *P* value is based on Student's *t*-test at day 20. **(E)** In HEP2 cells, *miR-21* was blocked by *miR-21* ASO or was overexpressed by pSilencer/sh-*miR-21* vector, and then the *miR-21* expression level was detected using real-time RT-PCR assays. The histogram shows the relative *miR-21* expression levels.

When the cells were transfected with *miR-21* ASO, the number of cells in the G1 phase significantly increased,

and G1-S arrest was obvious (Figure 2A). However, there was no significant increase of cell apoptosis in *miR-*

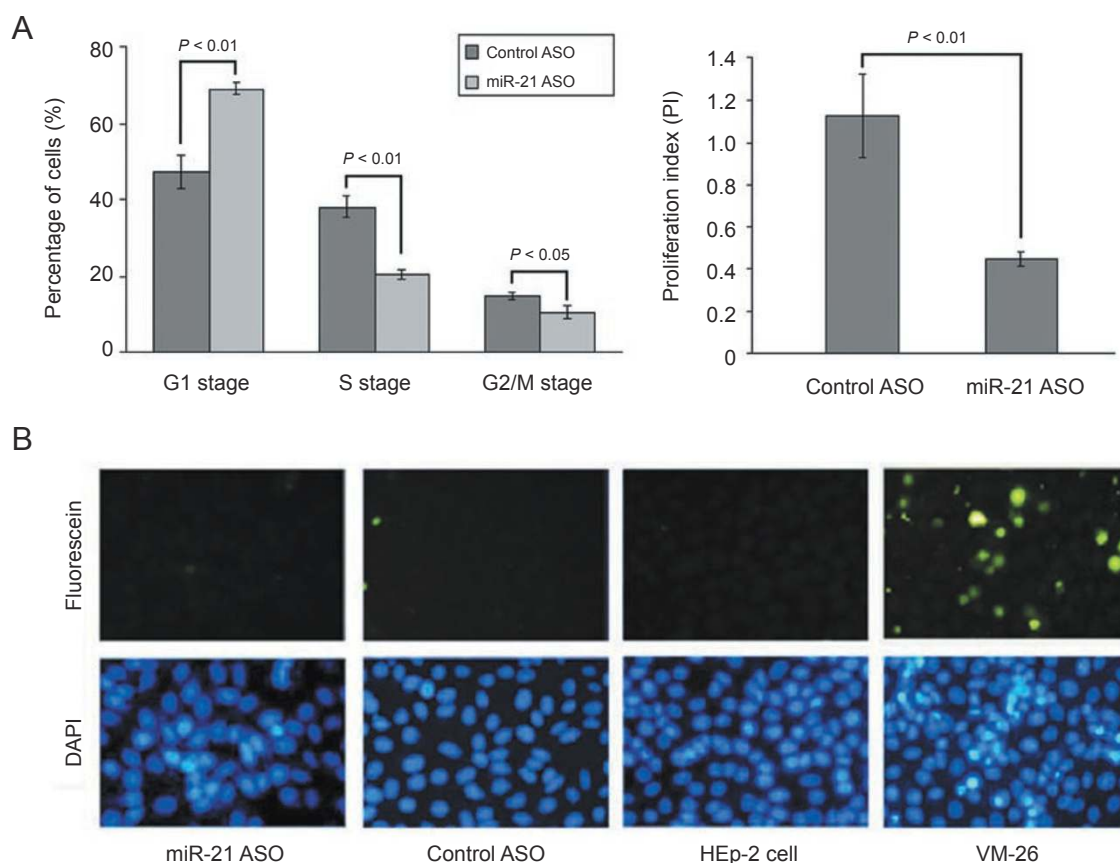


Figure 2 The effect of *miR-21* suppression on cell cycle and cell apoptosis. **(A)** At 48 h after transfection with *miR-21* ASO or control ASO, Hep-2 cells were detached, rinsed, fixed and stained. Quantification of the cell cycle phase distribution was analyzed by flow cytometry. The number of cells in the G1 phase was significantly increased in the *miR-21* ASO group, and G1-S arrest was obvious. The proliferation index (PI) decreased significantly in the *miR-21* ASO group. Data are shown as the mean±SD of three replicates and are representative of three independent experiments. **(B)** Hep-2 cells were transfected with *miR-21* ASO or control ASO, and detected by the *In situ* Cell Death Detection Kit at 48-h post-transfection. Fluorescein-stained cells indicated apoptotic cells. Hep-2 cells treated with 0.09 mg/ml teniposide (VM-26) were used as positive controls, and untreated Hep-2 cells were used as negative controls. DAPI staining was used to determine the number of nuclei and to assess the gross morphology.

21-ASO-transfected cells compared with control-ASO-transfected cells, although there were a few apoptotic cells due to liposome transfection (Figure 2B). These results indicate that the change of cell number in *miR-21*-ASO-transfected Hep-2 cells was due to the loss of control of the cell cycle instead of an increase in cell apoptosis. Thus, *miR-21* may contribute to laryngeal tumorigenesis by regulating cell cycle checkpoints and cellular proliferation.

BTG2 is downregulated in laryngeal carcinoma and is a putative target of *miR-21*

MicroRNAs regulate a variety of cellular activities through regulation of the expression of multiple target genes. To understand the mechanism of *miR-21*-mediated

cell cycle dysregulation in laryngeal carcinoma, we next focused on the identification of target genes that may participate in this process. As *miR-21* has been validated as being elevated in laryngeal carcinoma, its target genes should be correspondingly downregulated in laryngeal carcinoma. The 7 267-spot human cDNA microarray was used to investigate the gene expression profiles of the three paired laryngeal carcinoma tissues compared with the adjacent normal tissues. As a result, 90 genes were found to be differentially expressed in the laryngeal carcinoma tissue. Among these genes, 29 were upregulated and 61, including *BTG2*, were downregulated. In order to find *miR-21* target genes, we combined the datasets obtained from our microarray analysis with the datasets obtained from publicly available databases. It is well known

that miRNAs usually bind their targets with incomplete complementarity within the 3' untranslated region (UTR) of the mRNA target. Most bioinformatics methods use the first 2-8 bases of the mature miRNA sequence to search for sequences complementary to the 3'UTR of all expressed genes. Publicly available databases (PicTar and TargetScan) were used for target prediction [12, 13]. *BTG2*, a pan-cell cycle regulator that is confirmed to be downregulated in laryngeal carcinoma using semi-quantitative RT-PCR, was predicted to be a target gene of *miR-21* (Figure 3A).

MiR-21 regulates *BTG2* at both the mRNA and the protein levels

To determine the effect of *miR-21* on *BTG2* expression levels, total RNA and protein were isolated from HEP-2 cells transfected with *miR-21* ASO or control ASO. The mRNA level of *BTG2* was quantified using semi-quantitative RT-PCR, and the protein level of *BTG2* was detected by western blot. The results showed that both the mRNA and the protein levels of *BTG2* were elevated in HEP-2 cells transfected with *miR-21* ASO compared with cells transfected with control ASO (Figure

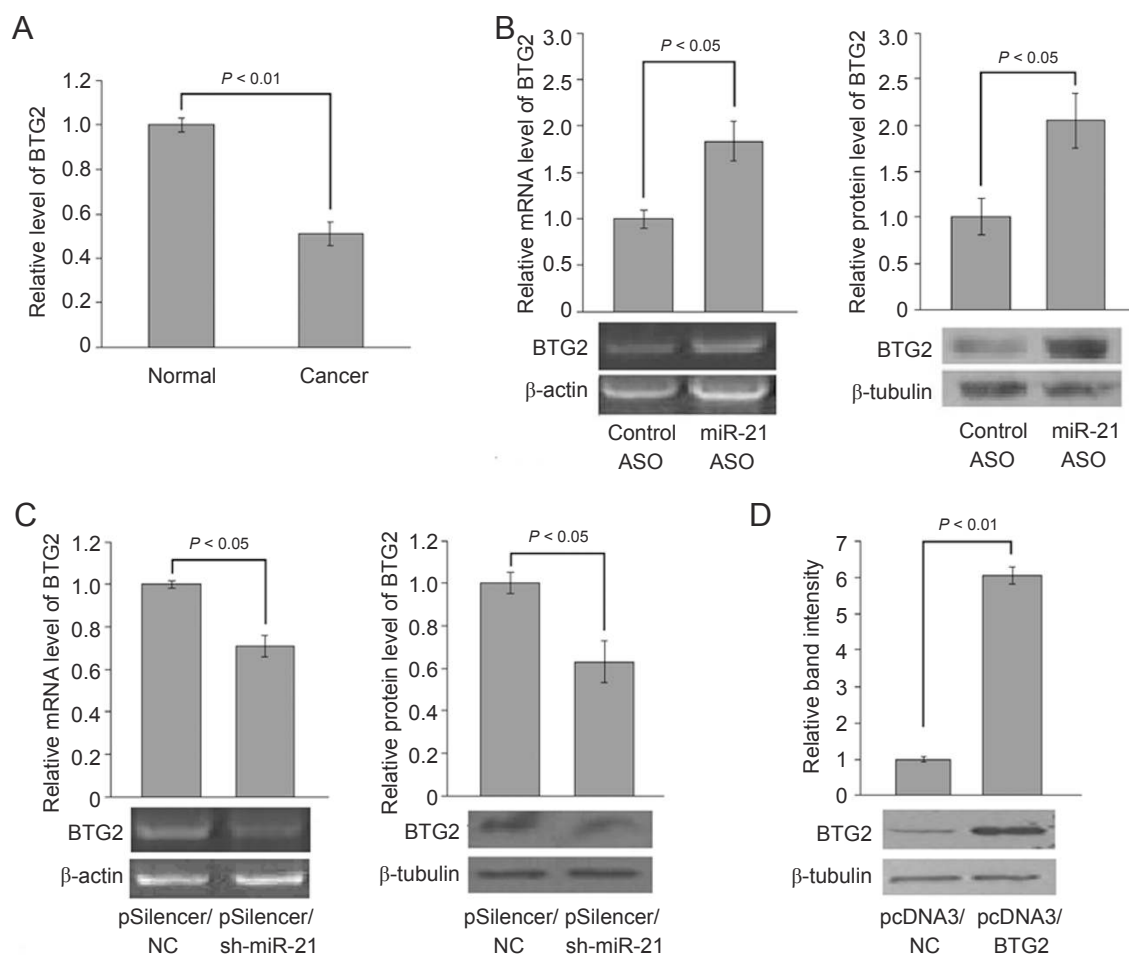


Figure 3 Regulation of *BTG2* expression by *miR-21*. **(A)** The differential expression of *BTG2* in three paired clinical samples from laryngeal carcinoma tissues and adjacent normal tissues were validated using semi-quantitative RT-PCR. The data represents the mean value±SD, and the normal group was set to 1. **(B)** HEP-2 cells were transfected with either *miR-21* ASO or control ASO. At 48-h post-transfection, the mRNA level of *BTG2* was determined by semi-quantitative RT-PCR and the *BTG2* protein level was detected by western blot. The histograms show the quantification results for semi-quantitative RT-PCR or western blot. The expression level of *BTG2* in the control ASO group was set to 1. **(C)** pSilencer/sh-miR-21 or control vector was transfected into HEP-2 cells. At 48-h post-transfection, the *BTG2* RNA and protein levels were assessed. The expression level of *BTG2* in the control group was set to 1. **(D)** The *BTG2* protein expression vector, pcDNA3/*BTG2*, as well as the control vector was transfected into HEP-2 cells and the *BTG2* level of these cells was detected with western blot assay to determine the efficacy of the anti-*BTG2* antibody. The expression level of *BTG2* in the control group was set to 1. All the data are shown as the mean value±SD of three independent experiments.

3B). Similarly, HEP-2 cells were transfected with either a *miR-21*-expressing vector (pSilencer/sh-*miR-21*) or a control vector (pSilencer/NC). At 48-h post-transfection, *BTG2* RNA and protein levels were assessed. We found that overexpression of *miR-21* in HEP-2 cells could reduce the expression of *BTG2* at both the mRNA and the protein levels (Figure 3C). In addition, when *BTG2* protein was overexpressed in HEP-2 cells, a band with higher intensity was detected in western blot, demonstrating the specificity of the anti-*BTG2* antibody (Figure 3D).

Direct effect of *miR-21* on the 3'UTR of *BTG2*

An EGFP (enhanced green fluorescent protein) reporter assay was used to demonstrate that the negative regulation of *miR-21* on *BTG2* expression was direct. The alignment of *miR-21* with the *BTG2* 3'UTR is illustrated in Figure 4A. The 3'UTR sequence of *BTG2* was cloned downstream of the EGFP gene. We found that the expression of EGFP was strongly increased when *miR-21* function was inhibited by transfecting cells with an antisense oligonucleotide (Figure 4B), whereas *miR-21* overexpression reduced EGFP expression (Figure 4C). To further substantiate a direct effect of *miR-21* on the *BTG2* 3'UTR, we introduced a triple mutation in the seed sequence of the *BTG2* 3'UTR complementary to *miR-21*. The EGFP expression from the mutated vector, pcDNA3/EGFP-*BTG2*MUT, was not affected by *miR-21*, regardless of its expression levels (Figure 4B and 4C). We thus conclude that *miR-21* regulates *BTG2* expression directly through the UTR-binding region.

Discussion

MicroRNAs are a novel class of regulatory molecules with the ability to control gene expression at the post-transcriptional level. MiRNAs appear to decrease protein production either by increasing the degradation of mRNA or by blocking translation of mRNA into protein. Hence, changes in the expression of even a single miRNA may lead to changes in diverse cellular activities, which may contribute to the development of diseases. In the present study, miRNA microarray assay was used to assess the miRNA expression profiles of three laryngeal carcinoma tissues compared to their matched normal tissues. A total of 13 miRNAs were identified to be differentially expressed. Of the six upregulated miRNAs, *miR-21* is known as an oncomir. It is overexpressed in many types of tumor tissues, and contributes to malignant phenotypes [8-11]. High expression of *miR-21* in laryngeal carcinoma tissues may also correlate with tumor development and progression. In our study, suppression of *miR-21* in cultured HEP2 cells decreased tumor cell

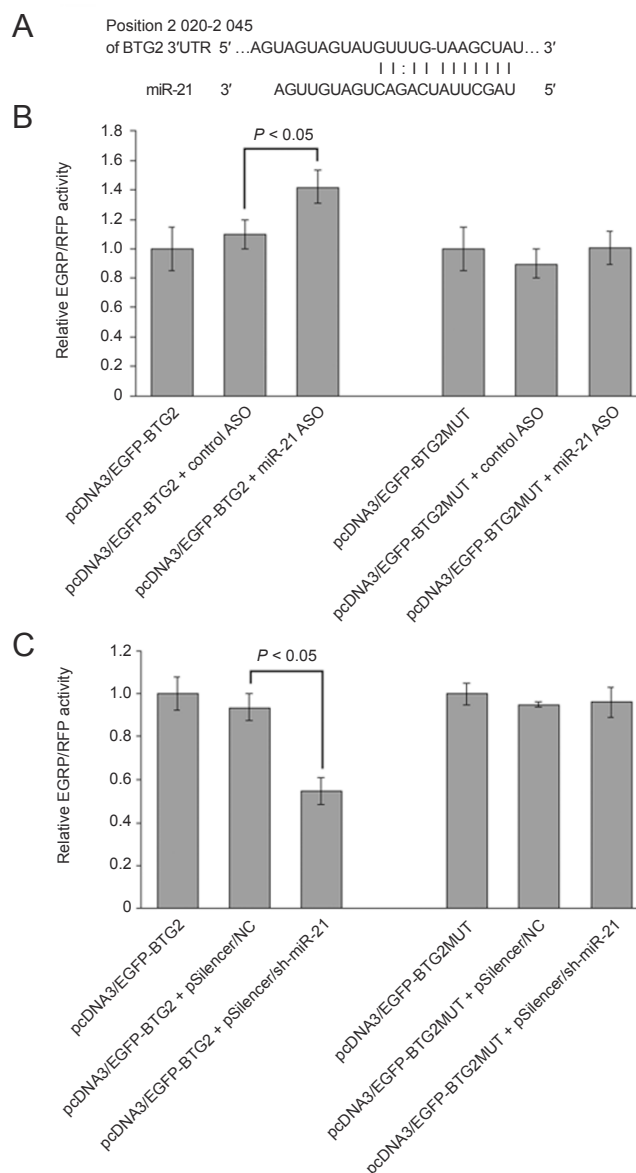


Figure 4 Direct effect of *miR-21* on the 3'UTR of *BTG2*. **(A)** A complementary sequence of *BTG2* to *miR-21* was obtained from publicly available algorithms. **(B)** HEP-2 cells were co-transfected with an EGFP reporter plasmid (pcDNA3/EGFP-*BTG2* or pcDNA3/EGFP-*BTG2*MUT) and a pDsRed2-N1 plasmid, either alone or together with oligonucleotides. The fluorescence value in the control group was set to 1. **(C)** HEP-2 cells were co-transfected with EGFP reporter plasmid and pDsRed2-N1 plasmid, either alone or together with a miRNA-expressing vector. At 48 h after transfection, EGFP and RFP activities were measured with an F-4500 fluorescence spectrophotometer. The fluorescence value in the control group was set to 1. All the histograms show normalized mean values \pm SD of fluorescence intensity from three independent experiments.

proliferation *in vitro* and *in vivo*, whereas overexpression of *miR-21* elevated the proliferation potential. The

growth suppression by *miR-21* inhibition was found to be due to the loss of control of the G1-S phase transition, and not due to an increase in cell apoptosis, as shown by flow cytometry and cell apoptosis assay.

It is now well known that miRNAs regulate a variety of cellular activities through their effect on the expression of multiple target genes [14]. Identification of the target genes of miRNAs may help to characterize the diverse functions of miRNAs. Recent reports have found that *PTEN*, *TPM1* and *PDCD4* are target genes of *miR-21* [15-18]. However, given that a single miRNA has multiple targets, we believe that *miR-21* also has other targets. Target prediction and validation are still challenges in miRNA research. Since the regulation of gene expression at the mRNA level may be a common mechanism for miRNA function, it is convenient to monitor transcriptional changes using a high-throughput approach such as a microarray [19]. Combining target prediction and expression profiling has been shown to be effective [20]. According to the results of the gene expression array from the three paired normal and cancer tissues, 29 genes were upregulated and 61 genes were downregulated in laryngeal carcinoma. Furthermore, the previously confirmed targets of *miR-21*, including *PTEN*, *TPM1* and *PDCD4*, also showed a downregulation trend in at least one of the cDNA microarrays, demonstrating that microarray analysis is a valid method to help find miRNA targets. As a new candidate target gene of *miR-21*, *BTG2* is downregulated in laryngeal carcinoma, and meanwhile, it is predicted to be a target gene of *miR-21* according to publicly available databases.

Several results indicate that *BTG2* is a target of *miR-21*. First, the 3'UTR of *BTG2* contains a sequence matching the *miR-21* seed region. Second, suppression of *miR-21* caused enhanced expression of a *BTG2* 3'UTR-containing fluorescent reporter in HEp2 cells, whereas *miR-21* overexpression reduced the EGFP level. Third, a site mutation in the *BTG2* 3'UTR complementary to *miR-21* abolished *miR-21* regulation. Finally, the mRNA and protein levels of *BTG2* in HEp-2 cells were specifically correlated with the expression level of *miR-21*. An interesting fact is that the extent of change of *BTG2* protein is more obvious than that of *BTG2* mRNA. A possible explanation is that aside from mRNA degradation, the transcriptional repression mechanism also contributes to the regulation of *BTG2* by *miR-21*. Recently, using array expression analysis, Frankel *et al.* [17] also found upregulation of *BTG2* in MCF-7 cells depleted of *miR-21*. In short, *miR-21* regulates *BTG2* expression directly by binding to its 3'UTR.

The *BTG2* belongs to an anti-proliferative gene family, which is highly conserved among various species.

Many potential roles of *BTG2* have been suggested, such as a transcriptional co-regulator, a differentiation and anti-apoptotic factor in neurogenesis, a key mediator of the stage-specific expansion of thymocytes, a negative regulator of hematopoietic progenitor expansion, and a tumor suppressor in both mice and humans [21]. It has been found that the expression of *BTG2* is constitutive and high in the thymus, lung alveolar epithelium, proximal tubule of the kidney and basal cell layer of prostate acini, whereas the mRNA level of *BTG2* is usually low in their corresponding tumors [21-25]. Our research also validated the downregulation of *BTG2* in laryngeal carcinomas by microarray and semi-quantitative RT-PCR. The loss of *BTG2* expression may play an important role in the carcinogenic process. Aberrant expression of *miR-21* may account for the repression of *BTG2* levels in laryngeal carcinoma, although other mechanisms cannot be excluded.

In addition, *BTG2*, as a pan-cell cycle regulator, induces G1-S arrest via *pRB*-dependent and *pRB*-independent pathways [26, 27]. The former is carried out through the inhibition of cyclin D1 expression, and the latter by delayed biosynthesis of cyclin E and CDK4 proteins [21, 26, 27]. Thus, the identification of *BTG2* as a *miR-21* target gene provides a possible explanation as to why the suppression of *miR-21* can inhibit the growth of tumor cells through influencing their cell cycles. As low *pRB* expression is frequently found in laryngeal carcinoma, suppression of *miR-21* may induce G1-S arrest, mainly through a *pRB*-independent pathway, by upregulating the levels of *BTG2* [27, 28]. However, further study is necessary to gain a full understanding of the underlying molecular mechanism.

Recently, aberrant expression of *miR-21* has been found to contribute to the malignant phenotype by blocking the expression of critical apoptosis-related genes [10, 11]. Our research indicates that *miR-21* suppression inhibits the growth of HEp2 cells without affecting apoptosis. Thus, loss of cell cycle control may be the potential mechanism of *miR-21*-mediated tumorigenesis in laryngeal carcinoma. Emerging evidence suggests that some miRNA activities influence cell cycle progression [5-7, 29]. Alteration of miRNA levels can thus contribute to pathological phenotypes by regulating cell cycle-related genes.

In conclusion, *miR-21* functions as an oncomir and is overexpressed in laryngeal carcinoma tissues compared to their adjacent normal tissues. Suppression of *miR-21* represses cell growth, possibly through inhibition of the cell cycle by targeting *BTG2*, but not through a marked increase in apoptosis. Thus, the identification of the oncogene, *miR-21*, and its target gene, *BTG2*, in laryngeal

carcinoma may help us to understand the molecular mechanism of tumorigenesis, and may have diagnostic and therapeutic values in the future.

Materials and Methods

Laryngeal carcinoma specimens, cell lines and isolation of RNAs

Laryngeal carcinoma specimens were collected from the Tianjin First Center Hospital. The paired fresh samples from tumor tissues and adjacent normal tissues were obtained from patients with sporadic laryngeal carcinoma undergoing surgical resection. The samples were snap-frozen in liquid nitrogen and stored at -80°C . The laryngeal carcinoma cell line, HEP-2, was cultured at 37°C with 5% CO_2 in RPMI-1640 media supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Total RNA and enriched small RNAs were obtained from the tissues using the *mirVana* miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. Total RNA was extracted from HEP-2 cells using the TRIzol reagent (Invitrogen, CA, USA).

MiRNA microarray analysis

A total of 210 DNA oligonucleotide probes from the *mirVana* miRNA Probe Set (Ambion) were designed according to the sequence of their respective mature miRNAs. Probes were resuspended at 50 mM in $3\times$ saline sodium citrate (SSC) and spotted on MICROMAX Glass Slides SuperChip I (PerkinElmer, MA, USA) in duplicates at 50–60% humidity by the SpotArray 24 Microarray Printing System (PerkinElmer). Small RNAs were labeled with Cy5 or Cy3 dyes (Amersham Biosciences, Piscataway, NJ, USA) using the *mirVana* miRNA Labeling Kit (Ambion). After hybridization at 42°C overnight (12–16 h), the slides were washed in SSC, and scanned with a ScanArray Express Microarray Scanner using ScanArray 3.0 software (PerkinElmer).

cDNA microarray analysis

The mRNAs from the three paired tumor and normal tissues were reverse transcribed using the MICROMAX TSA Labeling and Detection Kit (PerkinElmer), and the resulting cDNAs were labeled with fluorescein or biotin. Labeled cDNAs were purified and hybridized to DNA arrays containing 7 267 human genes relating to human cancer, apoptosis, signal transduction and liver enzymes (Qiagen Inc., CA, USA). Data normalization and calculation of expression values were performed using the ScanArray Express Microarray Analysis System (PerkinElmer).

Vector construction

The *miR-21*-expressing vector (pSilencer/sh-*miR-21*) was constructed according to the manufacturer's instructions for pSilencer 2.1 neo (Ambion). The plasmid expresses a miRNA hairpin that can be processed by the cellular machinery into mature *miR-21*. The negative control pSilencer 2.1 neo vector (pSilencer/NC) expresses a hairpin siRNA with limited homology to all known sequences in the human genome.

To construct the *BTG2* expression vector, the fragment of *BTG2* (GenBank accession No. NM_006763) coding region was amplified by RT-PCR from the cDNA library of normal laryngeal tissues

using primers *BTG2* sense, 5'-CGG AAT TCC GCG ACA TGA GCC ACG GGA AG-3'; and *BTG2* antisense, 5'-GCA GCT CGA GGC CTA GCT GGA GAC TGC CAT C-3'. The amplified fragment was cloned into pcDNA3 at *EcoRI* and *XhoI* sites to form the pcDNA3/*BTG2* vector.

To construct the fluorescence reporter plasmids, the EGFP gene from the pEGFP-N2 vector (Clontech, Mountain View, CA, USA) was subcloned into pcDNA3 and this is referred to as pcDNA3/EGFP. The pcDNA3/EGFP-*BTG2* reporter plasmid was constructed as follows: the 3'UTR sequence of *BTG2* was obtained by RT-PCR from cDNA library of normal laryngeal tissues using specific primers (*BTG2* UTR forward: 5'-CTA GGA TCC AGC CTC ATG GTC TC-3'; and *BTG2* UTR reverse: 5'-GGC GAA TTC AGT AAC CTT TGT CTT-3') containing the *EcoRI* and *BamHI* endonuclease recognition sites. This fragment was cloned into the pcDNA3/EGFP vector at the *EcoRI* and *BamHI* sites, downstream of the EGFP gene. We also constructed pcDNA3/EGFP-*BTG2MUT*, a plasmid with a triple point mutation (TAAGCTA was mutated to TTACGTA) in the seed sequence of pcDNA3/EGFP-*BTG2*.

Transfection

The HEP-2 cells were transfected with oligonucleotides or plasmids using Lipofectamine 2000 reagent (Invitrogen) at 24 h after plating. Transfection complexes were prepared according to the manufacturer's instructions. The final oligonucleotide concentration was 10 nM, and the final plasmid concentration was 0.5 $\mu\text{g/l}$. The transfection medium was replaced at 4-h post-transfection. The oligonucleotides complementary to miRNAs were synthesized by IDT (Coralville, IA, USA) and their sequences were as follows: *miR-21* antisense oligonucleotide (*miR-21* ASO): 5'-TCA ACA TCA GTC TGA TAA GCT A-3'; and negative control oligonucleotide (control ASO): 5'-GTG GAT ATT GTT GCC ATC A-3'.

Cell proliferation assay

After transfection, the HEP-2 cells were seeded in 96-well plates at 5 000 cells per well. The MTT assay was used to determine relative cell viability at 24 h, 48 h and at 96 h. 10 μl of MTT solution was added to 100 μl of culture media and incubated for 4 h at 37°C , and the optical density was measured at 570 nm. Each experiment was performed in triplicates.

Soft agar colony formation assay

Anchorage-independent growth was determined by soft agar analysis as follows. 48 h after transfection, the HEP-2 cells (500 cells per well) were suspended in soft agar medium (RPMI-1640 media containing 0.3% agar) and seeded onto a base of RPMI-1640 media containing 0.5% agar in six-well plates. Plates were incubated at 37°C , 5% CO_2 in a humidified incubator for 2 weeks. Colonies were counted under a microscopic field at $\times 100$ magnification. Each assay was performed in triplicates.

Flow cytometry analysis

At 48 h after transfection with *miR-21* ASO or control ASO, the HEP-2 cells were detached from the plates by trypsin incubation, rinsed with PBS and fixed in 70% (v/v) ethanol. The cells were then rehydrated in PBS and incubated with RNase (100 $\mu\text{g/ml}$) and propidium iodide (60 $\mu\text{g/ml}$) (Sigma-Aldrich, MO, USA). Cells were analyzed using the FACSCalibur System (BD Biosciences, San Jose, CA, USA), and the cell cycle phase was determined by

Cell Quest analysis. The proliferation index (PI) was calculated as follows: $PI = (S+G2/M)/G1$ (S, G2/M and G1 refer to the percentage of cells in S phase, G2/M phase and G1 phase, respectively.) [30].

Cell apoptosis analysis

HEp-2 cells were transfected with *miR-21* ASO or control ASO, and seeded into triplicate wells on a 14-well slide (Cell-Line/Erie Scientific Co., NH, USA) at 48-h post-transfection. HEp-2 cells cultured in complete medium, including 0.09 mg/ml teniposide (VM-26), an apoptosis inducer, were taken as positive control. HEp-2 cells cultured only in complete medium were taken as negative control. Cell apoptosis was detected by the *In situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, IN, USA) according to the manufacturer's instructions. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology.

In vivo assay

Female BALB/c athymic nude mice (6-week-old) (Institute of Zoology, Chinese Academy of Sciences) were used for *in vivo* study. A total of 1×10^6 HEp-2 cells transfected with *miR-21* ASO or control ASO, as well as the non-transfected HEp-2 cells, were implanted subcutaneously in the right armpit of nude mice. Each group consisted of six mice. Tumor volumes were measured from the first 6 days, and once every other day. Tumor volume was calculated from $(\text{length} \times \text{width}^2)/2$ and presented as mean \pm standard deviation (SD) mm^3 [31]. Statistical analysis was performed using the Student's *t*-test to compare the tumor volume between different treatment groups.

Semi-quantitative RT-PCR

Total RNAs were extracted from transfected HEp-2 cells for semi-quantitative RT-PCR (sqRT-PCR) at 48 h post-transfection. Target genes and the endogenous control gene, β -actin, were included in the same reaction. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transilluminator. Bands were quantified with Labworks 4.0 software.

Western blot

Total protein from HEp-2 cells transfected with either oligonucleotides or plasmids was extracted using RIPA buffer (1 mM MgCl_2 , 10mM Tris-HCl pH 7.4, 1% Triton X-100, 0.1% SDS, 1%NP-40), and protein expression was analyzed by western blot. β -Tubulin was served as a loading control. Total protein extracts were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. The level of *BTG2* expression was evaluated using the rabbit polyclonal anti-BTG2 antibody (Saier Biotech, Tianjin, China), which is immunogen affinity purified. As a loading control, the β -tubulin expression level was measured using rabbit polyclonal anti- β -tubulin antibody (Sigma-Aldrich, MO, USA). Bands were quantified with Labworks 4.0 software.

EGFP reporter assay

The HEp-2 cells were seeded in 24-well plates and cotransfected with 700 ng of the fluorescence reporter plasmid, 300 ng of the pDsRed2-N1 plasmid (as a control for transfection efficiency), and 100 pmol of oligonucleotides or 500 ng of the miRNA-expressing

construct using Lipofectamine 2000 reagent (Invitrogen). At 48 h after transfection, EGFP and RFP (red fluorescent protein) activities were measured with the F-4500 fluorescence spectrophotometer (HITACHI, Japan). The EGFP value was normalized by the RFP value.

Statistical analysis

Most *in vitro* experiments were performed thrice. Where appropriate, the results are expressed as mean \pm standard deviation (SD) and statistically analyzed using the Student's *t*-test for the comparison of two groups, with $P < 0.05$ considered to be significant.

Conflict of Interest

The authors declare no competing financial interests.

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