Promoter mutation of tumor suppressor microRNA-7 is associated with poor prognosis of lung cancer

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Abstract. The significance of promoter mutations of microRNAs (miRNAs) in lung cancer is poorly understood. Recent evidence demonstrated that miRNA-7 (miR-7), a unique member of the miRNA family, exhibited decreased expression and has emerged as an important regulator in lung tumorigenesis. However, the mechanism underlying the down-regulation of miR-7 in lung cancer remains largely unknown. In this study, we investigated the sites of mutation of the miR-7 promoter in lung cancer tissues using DNA sequencing. We identified a G→C change at the -617 site (25/39, 64.1%) and an A→G change at the -604 site (20/39, 51.3%) in the miR-7 promoter region in lung cancer tissues. Moreover, the expression of miR-7 in cancer tissue with promoter site mutations was lower compared with that in cancer tissue without mutations (P<0.05). Furthermore, we demonstrated that mutations at these sites may decrease the activity of the miR-7 promoter and alter the expression of miR-7. Notably, mutations at these sites of the miR-7 promoter were found to be closely associated with poor prognosis of lung cancer patients (P=0.037). These data may provide novel insight on the altered expression of specific miRNA molecules in lung cancer and ultimately prove to be helpful in the development of prognostic and therapeutic strategies against lung cancer.

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Abbreviations: miRNA, microRNA; miR-7, miRNA-7; HuR, human R antigen; Mu, mutation; WT, wild-type

Key words: promoter mutation, microRNA-7, lung cancer, prognosis

Introduction

Lung cancer is one of the most common causes of cancer-related mortality. To date, multiple genes have been found to exhibit altered expression in the pathogenesis of lung cancer (1,2). Furthermore, the altered expression of these genes was found to be closely associated with the mutation or methylation of promoter sites of these genes (3,4). The overall 5-year survival rate of lung cancer patients receiving traditional treatment remains poor (5). Thus, it is crucial to identify and characterize novel molecular markers and gene targets, as well as investigate the mechanism underlying their altered expression, in order to improve the accuracy of prognosis and develop optimal targeted treatment strategies to improve the clinical outcome of lung cancer patients.

Recently, a growing body of literature has demonstrated that microRNAs (miRNAs), a class of non-coding RNA molecules that regulate the expression of protein-coding genes through binding to the 3'-untranslated regions of target mRNAs, have emerged as important regulators in the development of lung cancer and a novel potential target for the development of therapeutic strategies for lung cancer patients (6,7). Various specific miRNA molecules were reported to exhibit repressed expression in lung cancer tissues and to be closely associated with patient prognosis (8,9). However, the mechanism underlying the altered expression of these specific miRNA molecules in lung cancer, including mutation or methylation of their promoter regions, remains largely unknown.

miRNA (miR)-7, a distinct member of the miRNA family, was reported to be able to regulate the biology of various tumor cells through repressing the expression of different target molecules (10-13). miR-7 expression was found to be repressed in lung cancer tissues (7,14). Moreover, miR-7 was able to suppress the growth and metastatic potential of human lung cancer cells *in vitro* (15). Our previous studies also demonstrated that overexpression of miR-7 reduced the growth and metastasis of human lung cancer cells *in vivo* (16,17). These findings suggested that miR-7, as a tumor suppressor, may be

a potential candidate as a prognostic marker and a therapeutic target in lung cancer. However, the presence of mutations in the miR-7 promoter and their possible association with the prognosis of lung cancer remain to be elucidated.

The aim of this study was to investigate the presence of promoter mutations of miR-7 in lung cancer and their possible association with the prognosis of the patients.

Patients and methods

Patients and tissue samples. A total of 39 Chinese patients who were diagnosed with lung cancer between 2007 and 2008 were included in the present study. Clinical and pathological information, including age, gender, smoking status, type of tumor and disease stage, were collected. Paraffin blocks and fresh-frozen tumor specimens of tumor samples from all 39 patients were prepared. In addition, 8 samples from normal tissues adjacent to the tumors were also collected. All the patients were followed up until December, 2012. This study was approved by the Ethics Committee of the First Hospital of Zunyi Medical College (Guizhou, China) and written informed consent was obtained from all the participants.

Sample preparation. Tissue sections (4-5 μm) were cut from the paraffin blocks for pathological analysis. Total RNA was extracted from the fresh-frozen tumor specimens for the detection of miR-7 expression in lung cancer or normal lung tissues. Genomic DNA was isolated from the frozen specimens using a NucleoSpin Tissue kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The DNA samples were frozen at -70˚C until use.

Amplification of the 5'-flanking region of the human miR-7 gene by polymerase chain reaction (PCR) analysis. PCR primer sets were designed to amplify a 1.3-kb product containing -1068 and +234 sites from the 5'-flanking region of the human miR-7-2 gene. The sequences of the primer sets were as follows: Sense (-1068 to -1051): 5'-AGCACCAATAGG GAAGGG-3'; and antisense (+217 to +234): 5'-GAGTCTGCC GATGGGTGT-3'. PCR was performed in 50 μl of reaction mixture containing 20 mmol/l Tris-HCl, pH 8.8, 2 mmol/l $MgSO_4$, 10 mmol/l KCl, 10 mmol/l $(NH_4)_2SO_4$, 0.1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin, 10 mmol/l each of dATP, dCTP, dGTP and dTTP,, 0.2 pM of each primer, 6% dimethyl sulfoxide, 1 μg genomic DNA and 2.5 U *Pyrococcus furiosus* DNA polymerase. The thermal cycling settings for PCR included a 5-min initial denaturation at 95˚C followed by 35 amplification cycles (denaturation for 1 min at 94˚C, annealing for 1 min at 58˚C and extension for 1.5 min at 72˚C, with a final extension step at 72˚C for 10 min). The PCR products were analyzed on a 1% agarose gel in Tris-acetate buffer with ethidium bromide staining. The PCR products were then purified from the agarose gels using the GeneClean kit (Bio101 Inc., Vista, CA, USA) according to the manufacturer's recommendations.

Sequence analysis. To investigate mutations in the promoter region of the human miR-7 gene, the above products of PCR templates were prepared and the nucleotide sequence was determined on both strands by Sanger's dideoxynucleotide chain-termination method with Sequenase 2.0 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Multiple overlapping fragments were sequenced at least twice in each direction and the DNA sequence was analyzed using MacVector software (Eastman Kodak Co., Rochester, NY, USA). DNA sequence analysis was performed by a manual method (Thermo Sequenase Cycle Sequencing kit; Amersham Pharmacia Biotech) and an automatic sequence method (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Construction of the eukaryotic vector. PCR primer sets were designed to amplify a 1.3-kb product containing -1068 and +234 sites from the 5'-flanking region of the human miR-7-2 gene. The sequences of the primer sets were as follows: Sense (-1068 to -1051): 5'-CTAGCTAGCTAGAGCACCAATAGG GAAGGG-3'; and antisense (+217 to +234): 5'-GAAGATCTT CGAGTCTGCCGATGGGTGT-3'. The PCR products were amplified from DNA derived from lung cancer tissues with or without miR-7 promoter mutations, then subcloned into *Nhe*I and *Bgl*II sites of the pGL3.0 basic vector (Invitrogen Corporation, San Diego, CA, USA) to generate the pGL3.0-miR-7 expression plasmid [referred to as p-wild-type (WT)-miR-7 or p-mutation (Mu)-miR-7, respectively]. For the construction of plasmids pGL-miR-7 promoter Luc, the promoter region (-1068 to 0 bp) of miR-7 was amplified from DNA derived from lung cancer tissues with or without miR-7 promoter mutations using a forward primer (5'-CTAGCTAGC TAGAGCACCAATAGGGAAGGG) and a reverse primer (5'-GAAGATCTTCGCCAGTTCTGCAAGGCGT) and subcloned into *Nhe*I and *Bgl*II sites of the pGL basic vector (referred to as p-WT-promoter or p-Mu-promoter, respectively). Clone identity was verified using restriction digest analysis and plasmid DNA sequencing. Endotoxin-free plasmids were obtained using the EndoFree Plasmid Mega kit (Qiagen, Hilden, Germany). The plasmids were then transiently transferred into the 95D human lung cancer cells using Lipofectamine®-2000 (Invitrogen Corporation) in the following experiments according to the manufacturer's instructions.

Luciferase reporter assay. The 95D cells were transiently co-transfected with the p-WT-promoter or p-Mu-promoter and pCMV-lacZ plasmids using Lipofectamine-2000 (Invitrogen Corporation) according to the manufacturer's instructions and cultured in 37˚C. After 24 h, luciferase and β-galactosidase (β-gal) activity in 100 μl of cell lysate were measured using the Luciferase Assay system and the β-Galactosidase Enzyme Assay system (Promega Corporation, Madison, WI, USA), respectively. Transfection efficiency was normalized using β-gal activity.

Quantitative PCR (qPCR) assay. All the reagents, primers and probes were obtained from Applied Biosystems. The relative expression of miR-7 was determined as previously described (17). Briefly, a β-actin endogenous control was used for normalization. Reverse transcription (RT) reactions and qPCR were performed according to the manufacturer's protocols (Applied Biosystems). RNA concentrations were determined with a NanoDrop instrument (NanoDrop

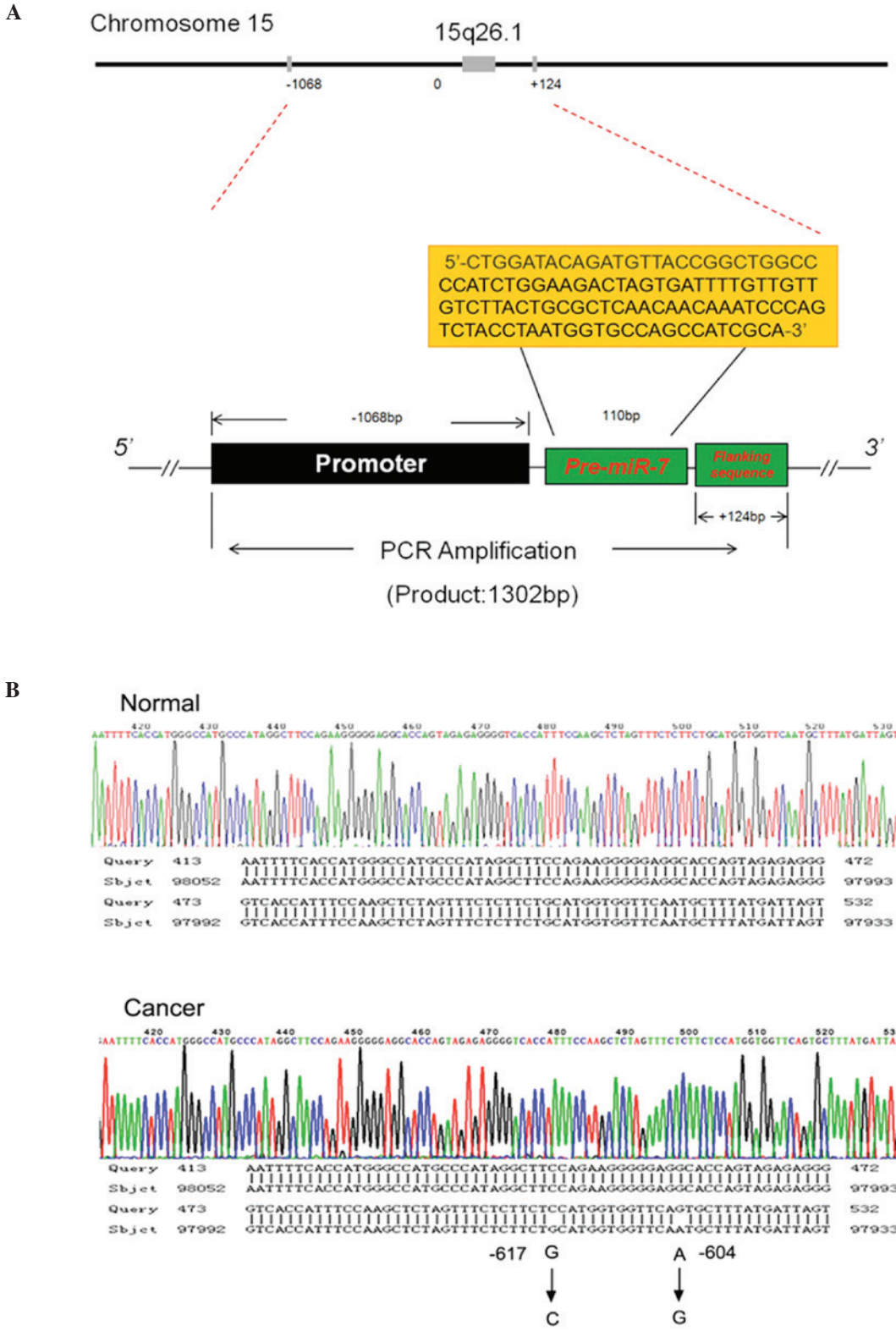

Figure 1. MicroRNA-7 (miR-7) promoter mutation in lung cancer. (A) Schematic representation of the human miR-7 gene and sequencing strategy for the 5'-flanking region of the gene. The coding sequences are depicted in the yellow box. The fragment containing the 5'-flanking region and 234 bp downstream from the initial site of the human pre-miR-7 gene was amplified by polymerase chain reaction (PCR) from lung cancer or normal lung tissues. (B) DNA sequence analysis of the PCR products from lung cancer or normal lung tissues. The wild-type sequence were isolated from normal lung tissues. Mutations in lung cancer tissues are identified at the indicated positions. G→C at -617 and A→G at -604.

Technologies, Wilmington, DE, USA). One nanogram of RNA per sample was used for the assays. All RT reactions, including no-template controls and RT minus controls, were run in triplicate in GeneAmp® PCR 9700 thermal cycler (Applied Biosystems). The gene expression levels were quantified using the ABI PRISM® 7900HT Sequence Detection system (Applied Biosystems). Relative expression was calculated using the comparative threshold cycle method.

Cell proliferation assays. 95D cells transiently transfected with 10 nmol p-WT-miR-7 or p-Mu-miR-7 vector using Lipofectamine-2000 (Invitrogen Corporation) were seeded at $3x10^3$ cells per well and incubated at 37˚C in 5% CO_2 in 96-well plates for 72 h. Cell proliferation was measured in terms of optical absorbance per well by a semi-automated tetrazolium-based colorimetric assay using MTT.

Statistical analysis. Statistical evaluation was performed using one-way analysis of variance (P<0.05). All comparisons between categorical variables were performed by the Fisher's exact Chi-square test. Relapse-free survival was calculated using the Kaplan-Meier survival estimates and the log-rank test from the date of diagnosis until the last contact or relapse. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were conducted using SPSS 13.0 software for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Promoter mutation of miR-7 in lung cancer. To elucidate the mechanism underlying the reduced expression of human miR-7 in lung cancer, we first searched for the putative promoter region in the 5'-flanking region, which may alter miR-7 gene transcription according to a previous report (13). Sequence analysis revealed putative transcription factor binding sites for SRY, c-Myc, Gfi-1 and CdxA in the 5'-flanking region, suggesting that the expression of the human miR-7 gene may be controlled by these regulatory elements (data not shown). Primer sets were used to amplify overlapping fragments from the 5'-flanking region from -1068 bp upstream to +234 bp downstream from the initiation site of pre-miR-7 (Fig. 1A) from the genomic DNA. The sizes of PCR products amplified from the genomic DNA of lung cancer and normal lung tissues were identical and corresponded to the expected sizes, indicating that there were no major additions or deletions in the 5'-flanking region of the human miR-7 gene in the lung cancer tissues examined. Subsequently, the PCR products were sequenced to assess the promoter mutation of miR-7 of lung cancer tissues from 39 lung cancer patients and normal lung tissues from 8 healthy donors, respectively. Detailed DNA sequence analysis identified two mutations in the PCR region in lung cancer tissues, namely a G→C change at -617 and a A→G change at -604 (Fig. 1B). Moreover, as shown in Table I, the promoter mutation at the -617 site of miR-7 was detected in 25 patients (64.1%) and at the -604 site in 20 patients (51.3%). All A→G mutations were found in combination with the G→C mutation; the A→G mutation alone was not detected in any patients (0%), whereas the G→C mutation alone was detected in 5 patients (12.8%). However, promoter mutations were not detected in normal lung tissues (0%). We then investigated the association between mutation sites of the miR-7 promoter and a significant correlation was observed between the G→C and A→G sites (P<0.05).

The mutation sites alter the activity of the miR-7 promoter. To determine whether the mutations affected the activity of the miR-7 promoter, PCR-amplified promoter fragments (-1068 to 0), with or without the two mutations, were obtained from lung cancer and normal lung tissues and subcloned into the pGL3-basic vector (referred to as the p-Mu promoter and the p-WT promoter, respectively). The promoter activities were then evaluated by firefly luciferase reporter gene expression normalized to a co-transfected pCMV-lacZ as a control for transfection efficiency. The normalized luciferase reporter activities indicated that the mutations in the promoter significantly reduced promoter activity (Fig. 2, P<0.05). Compared with normal promoter activity, the mutated promoter activity decreased by >50% in the pGL3 constructs. These data suggested that the mutation sites reduced the activity of the miR-7 promoter.

Table I. Mutation in the promoter region of the human microRNA-7 gene in lung cancer (n=39).

Mutation	Position	No. (%)
G→C	-617	25 (64.1)
A→G	-604	20 (51.3)
G→C and A→G	-617 and -604	25 (64.1)
No mutation		14 (35.9)

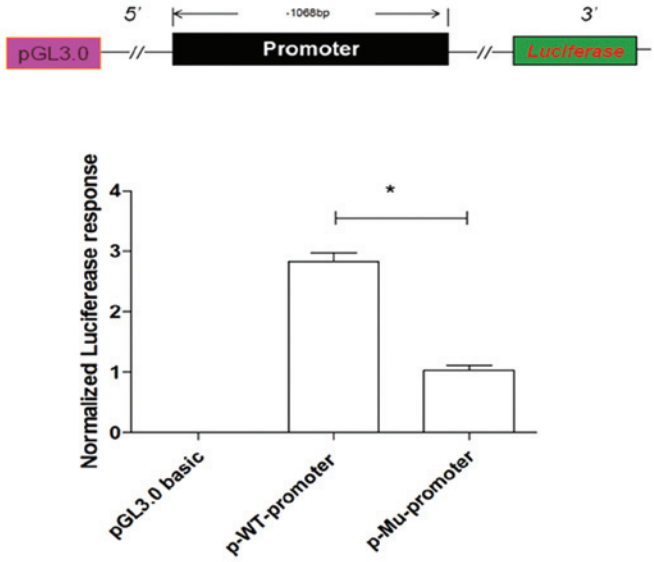

Figure 2. The mutation sites altered the activity of the microRNA-7 (miR-7) promoter. The 95D human lung cancer cells were transfected with plasmids cloned in a pGL3 basic vector containing the human miR-7 promoter [wild-type (WT), or mutation (Mu)] and the firefly luciferase reporter gene. The activities were normalized by a co-transfected pCMV-lacZ to correct for variations in transfection efficiencies. Representative data from one of three independent experiments is shown. *P<0.05.

Promoter mutation reduces miR-7 expression in lung cancer cells. To further determine whether these mutations affected the activity of the miR-7 promoter, subsequently altering the expression of miR-7, the fragment containing the promoter region and the pre-miR-7 region (-1068 to +234), with or without the two mutations, from lung cancer and normal lung tissues, was further amplified and subcloned into the pGL3-basic vector to construct miR-7 expression vectors

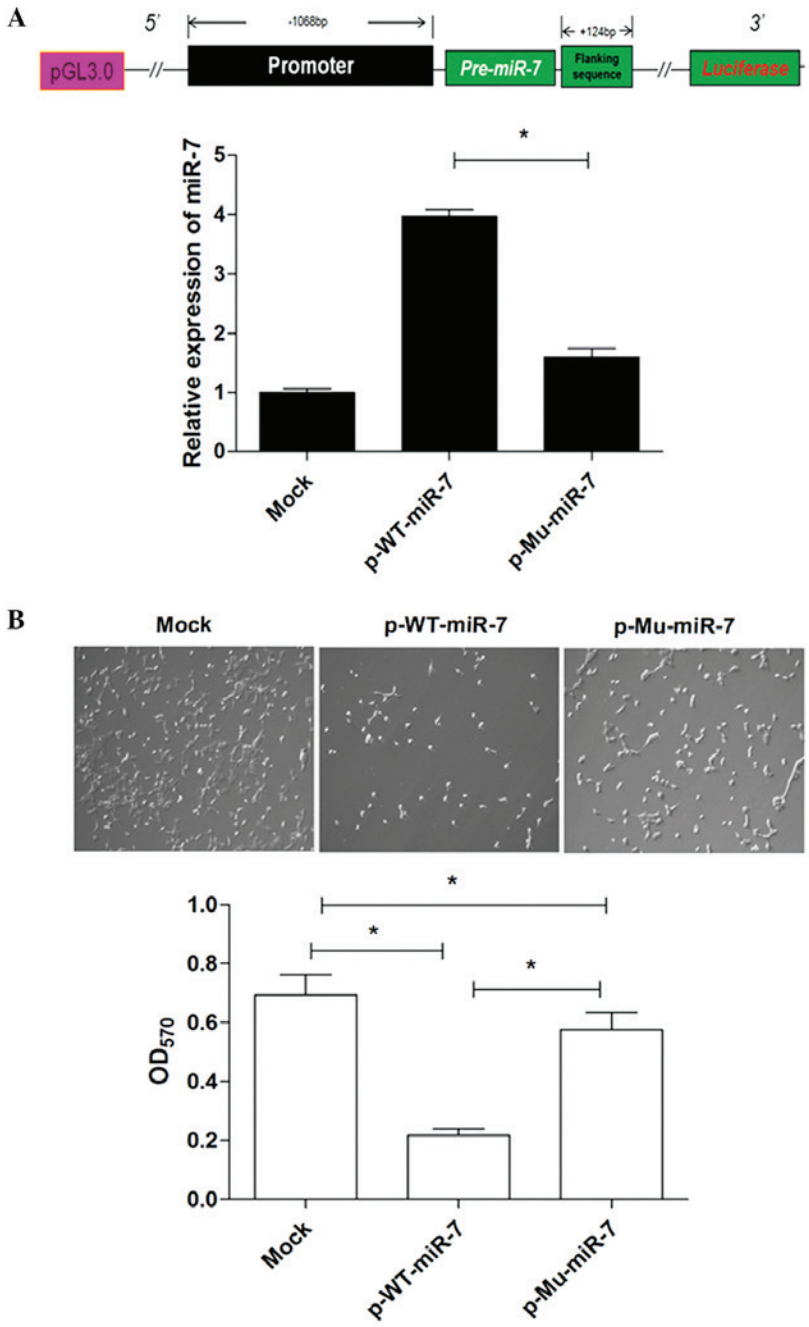

Figure 3. The mutation sites reduced the expression of microRNA-7 (miR-7) in lung cancer cells. (A) 95D cells were transfected with plasmids cloned in a pGL3 basic vector containing the human miR-7 promoter [wild-type (WT), or mutation (Mu)] and pre-miR-7. The relative expression of miR-7 was analyzed by quantitative polymerase chain reaction analysis. (B) The proliferation of 95D cells was also determined by the MTT assay. Magnification, x100. Representative data from one of three independent experiments is shown. *P<0.05. OD, optical density.

(referred to as p-Mu-miR-7 and p-WT-miR-7, respectively). Human lung cancer cells were transiently transfected with these constructed plasmids. The relative expression level of miR-7 was then determined by qPCR analysis. The data revealed that the expression level of miR-7 was significantly decreased in the p-Mu-miR-7 vector transfection group compared with that in the p-WT-miR-7 vector transfection group (Fig. 3A, P<0.05), indicating that the mutation sites reduced the expression of miR-7. Consistently, the growth of 95D cells in the p-Mu-miR-7 vector transfection group was higher compared with that in the p-WT-miR-7 vector transfection group (Fig. 3B, P<0.05), which was consistent with our previous findings (17). Combining these data demonstrated that the mutation sites significantly reduced the activity of the miR-7 promoter and altered the expression of miR-7, subsequently affecting its biological activity.

Promoter mutation is associated with repressed expression of miR-7 in lung cancer tissues. We next sought to determine whether the mutation of the miR-7 promoter was associated with the expression of miR-7 in lung cancer tissues. The relative expression of miR-7 was also assessed by qPCR in

Table II. Association of miR-7 promoter mutations in cancer tissue with the clinicopathological characteristics of lung cancer patients (n=39).

Clinicopathological characteristics	Promoter mutation status, patient no. (%)		P-value
	Positive	Negative	
Age, years			0.396
<60 (n=15)	10 (66.7)	5 (33.3)	
>60 (n=24)	15 (62.5)	9 (37.5)	
Gender			0.635
Male (n=33)	21 (63.6)	12 (36.4)	
Female (n=6)	4 (66.7)	2 (33.3)	
Smoking status			0.493
Ever (n=32)	21 (65.6)	11 (34.4)	
Never (n=7)	4 (57.1)	3 (42.9)	
Histologic types			0.357
SCC (n=13)	7 (53.8)	6 (46.2)	
ADC (n=17)	13 (76.5)	4 (23.3)	
LCC (n=9)	5 (55.6)	4 (44.4)	
Pathological stage			0.020
I (n=7)	2 (28.6)	5 (71.4)	
II-IV (n=32)	25 (78.1)	7 (21.9)	

miR-7, microRNA-7; SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large-cell carcinoma.

39 lung cancer and 8 normal lung tissue specimens. Consistent with previous findings (14), the relative expression of miR-7 decreased significantly in lung cancer tissues compared with that in normal lung tissues (data not shown). Notably, we found that the expression level of miR-7 in lung cancer tissues with mutation sites was lower compared with that in lung cancer tissues without mutation sites (Fig. 4, P<0.05), indicating that the promoter mutation is closely associated with the repressed expression of miR-7 in lung cancer tissues.

Promoter mutation of miR-7 is associated with poor survival of lung cancer patients. Finally, the possible value of miR-7 promoter mutations in the prognosis of lung cancer patients was analyzed. There was no association between mutation and age at diagnosis, gender, or smoking status (Table II). miR-7 promoter mutations were more common in adenocarcinoma (ADC) (13/17, 76.5%) compared with squamous cell carcinoma (7/13, 53.8%) and large-cell carcinoma (5/9, 55.6%), although the difference was not statistically significant (P=0.357). However, a statistically significant association was observed between the mutations and stage II-IV disease according to the American Joint Committee on Cancer (AJCC), with miR-7 promoter mutations being present at a higher frequency in stage II-IV (25/32, 78.1%) compared with stage I disease (2/7, 40.0%) (P=0.020). Finally, the Kaplan-Meier long-rank analysis revealed that the presence of promoter mutation of miR-7 was associated with poorer overall survival (Fig. 5, P=0.037).

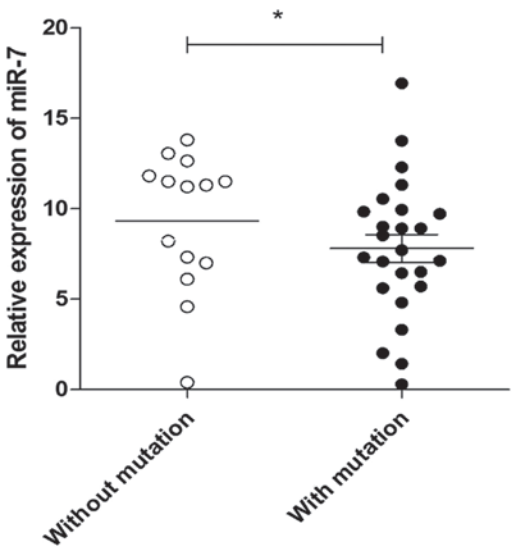

Figure 4. The mutation sites altered the expression of microRNA-7 (miR-7) in lung cancer cells. The relative expression of miR-7 in lung cancer tissues with mutation (n=25) or without mutation (n=14) was determined by quantitative polymerase chain reaction analysis. *P<0.05.

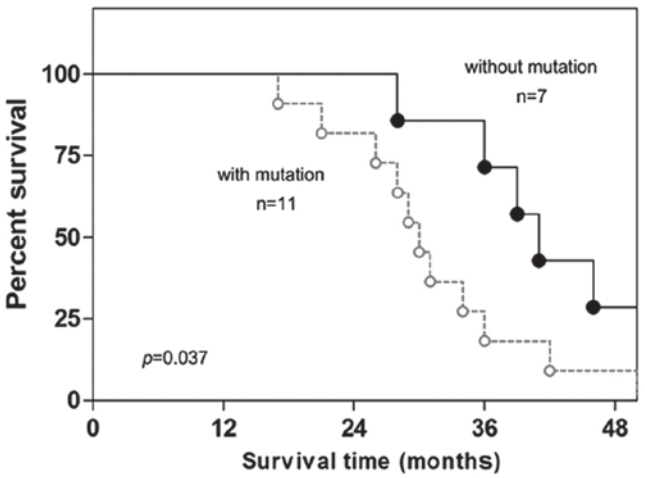

Figure 5. Promoter mutation of microRNA-7 (miR-7) was associated with poor survival of lung cancer patients. A total of 18 lung cancer patients were divided into two groups by promoter mutation of miR-7 (with mutation, n=11; and without mutation, n=7). The overall survival rate was analyzed by the Kaplan-Meier method. The log-rank test was applied to compare survival between the groups.

Discussion

miR-7, a unique member of the miRNA family, has been proposed to be a new type of tumor suppressor gene, or oncomiRNA, in several tumor types (13,18-20). In lung cancer, recent evidence demonstrated that the expression of miR-7 is decreased in cancer tissues (14). Moreover, miR-7 was found to inhibit the growth and metastasis of lung cancer cells and induce their apoptosis (21). Mechanistic evidence demonstrated that miR-7 was able to regulate the transduction of the Akt pathway, which is crucial for the growth and metastasis of tumor cells (22,23). In line with these findings, our previous study also demonstrated that the overexpression of miR-7 inhibited the growth and metastasis of lung cancer cells (17).

Consistently, more recent evidence further demonstrated that restoration of miR-7 expression suppressed the tumorigenicity of lung cancer cells *in vivo* (24) and overexpression of miR-7 improved the sensitivity of lung cancer cells to paclitaxel (25). These data suggested that miR-7 may be an important regulator and have multiple functions in the development of lung cancer. Of note, a recent study reported that stable overexpression of miR-7 promoted the growth and migration of a lung cancer cell clone by regulating the expression of Kruppel-like factor 4 (26). Combing these data indicated that the precise role of miR-7 in the development of lung cancer is complex, and closely associated with expression level, cellular context and growth conditions, as well as the specific targeted genes. Therefore, investigating the regulation of expression of miR-7 in cancers may be of significant value. However, to date, the mechanism underlying the altered expression of miR-7 remains largely unknown.

In the present study, we identified two mutation sites in the miR-7 promoter in lung cancer. Moreover, there was a positive association between the -617 and -604 sites. Importantly, the expression level of miR-7 in lung cancer with mutations of the promoter sites was clearly decreased. Moreover, the transcriptional activity driven by the mutated promoter from lung cancer tissues was significantly reduced. These results suggested that the reduced miR-7 expression in lung cancer is, at least in part, due to a defect in the promoter of the gene. In addition, mutations were not detected in all cancer tissues; in fact, no mutation was found in ~35.9% of lung cancer patients. This finding suggests that other contributing factors are also involved in the altered expression of the miR-7 gene in lung cancer. Possible non-mutational causes for the reduced expression of miR-7 in lung cancer include the presence of transcription activators or repressors, as well as post-transcriptional factors. In fact, it was previously reported that c-Myc may bind to the promoter region of miR-7 and enhance miR-7 expression in lung cancer cells (13). Our recent findings further demonstrated that the human R antigen post-transcriptionally regulates the expression of miR-7 (17). In addition, recent evidence indicated that histone methylation or CpG methylation may affect the expression of distinct miRNA molecules in certain types of cancers (27,28). Therefore, the predominant mechanism involved in the repressed expression of the miR-7 gene remains to be fully elucidated in future studies.

Accumulating data suggests that the mutation or methylation of the promoter region of certain molecules, which was found to be associated with major prognostic factors, has emerged as a useful novel biological marker for the prognosis of cancer patients. Similarly, miR-7 was reported to be down-regulated in various cancers, including pancreatic and breast cancer, and was closely associated with the metastatic status of the patients (12,29). In the present study, we observed that the frequency of miR-7 promoter mutation was higher in the ADC type of lung cancer, although the difference was not statistically significant. Moreover, miR-7 promoter mutations were also associated with stage II-IV of lung cancer. Finally, we further demonstrated a significant correlation between miR-7 promoter mutation and poor prognosis of lung cancer patients, indicating that miR-7 promoter mutation is of significant prognostic value. Similarly, it has been reported that miR-7 exhibits high diagnostic accuracy and may be a helpful adjunct to thyroid fine-needle aspiration biopsy (30). In addition, we also noted that there was no significant association between miR-7 promoter mutation and other factors, such as smoking status. Therefore, a Cox regression analysis including other prognostic factors, such as tumor size, lymph node status and adjuvant therapy, which were not investigated in the present study, may be of value for validation of the significance of promoter mutation of miR-7 in the prognosis of lung cancer patients.

In conclusion, the present study demonstrated that there are mutation sites in the miR-7 promoter region in lung cancer tissues, which alter the expression of miR-7. Moreover, the mutation sites of the miR-7 promoter are closely associated with the poor prognosis of lung cancer patients. These data may provide a novel insight in the mechanism underlying the altered expression of distinct miRNA molecules in lung cancer, and may be helpful in the development of novel prognostic methods and therapeutic targets against lung cancer.

Acknowledgements

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