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Regulation of tumor suppressor gene FUS1 expression by the untranslated regions of mRNA in human lung cancer cells

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Abstract

FUS1, also known as tumor suppressor candidate 2 (TUSC2), is a tumor suppressor gene located in the human chromosome 3p21.3 region. FUS1 mRNA transcripts could be detected on Northern blots in both normal lung and some lung cancer cell lines, but no endogenous FUS1 protein could be detected in a majority of lung cancer cell lines and small cell and non-small cell lung tumor tissues. However, mechanisms regulating FUS1 protein expression and its inactivation in primary lung cancer cells are largely unknown. In this study, we investigated the role of the 5'- and 3'- untranslated regions (UTRs) of the FUS1 gene transcript in the regulation of FUS1 protein expression. We identified RNA sequence elements in FUS1 UTRs that regulate FUS1 protein expression. We found that two small upstream open-reading frames in the 5' UTR of FUS1 mRNA could inhibit the translational initiation of FUS1 protein by interfering with the "scanning" of the ribosome initiation complexes. Several secondary RNA structural elements/motifs on the 3'UTR of FUS1 also exhibited a significant inhibitory effect on FUS1 protein expression. The 3'UTR-mediated regulatory effect on FUS1 protein expression was also differentially detected in normal lung epithelial and fibroblast cells compared with lung cancer cells. Our results provide new insight into the molecular mechanisms involved in the regulation of FUS1 expression.

Keywords

FUS1/TUSC2; Tumor suppressor gene; Lung cancer; untranslated region (UTR); upstream open reading frame (uORF); expression regulation

1. Introduction

The *FUS1* gene is one of the candidate tumor suppressor genes identified in the 3p21.3 region [1–3]. Ectopic expression of the FUS1 protein by either adenoviral vector- or plasmid vector-mediated gene transfer in human non-small cell lung cancer (NSCLC) cells with aberrations at 3p21.3 region significantly inhibited tumor cell growth by inducing apoptosis [4;5]. Systemic administration of FUS1-expression plasmid DNA complexed with DOTAP (1,2-dioleoyl-3-trimethylammonium propane):cholesterol (DC) nanoparticles (DC-FUS1 nanoparticles) significantly suppressed tumor growth, inhibited lung metastases, and

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increased survival in mouse models bearing human lung cancer tumor xenografts [5;6]. Furthermore, anti-tumor efficacy was observed in a phase I clinical trial with systemic administration of DC-FUS1 nanoparticles in patients with disseminated lung cancer. These findings suggest a role for FUS1 as a tumor suppressor in human lung cancer.

FUS1 mRNA transcripts were detected on Northern blot analysis in both normal lung and some lung cancer cell lines, but no endogenous FUS1 protein was detected in most of the lung cancer cell lines and tumor tissue samples[4;7]. Loss or reduction of FUS1 expression was observed in almost all small cell lung cancers (SCLCs) and in more than 70 % of NSCLCs. Loss or reduction of FUS1 protein expression was associated with worse overall survival [4;7]. Both loss of function mutations in the FUS1 gene and epigenetic inactivation by hypermethylation in the FUS1 gene promoter and coding regions are rare events in human lung cancer and in other cancers [2;3;8]. Therefore, mechanisms other than genetic mutations and epigenetic silencing may work separately or collectively to regulate FUS1 gene and protein expression at transcription, post-transcription, translation, and post-translation levels in both normal and tumor cells. Particularly, the inactivation of FUS1 may occur through aberrant translational control of FUS1 mRNA.

Although many structural and functional features of an mRNA can contribute to its translation, some key regulatory structural elements or motifs are mostly found in the untranslated regions (UTRs) of mRNA transcripts. The FUS1 mRNA contains highly conserved and complex 5'UTR [150 bp] and 3'UTR [1100 bp], suggesting that these UTRs might play an important role in regulating FUS1 protein expression. However, the UTR structural and functional elements of FUS1 transcripts have not yet been identified and characterized. The purpose of this study was to identify critical UTR elements that regulate FUS1 expression in human lung cancer. We determined the potential structural and functional elements and motifs in FUS1 UTR regions by serial deletions and computer-based prediction of functional structure motifs and we investigated the biological roles of these elements in the regulation of FUS1 protein expression and mechanisms that lead to the inactivation of FUS1 expression in human lung cancer cells.

2. Materials and methods

2.1. Cell cultures

The human NSCLC cell lines H1299 and A549 were obtained from American Type Culture Collection (Manassas, VA). Immortalized normal human bronchial epithelial (HBE) cells were obtained from Dr. John Minna's laboratory at The University of Texas Southwestern Medical Center, Dallas, TX. H1299 and A549 cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum. The HBE cells were grown in Keratinocyte-SFM (Invitrogen, Carlsbad, CA) in an atmosphere of humidified air containing 5 % CO₂.

2.2. Preparation of the plasmids

A full length (1703 bp) of FUS1 mRNA containing 5'UTR (149 bp), FUS1 coding sequence (CDS, 333 bp), and 3'UTR (1221 bp) regions was cloned into an expression plasmid vector with an expression cassette consisting a CMV promoter and BGH poly A signaling sequences. Various deletion mutants were constructed by replacing either the 5'UTR or the 3'UTR sequences as illustrated in Figure 1A, and these deletion mutants conferred the following sequence of elements: 5'UTR-FUS1-3'UTR, 5'UTR-FUS1, FUS1-3'UTR, and FUS1. All constructs were confirmed by DNA sequencing. We also constructed similar reporter plasmids containing a eukaryotic green fluorescent protein (EGFP) CDS in place of the FUS1 CDS to facilitate the characterization of the function of FUS1 UTRs in lung cancer cells.

2.3. Transfection of the NSCLC cells

Cells were seeded in a 6-well tissue culture plate and cultured overnight until they achieved approximately 70 % confluence and then were transfected with DC-encapsulated plasmid DNA (DC-DNA) nanoparticles containing various FUS1 or EGFP expression vectors as described previously. In brief, 2 μ L of DC reagent and 2 μ g of plasmid DNA were mixed and the DC-DNA mixture was then added into each well in the 6-well plate. Transfected cells were incubated and harvested at designated time points. Transfection efficiency was assessed by parallel transfection with an equal amount of corresponding EGFP-expression plasmid reporter vector.

2.4. Quantitative real-time reverse-transcription and polymerase chain reaction (PCR)

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) and treated with DNase I (RNase-free, Qiagen). cDNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Carlsbad, CA). A quantitative real-time PCR (qRT-PCR) was performed in triplicate. Samples without reverse-transcription were used as negative controls to avoid any potential DNA contaminants in the RNA preparations. Melting curve analysis was used to confirm a single PCR product in each reaction. The nucleotide sequences of the qRT-PCR primers are: FUS1: sense primer 5'-TCAGAGGCAGCAGGAGCTGA-3', anti-sense primer 5'-CATAGAACATAGAGCCGCGG; GAPDH: sense primer 5'-TGCACCACCAACTGCTTAGC-3', anti-sense primer 5'-GGCATGGACTGTGGTCATGAG; EGFP: sense primer 5'-TGAGCAAGGGCGAGGAGCTGTT-3', anti-sense primer 5'-CACGCTGAACCTGTGGCCGT.

2.5. Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and cell lysates were prepared by incubating cells in laemini/urea lysis buffer on ice and scratching them off of the 6-well plate. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C and then the pellets were discarded. For electrophoresis, equal amounts of crude protein lysates (50 μ g) were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated protein bands were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The FUS1 protein was detected by immunoblot analysis with rabbit anti-FUS1 antibodies produced in our laboratory. The monoclonal anti-EGFP and anti- β -actin antibodies were purchased from Clontech (Palo Alto, CA).

2.6. Prediction of the secondary structure RNA segments

The lowest free-energy secondary structure for FUS1 RNA was predicted by using Web-based RNAfold software (Vienna RNA at www.tbi.univie.ac.at)

2.7. Statistical analysis

All experiments were repeated at least two times with duplicate or triplicate samples. A two-tailed *t*-test was used to compare the values of the test and control samples. $P < 0.05$ was considered statistically significant. STATISTICA software (StatSoft, Tulsa, OK) was used for all statistical analyses. All data presented are expressed as mean $\pm$ SEM.

3. Results

3.1. Role of the UTRs in regulating FUS1 expression

To determine the role of FUS1 UTRs in regulation of FUS1 gene and protein expression in normal lung and lung cancer cells, we transfected NSCLC H1299 cells and HBE cells with

four FUS1 expression plasmids that contained the same plasmid DNA backbone and differed only in the UTRs as illustrated in Figure 1A. A significant downregulation of FUS1 mRNA expression was detected in both H1299 cells ($p = 0.024$) and HBE cells ($p = 0.038$) transfected with FUS1 expression plasmids containing either 5'UTR, 3'UTR or both UTRs by using quantitative real-time RT-PCR (Fig. 1B). Using the FUS1 transcript level in H1299 cells transfected by the control vector containing only FUS1 CDS as baseline, a significantly less than one third ($p = 0.038$) and one tenth ($p = 0.025$) of baseline transcription level was detected in H1299 cells transfected by the 5'UTR-FUS1 and the FUS1-3'UTR expression plasmids, respectively (Fig. 1B, H1299). We also detected approximately one half ($p = 0.0778$) and one fourth ($p = 0.0319$) of the FUS1 transcript level in HBE cells transfected by the 5'UTR-FUS1 and the FUS1-3'UTR vectors, respectively (Fig. 1B, HBE). The 3'UTR mediated a higher degree of FUS1 transcription downregulation in tumor cells than in normal cells. These observations suggest that FUS1 UTRs could negatively-regulate FUS1 mRNA expression by either downregulating the FUS1 transcription or degrading the newly synthesized FUS1 mRNA transcripts.

To determine the effect of these FUS1 UTRs on FUS1 protein expression, we used Western-blot analysis to detect FUS1 protein expression in both NSCLC H1299 and normal HBE cell transfectants (Fig. 1C). A varied level of downregulation in FUS1 protein expression (the upper bands, as indicated by arrows in Fig. 1C) was detected in both H1299 and HBE cells transfected by these FUS1-UTR constructs 48 h after transient transfection. The 3'UTR exhibited a more pronounced inhibitory effect on FUS1 protein expression in the tumor cell line H1299 than that in the normal HBE cells. The levels of the down-regulated FUS1 protein expression by the 3'UTR in both tumor and normal cells were well correlated with those of decreased mRNA transcription (Figs. 1B and 1C). These observations suggest that FUS1 UTRs may regulate FUS1 expression at both transcriptional and translational levels. We also used an EGFP expression reporter system to further confirm the role of these FUS1 UTRs in regulating protein expression in NSCLC H1299 cells (Fig. 1D), H1299 cells were transiently co-transfected with plasmids carrying an EGFP coding region with or without FUS1 UTRs and plasmid vectors expressing red fluorescence protein (RFP). The CMV promoter-driven RFP expression plasmid was used to internally normalize plasmid transfection efficiency. A marked inhibition of EGFP protein expression by these FUS1 UTRs was also detected in those H1299 transfectants 48h after transfection (Fig. 1D). These results confirmed the roles of these FUS1 UTRs in regulating gene transcription and translation.

3.2. Repression of the FUS1 translation by the 5'UTR

The FUS1 5'UTR region contains two small uORFs that encode an 8-amino-acid peptide (uORF1) and a 6-amino-acid peptide (uORF2). To determine the effects of these uORFs on FUS1 translation, we constructed expression plasmids containing three site-directed point mutations in the FUS 5'UTR region as illustrated in Fig. 2A. The predicted secondary structure of 5'UTR of FUS1 mRNA is shown in Fig. 2B. We analyzed the effects of these uORF mutants on FUS1 protein expression in both NSCLC H1299 and normal HBE cells by transient transfection and Western-blotting (Fig. 2C). The elimination of either uORF1 or uORF2 showed no marked restoration of the 5'UTR-mediated downregulation of FUS1 protein expression but the inactivation of both uORFs in Mut-uORF1-2 significantly abolished the 5'UTR-mediated downregulation in FUS1 protein expression in both H1299 and HBE cell transfectants (Fig. 2C). To clarify whether the observed effects on protein expression by these uORF mutants acted on the level of FUS1 transcription or translation, we performed a quantitative real-time RT-PCR to analyze the potential effects of these uORF mutants on FUS1 mRNA transcription in both H1299 and HBE cells (Fig. 2D). No significant differences in levels of FUS1 mRNA expression were detected between the wild

type-5'UTR and the uORF mutants in those H1299 and HBE cell transfectants. These results demonstrate that both uORFs in the 5'UTR region can repress FUS1 protein expression by interfering with translation but not transcription.

3.3. Regulation of the FUS1 expression by the 3'UTR

To identify the structural and functional nucleotide sequences and motifs of the FUS1 3'UTR region and to elucidate their mechanisms of action, we performed a series of deletions in the 3'UTR region based on a computer-aided mRNA secondary structural and functional prediction, as demonstrated in Fig. 3A, and we analyzed the effects of these 3'UTR mutants on FUS1 gene transcription and protein expression in both the normal lung and cancer cells in vitro. The predicted secondary structures of the resulting 3'UTR deletion mutants are shown in Fig. 3B. We analyzed FUS1 protein expression by Western-blotting (Fig. 3C) and gene transcription by a quantitative RT-PCR (Fig. 3D and 3E) in NSCLC H1299 and normal HBE cells transfected by these 3'UTR deletion mutant plasmids.

A dramatic increase in the level of FUS1 protein expression was detected in H1299 cells transfected by the 3'UTR- Δ mut4, which had RNA nucleotides deleted at positions 764–1476 (Fig. 3C, lane Δ mt4) and a moderate increase by the 3'UTR- Δ mut3 with a deletion of positions 1080–1461 (Fig. 3C, lane Δ mt3), compared with that in H1299 cells transfected by the control plasmid containing an intact 3'UTR sequence (Fig. 3C, lane 3'UTR) 48 h after transfection. The 3'UTR deletions at the individual regions of 334–504 (Δ mut1), 776–1102 (Δ mut2), and 1163–1248 (Δ mut5) showed no significant changes in the levels of FUS1 protein expression in the H1299 cells transfected by these deletion mutants compared with that transfected by the intact 3'UTR-FUS1 expression plasmid. These results indicate that the two 3'UTR regions at 764–1476 (Δ mut4) and 1080–1461 (Δ mut3) are critical in down-regulation of FUS1 translation. A similarly significant increase in FUS1 transcription levels was also observed in H1299 cells transfected by these two 3'UTR deletion mutants (Δ mut3 and Δ mut4) compared with that in cells transfected by the intact 3'UTR-FUS1 expression plasmid by qRT-PCR (Fig. 3D), suggesting that these two 3'UTR regions may regulate FUS1 expression on both transcription and translation levels in tumor cells. A significant increase in FUS1 mRNA expression (Fig. 3D) but not in protein expression (Fig. 3C) was also detected in H1299 cells transfected with the mutant bearing a deletion at the 3'UTR 1163–1248 region (Δ mut5). This observation suggests that this region may be involved in regulating FUS1 transcription or mRNA stability that leads to the overall downregulation of FUS1 protein expression. Furthermore, although a significantly increased mRNA level was detected in NSCLC H1299 cells transfected by Δ mut4 (Fig. 3D and 3E, H1299) no significant changes in FUS1 mRNA levels were detected in normal HBE cells transfected by the same deletion mutant (Fig. 3E, HBE) in comparison with that in those cells transfected by FUS1 CDS-3'UTR plasmid, suggesting that a regulatory mechanism may operate differentially on this critical 3'UTR region in the normal lung and the cancer cells.

4. Discussion

The UTR regions of mRNAs can determine gene expression by influencing mRNA stability and translational efficiency [9]. Gene transcription and protein translation can be regulated by the differential use of alternative UTRs, enabling developmental, physiological, and pathological regulation [9;10]. Understanding the mechanism in the regulation of FUS1 gene and protein expression by UTRs will provide new insights into the mechanism of FUS1 inactivation in cancer pathogenesis. In this study, we used biochemical and molecular biology approaches to identify the critical structural and functional elements in the 5'- and 3'UTRs of transcript of a novel tumor suppressor gene FUS1, and we investigated the roles of these UTRs in the regulation of FUS1 gene and protein expression in lung cancer and HBE cells.

We identified two uORFs in the 5'UTR region of the FUS1 transcript and demonstrated that both uORFs could significantly downregulate FUS1 protein expression. These uORFs in the FUS1 5'UTR could potentially inhibit the translational initiation of the main ORF by interfering with the “scanning” of the ribosome subunit on mRNA during the translation initiation [11;12]. Selection of the translational initiation site in most eukaryotic mRNAs usually occurs via a scanning mechanism, in which the small ribosome subunit scans linearly from the 5' end of the mRNA to identifying the translation start codon [11]. The initiation scanning model predicts that the proximity to the 5' end of a transcript plays a dominant role in identifying the start codon [11;13] and serves as the primary target of translational control in both prokaryotic and eukaryotic cells [13;14]. However, the “position effect” or potential scanning interference could occur in cases where alternative AUGs present to form short ORFs, or a silent internal AUG codon is activated upon being relocated closer to the 5' end, or a mutation creates an AUG codon upstream from the normal start site and translation shifts to the upstream site [11;15].

The 5'UTR of FUS1 mRNA contains two alternative uORFs, which are highly conserved in mice, chimpanzee, and humans. The two uAUG codons in the two uORFs are far from the cap of the mRNA: the first uAUG is flanked by a guanine at position -3 and by a guanine at position +4, and the second uAUG is flanked by a guanine at position -3 and by a cytosine at position +4, thus, there is in an ‘adequate’ consensus context for uORF translation initiation [13]. Our observations that two uORFs significantly influenced FUS1 translation from the normal start codon and mutations of uAUGs to AAGs significantly upregulated FUS1 translation is in agreement with this prediction [13;15]. Our results suggest that the repression of FUS1 translation is possibly mediated by a strong uAUG context, an evolutionary conservation, an increased distance from the cap, and the multiple alternative uORFs in the 5'UTR of FUS1 mRNA. In addition, a computer-aided RNA folding analysis predicted that the FUS1 5'UTR RNA sequences consist of a highly stable secondary structure with a free energy of -72.18 kcal/mol, which is sufficient to block ribosomal scanning in 5'UTR and therefore inhibit translation [32]. Because the observed inhibitory effects of 5'UTR on FUS1 protein expression appeared not to completely result from the alternative uORFs the stable secondary structure of 5'UTR might partially contribute to the repression of FUS1 translation initiation by blocking ribosomal scanning might impede ribosome scanning.

We also identified several regulatory elements or motifs in the 3'UTR region of FUS1 transcripts by serial deletions and biological characterization of these 3'UTR deletion mutants in normal lung and lung cancer cells. FUS1 mRNA contains a highly conserved and complex 3'UTR that is more than 1200 RNA nucleotides in length. We found that the RNA nucleotide sequence elements at positions 764–1479 in the 3'UTR region are critical in downregulation of FUS1 mRNA expression, stability, and protein translation in both normal lung and tumor cells. However, the FUS1 3'UTR lacks typical AU-rich elements that are well-known targets for mRNA degradation. Lee *et al.* [16] recently showed that the miR-378 could target FUS1 3'UTR and repress the FUS1 expression, leading to enhanced cell survival, tumor growth, and angiogenesis in human NSCLC cells. The 3'UTR of FUS1 has also been shown to be a target of other miRNAs including miR-93, miR-98, and miR-197 [17]. Downregulation of FUS1 expression in both SCLC and NSCLC cell lines and primary tumors correlates with elevated miR-93 and miR-197 expression [17]. The predicted miR-98 targeting site is located in Δ 334–504 region of 3'UTR. These results suggest that differentially expressed miRNAs may target the 3'UTR of FUS1 mRNA and act as negative regulators of FUS1 expression in lung cancers. The 3'UTR of mRNA has also been identified as “a molecular hotspot for pathology” and cis-acting determinants have been shown in the 3'UTR to interact with RNA trans-acting factors responsible for silencing and inactivating gene transcription and translation, altering biological function of gene products,

and leading to the pathogenesis of different diseases [18]. The level of FUS1 3'UTR-mediated downregulation of FUS1 gene and protein expression is significantly higher in lung cancer cells than in normal bronchial epithelial cells, suggesting that differentially expressed negative regulators such as miRNAs and trans-acting factors may interact with the potential cis-determinants in the FUS1 3'UTR to differentially inactivate FUS1 expression in tumor cells.

Our results demonstrate a critical role of the UTRs of FUS1 transcripts in downregulation of FUS1 transcription and translation in lung cancer cells and provide new insight into the mechanism of the regulation of FUS1 expression in a biological system. These findings warrant further investigation to identify the precise regulatory cis-determinants in FUS1 UTRs and putative transacting factors and to elucidate the detailed mechanisms for regulating differential FUS1 expression in response to oncogenic stresses and apoptotic stimuli in normal and tumor cells. Our findings also imply that the FUS1 UTRs can be used as potential therapeutic targets by developing small molecules and biological agents that can effectively block or interrupt the interaction of negative regulators with these cis-determinants in FUS1 UTRs to re-activate FUS1 expression and restore its tumor suppression function in tumor cells.

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References

1. Ji L, Minna JD, Roth JA. 3p21.3 tumor suppressor cluster: prospects for translational applications. *Future Oncol.* 2005; 1:79–92. [PubMed: 16555978]
2. Lerman MI, Minna JD. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer Res.* 2000; 60:6116–6133. [PubMed: 11085536]
3. Zabarovsky ER, Lerman MI, Minna JD. Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. *Oncogene.* 2002; 21:6915–6935. [PubMed: 12362274]
4. Ji L, Roth JA. Tumor suppressor FUS1 signaling pathway. *J Thor Oncol.* 2008; 3:327–330.
5. Uno F, Sasaki J, Nishizaki M, et al. Myristoylation of the FUS1 protein is required for tumor suppression in human lung cancer cells. *Cancer Res.* 2004; 64:2969–2976. [PubMed: 15126327]
6. Ito I, Ji L, Tanaka F, et al. Liposomal vector mediated delivery of the 3p FUS1 gene demonstrates potent antitumor activity against human lung cancer in vivo. *Cancer Gene Ther.* 2004; 11:733–739. [PubMed: 15486560]
7. Prudkin L, Behrens C, Liu DD, et al. Loss and reduction of Fus1 protein expression is a frequent phenomenon in the pathogenesis of lung cancer. *Clin Cancer Res.* 2008; 14:41–47. [PubMed: 18172250]
8. Wistuba II, Tang M, Maitra A, et al. Genome-wide allelotyping analysis reveals multiple sites of allelic loss in gallbladder carcinoma. *Cancer Res.* 2001; 61:3795–3800. [PubMed: 11325854]
9. Hughes TA. Regulation of gene expression by alternative untranslated regions. *Trends Genet.* 2006; 22:119–122. [PubMed: 16430990]
10. Hughes TA, Brady HJ. Regulation of axin2 expression at the levels of transcription, translation and protein stability in lung and colon cancer. *Cancer Lett.* 2006; 233:338–347. [PubMed: 15885887]
11. Kozak M. Pushing the limits of the scanning mechanism for initiation of translation. *Gene.* 2002; 299:1–34. [PubMed: 12459250]

12. Kozak M. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene*. 2005; 361:13–37. [PubMed: 16213112]
13. Kozak M. Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. *Nature*. 1984; 308:241–246. [PubMed: 6700727]
14. Kozak M. Emerging links between initiation of translation and human diseases. *Mamm Genome*. 2002; 13:401–410. [PubMed: 12226704]
15. Kozak M. Alternative ways to think about mRNA sequences and proteins that appear to promote internal initiation of translation. *Gene*. 2003; 318:1–23. [PubMed: 14585494]
16. Lee DY, Deng Z, Wang CH, Yang BB. MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc Natl Acad Sci USA*. 2007; 104:20350–20355. [PubMed: 18077375]
17. Du L, Schageman JJ, Subauste MC, et al. miR-93, miR-98, and miR-197 regulate expression of tumor suppressor gene FUS1. *Mol Cancer Res*. 2009; 7:1234–1243. [PubMed: 19671678]
18. Conne B, Stutz A, Vassalli JD. The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? *Nat Med*. 2000; 6:637–641. [PubMed: 10835679]

Highlights

- The UTRs of tumor suppressor gene FUS1/TUSC2 mRNA play an important role in regulation of FUS1 expression in human bronchial epithelia and lung cancer cells.
- Two uORFs in the 5'UTR inhibit FUS1 translation initiation by interfering with the scanning of the ribosome initiation complexes.
- Several RNA sequence elements/motifs in 3'UTR significantly downregulate FUS1 protein expression.
- The 3'UTR regulates FUS1 expression differentially in normal lung and tumor cells.

Fig. 1. Effects of UTRs of FUS1 mRNA on FUS1 gene and protein expressions in human NSCLC H1299 and normal HBE cells. (A) Schematic representations of the FUS1 expression plasmid constructs containing an expression cassette with a CMV promoter-FUS1 CDS-BGH polyA signal sequences in the presence or absence of intrinsic FUS1 5'UTR and/or 3'UTR. (B) Effects of UTRs on FUS1 mRNA expression in H1299 and HBE cells by a quantitative real-time RT-PCR assay. A significant difference ($p < 0.05$) in level of mRNA expression between the FUS1 expression constructs with and without UTRs is indicated by * an asterisk, and the standard errors are indicated by error bars. (C) Effects of UTRs on FUS1 protein expression in H1299 and HBE cells by Western-blot analysis. (D) Repression of reporter GFP protein expression by FUS1 UTRs in H1299 cells. H1299 cells were co-transfected with either a CMV-GFP [a] or a CMV-5'UTR-GFP-3'UTR [b] expression plasmid, together with a CMV-RFP expression plasmid, to normalize GFP expression in these H1299 transfectants. GFP [a and b] and RFP [c and d] protein expressions were visualized under a fluorescence microscope.

Fig. 2. Effects of FUS1 5'UTR regulatory elements on FUS1 expression in NSCLC H1299 and normal HBE cells. (A) Structures and sequences of the wild-type FUS1-5'UTR region and functional mutants of the uORFs. The positions of the uORFs and their corresponding point-mutation constructs are indicated by arrows. (B) The prediction of the secondary structure of FUS1-5'UTR. (C) Effects of the uORFs in the FUS1 5'UTR region on FUS1 protein expression in H1299 and HBE cells by Western-blot analysis. (D) Effects of the uORFs in the FUS1 5'UTR region on FUS1 mRNA expression in H1299 and HBE cells by a real-time quantitative RT-PCR assay. A significant difference ($p < 0.05$) in the level of mRNA expression between the FUS1 expression constructs with the intact 5'UTR and uORF mutants in the 5'UTR region are indicated by * an asterisk, and the standard errors are indicated by error bars.

Fig. 3. Effects of FUS1 3'UTR regulatory elements on FUS1 expression in NSCLC H1299 and normal HBE cells. (A) Constructs of deletion mutants of FUS1 3'UTR. Names, relative locations, nucleotide sequence lengths, and putative regulatory 3'UTR sequence elements of the deletion mutants in FUS1 3'UTR are schematically illustrated. (B) The prediction of secondary structures of the intact FUS1 CDS-3'UTR and deletion mutants. (C) Effects of the 3'UTR mutations on FUS1 protein expression by Western-blot analysis. Expression of GFP was used to normalize the transfection efficiency in H1299 cells co-transfected with a GFP expression plasmid and individual FUS1-3'UTR deletion mutants. (D) Effects of the 3'UTR deletions on FUS1 mRNA transcription in H1299 cells by a real-time quantitative RT-PCR assay. (E) Effects of the deletion at position 764–1476 in the FUS1 3'UTR region on FUS1 mRNA transcription in the H1299 and HBE cells. A significant difference ($p < 0.05$) in the level of mRNA expression between the intact 3'UTR and the 3'UTR deletion constructs are indicated by * an asterisk, and the standard errors are indicated by error bars.