Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Association between the ATF3 gene and non-small cell lung cancer

Xiaoxue Song[1,*], Fang Lu[1,*], Reng-Yun Liu[1], Zhe Lei[1], Jun Zhao[2], Qinghua Zhou[3] & Hong-Tao Zhang[1]

1 Soochow University Laboratory of Cancer Molecular Genetics, Medical College of Soochow University, Suzhou, China
2 Department of Thoraco-cardiac Surgery, The First Affiliated Hospital, Soochow University, Suzhou, China
3 Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Institute, Tianjin Medical University, Tianjin, China

Keywords
NSCLC; ATF3; mutation; methylation; risk

Correspondence
Hong-Tao Zhang, Soochow University Laboratory of Cancer Molecular Genetics, Medical College of Soochow University, 199 Ren'ai Road, Sino-Singapore Industrial Park, Suzhou 215123, China.
Tel: +86 512 6588 2809
Fax: +86 512 6588 2809
Email: htzhang@suda.edu.cn

Qinghua Zhou, Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Institute, Tianjin Medical University, Tianjin 300052, China.
Tel: +86 22 6036 6013
Fax: +86 22 6036 6013
Email: zhouqh1016@yahoo.com.cn

Received: 25 December 2011;
accepted 27 December 2011.

doi: 10.1111/j.1759-7714.2011.00110.x

*Xiaoxue Song & Fang Lu contributed equally to this work.

Abstract

Background: Non-small cell lung cancer (NSCLC) accounts for ~85% of all cases of lung cancer and has a poor prognosis. Activating transcription factor 3 (ATF3), a member of the ATF/cyclic adenosine monophosphate response element binding (ATF/cyclic response element binding) family of transcription factors, has been implicated in the pathogenesis of several types of cancer. However, whether the expression of ATF3 is aberrant in NSCLC and genetic variants, or DNA methylation of the gene contributes to the tumorigenesis of NSCLC, are largely unknown.
Methods: The expression of ATF3 in four NSCLC cell lines and normal human bronchial epithelial cell (HBEpiC) line was detected by Western blot analysis. The mutation of the 5′-flanking 1500-bp and coding sequence regions of the ATF3 gene were screened using DNA direct sequencing, and bisulfite-sodium modification sequencing was used to detect the promoter methylation status of the gene.
Results: up-regulated expression of ATF3 was observed in NSCLC cell lines, and there was no difference in the sequence regions of the ATF3 gene between NSCLC cells and HBEpiC cells, which were validated in lung cancer tissues and their corresponding paracarcinoma tissues.
Conclusion: Our findings suggest that the tumorigenesis of NSCLC may particularly attribute to increased expression of ATF3 but not the genetic variants of the gene.

Introduction

Lung cancer has been the main cause of cancer death worldwide and non-small cell lung cancer (NSCLC) cases account for nearly 85% of all lung cancer cases. Up to 86% of mortality associated with lung cancer is partially relevant to the lack of early detection methods.[1,2] It is widely accepted that several signaling pathways including the transforming growth factor-β (TGF-β) pathway can play key roles in the pathogenesis of NSCLC.[3–5] In response to TGF-β signaling, two cytosolic proteins, Smad2 and Smad3, are phosphorylated and activated by TGFβR1 kinase, and then heteromerize with Smad4. The latter complex translocates into the nucleus and incorporates with transcriptional complexes that can either activate or repress target genes.[6–9] TGF-β signaling can be regulated by multiple transcriptional factors, such as c-Myc, CBP/p300 and activating transcription factor 3 (ATF3).

ATF3 belongs to the member of the ATF/cyclic AMP response element binding (ATF/CREB) family of transcription factors,[10] and is a 22-KDa leucine zipper.[11] It can be viewed as an adaptive-response gene that participates in cellular processes to adapt to extra- and/or intracellular changes.[12] ATF3 is induced by TGF-β, upon induction, its gene product interacts directly with Smad3 and forms a

functional complex to further repress its target gene ID1,[9] indicating that there is a direct link between ATF3 and TGF-β.

ATF3 expression is maintained at low levels or undetectable in most cells,[13] and ATF3 alterations have been identified in several human malignancies, including colorectal cancer, prostate cancer, ovarian cancer and breast cancer.[14–17] Additionally, the accumulation of genetic variants and epigenetic changes, including DNA methylation, has been suggested to contribute to development and progression of human cancers.[2,4,18,19] However, whether ATF3 expression is altered and aberrant genetic variants or DNA methylation of the gene is involved in the tumorigenesis of NSCLC are largely unknown.

Materials and methods

Cell lines, cell culture and tumor specimens

Four NSCLC cell lines including A549, NCI-H292, 95C (Low metastatic) and 95D (High metastatic), and Human bronchial epithelial cell line (HBEpic) were purchased from the Cell Bank of the Chinese Academy of Science and were cultured at the Roswell Park Memorial Institute (RPMI) 1640 medium with 50 units/mL each of penicillin and streptomycin and 10% heat-inactivated fetal bovine serum (FBS). Fresh tumor and paired normal lung tissue specimens were obtained after informed consent from 60 patients who underwent pulmonary resection for primary NSCLC and had not received radiotherapy or chemotherapy at the First Affiliated Hospital of Soochow University. The specimens were snap-frozen and stored in liquid nitrogen immediately after operation, until use. The Academic Advisory Board of Soochow University approved this research.

Western blotting

Total protein was extracted from cells according to the manual. A BCA protein assay reagent was used to measure protein concentration with a prestained marker serving as an index; equal amounts of protein were loaded on a sodium dodecyl sulfate (SDS)-PAGE (12% gel) and transferred to nitrocellulose membrane. After being blocked with 2.5% gelatin for 1 hour, membranes were incubated with indicated primary ATF3-antibody overnight at 4°C, followed by treatment with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Here the primary antibody concentration was 1:900 and the secondary antibody was 1:2000. Detection was performed using an enhanced chemiluminescence kit, and β-actin was calculated as an internal control for the semi-quantitative analysis. Quantity1.0 software was carried out to estimate relative expression of ATF3 protein for these cell lines.

DNA bisulfite modification

Genomic DNA from cell lines and tumor tissues was extracted according to standard proteinase K digestion and phenol-chloroform extraction. Genomic DNA isolated from cell lines and tumor tissue specimens was treated with sodium bisulfite using EpiTect Bisulfite Kit (Qiagen Valencia, CA). Briefly, 1.0 ug DNA was denatured in a volume of 140 μL of Bisulfite Mix solution for 5 minutes at room temperature, and then the samples were incubated using Thermal Cycler for 5 hour. The modified DNA was purified according to the manufacturer's protocol. Finally, DNA was eluted with sterilized distilled water and stored at −20°C until use.

CpG islands selection and bulfite-sequencing PCR

A 2kb sequence was selected from the promoter region of the ATF3 gene. The CpG islands in ATF3 were analyzed by Methyl Primer Express v1.0 software (Applied Biosystems). Potential transcription factor-binding sites were predicted using the web-based TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). The two regions from −1175 to −1039bp and −496 to −288bp (the transcription initiation site of ATF3 was designated as 0) totally contain 27 CpG cites that are located in transcription factor (TF) binding sites, and were selected for bisulfite-sequencing polymerase chain reaction (PCR) (BSP).

BSP assay was performed to detect the methylation status of the 27 CpG sites. Two pairs of PCR primers were designed specifically to amplify bisulfite-converted DNA. For −1175 to −1039bp: 5'-ATTTAGTTTTGTTGAGGTTTT-3' (forward) and 5'-TTGGATGGGTTTGGATT TTTA-3' (reverse); for −496 to −288bp: 5'-GAGGAGGTTAGTGGGGGA-3' (forward) and 5'-GGAGAGAGAGGTATAGTTTTG-3' (reverse).

The cycling parameters for PCR reaction were as follows: denaturing at 95°C for 9 minutes, then 39 cycles of 94°C for 30 seconds, annealing temperature for 45 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The PCR products with the length of 243bp and 293bp, respectively, were sequenced.

Mutational analysis of the ATF3 gene

We used the Basic Local Alignment Search Tool (BLAST) function to search homologous sequences in the NCBI GenBank database. The gene (ID: 467) was used as reference sequence for ATF3 DNA sequence. Primers for 5′-flanking 1500 nucleotides and the coding sequence (CDS) regions of the ATF3 gene are designed using Oligo6.0 software, and their sequences are available in Tables 1 and 2, respectively.

Table 1 Primers used for amplification of promoter regions of the activating transcription factor 3 (ATF3) gene

Region		Primer sequences (5′→3′)	Product length (bp)	Annealing temp. (°C)
–1522 to –932	FP	TTACCAAACTGTGACCTTCGG	590	60.0
	RP	AATCAGAGGCAACCCGGT		
–947 to –391	FP	GGTTGCCTCTGATTCCTCCTG	556	65.0
	RP	GGCATCTGTCCCTCCGCT		
–485 to +43	FP	TTCCCAGCCTCACCTAGTC	528	64.0
	RP	GAGAGAAGAGAGCTGTGCAGT		

Bp, base pair; FP, forward primer; RP, reverse primer.

The PCR reaction for DNA direct sequencing were performed in a total volume of 25 μL, contain 50 to 100ng of genomic DNA, 1 unit of Ex Taq DNA polymerase (Takara, Japan), 0.2 mmol/L of each primer, 1 Ex Taq Buffer (Mg2+ Plus), 0.25 mmol/L of each deoxynucleotide triphosphate. PCR was carried out under the following conditions: 1 cycle at 94°C pre-denaturing for 5 minutes and 38 cycles of 95°C for 30 seconds and annealing temperature as indicated for 35 seconds and 72°C for 45 seconds, followed by a final extension at72°C for 10 minutes. DNA sequencing analysis was performed using the Invitrogen Kit (California, USA).

The PCR reaction for single strand conformation polymorphism (SSCP) was carried out in accordance with that used for DNA direct sequencing. SSCP was performed as described previously[5] and the conditions reached are demonstrated in Table 2.

Statistical analysis

Quantity1.0 software was used to evaluate the gray value of ATF3 and β-actin, and the ratio of ATF3/β-actin was used as relative expression. SPSS statistics software (version 16.0) was performed to test the significant difference in ATF3 expression between NSCLC cell lines and HBEpiC. Statistical differences were considered to be significant at $P < 0.05$.

Results

Western blot analysis of ATF3 expression in cell lines

Western blot analysis was applied to investigate the protein expression of ATF3 in four NSCLC cell lines (A549, NCI-H292, 95C, 95D) and HBEpiC (Fig 1a). It was identified that the expression of ATF3 at protein level was significantly higher in NSCLC cells than in HBEpiC ($P < 0.01$) (Fig 1b). This result suggested that ATF3 might be involved in tumorigenesis of NSCLC.

Analyses for DNA methylation and genetic variants in the promoter of the ATF3 gene

To determine whether DNA methylation status and genetic variants in the promoter of ATF3 are associated with NSCLC, we used the BSP method to detect methylation features of the ATF3 gene for these five cell lines, and explored gene mutation for 5′-flanking 1500 nucleotides of ATF3 DNA by direct sequencing analysis. As a result, BSP analysis indicated that the promoter regions of the ATF3 gene including –1175 to –1039bp and –496 to –288bp were completely unmethylated (Fig 2) in all detected cell lines, and no difference in sequence

Table 2 Primers used for amplification of coding sequence (CDS) regions of the activating transcription factor 3 (ATF3) gene and single strand conformation polymorphism (SSCP) conditions

Region		Primer sequence (5′→3′)	Product length (bp)	Annealing Temperature (°C)	Non-denaturing PAGE gel conc. (%)
+6247 to +6523	FP	GTTTCAATGTGTCTTTCAGC	276	61.0	8
	RP	TGATAGAACCCACCTCG			
+9297 to +9515	FP	TGGCAGTAAAAGGCAGTGTAT	218	62.0	8
	RP	CGAGAGGAAGGGTAAGGCT			
+10262 to +10492	FP	CTAAGCCACCATAAGTCTG	230	61.5	10
	RP	AGCTGCGAGAGGAAGAT			
+10475 to +10805	FP	ATCTTCCTCTCGCAGCT	330	54.0	8
	RP	GCTTAGCTCTGCAATGTT			

Bp, base pair; CDS, coding sequence; FP, forward primer; PAGE, polyacrylamide gel electrophoresis; RP, reverse primer; SSCP, single-strand conformation polymorphism.

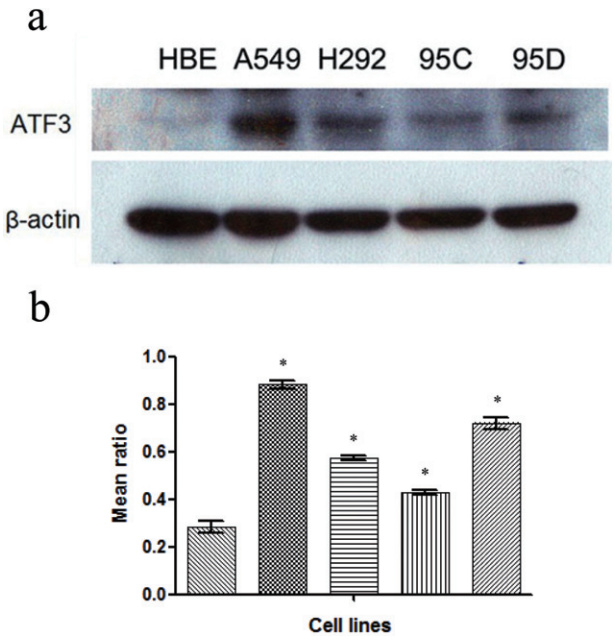

Figure 1 Western blot analysis for semi-quantitative expression levels of activating transcription factor 3 (ATF3) protein in human bronchial epithelial cell H(BEpiC) and non-small cell lung cancer (NSCLC) cell lines. (a) Schematic western blot analysis for ATF3 expression. (b) Relative estimate for ATF3 expression in cell lines. Quantity1.0 was used to evaluate the gray value of ATF3 and β-actin, and the ratio of ATF3/β-actin was used as relative expression. Significant difference was observed in ATF3 expression between each NSCLC cell line and HBEpiC. *, $P < 0.01$. ▨, HBEpiC; ▩, A549; ▤, NCI-H292; ▥, 95C; ▨, 95D.

of the promoter region was found between HBEpiC cells and NCSLC cell lines, according to the results of direct sequencing analysis. We next extended our analysis in 60 lung cancer tissues and their corresponding paracarcinoma tissues in an attempt to validate the data from the cell lines, and the results are consistent with those from the cell lines. This suggested that the promoter methylation status and genetic variants in the promoter of the ATF3 gene have no significant relationship with NSCLC tumorigenesis.

DNA abnormality analysis in CDS regions of the ATF3 gene

Although genetic variants in the promoter of the ATF3 gene were not associated with NSCLC, variants in CDS regions of the gene might be involved in the occurrence of NSCLC. To verify this hypothesis, CDS regions of the ATF3 gene were genetically screened by SSCP in 60 samples. As a result, no active mutation was detected in the coding region of the ATF3 gene (Fig 3).

Discussion

NSCLC accounts for ~85% of all cases of lung cancer. Early detection is the key to the treatment of NSCLC and understanding how NSCLC develops and progresses plays a critical role in this early detection and treatment. In the present study, we found that the expression of ATF3 in NSCLC cells was higher than that in normal bronchial epithelial cells, which is in accordance with previous evidence that the ATF3 expression is elevated in several other types of cancer, including prostate cancer,[20] Hodgkin Reed-Sternberg cells and non-malignant tissue[21] and breast cancer.[22] Our finding for the first time suggested that ATF3 might play an oncogenic role in the process of NSCLC occurrence.

Transgenic ectopic expression of ATF3 in basal epithelial cells causes basal cell carcinoma in mouse and mammary carcinomas.[23,24] Based upon these findings, we raised a hypothesis that up-regulated expression of ATF3 could be involved in the tumorigenesis and development of NSCLC. Therefore, we next tested whether the increased expression of ATF3 in NSCLC cells was attributed to genetic and/or epigenetic variants in the promoter of the gene. However, the present study failed to find any difference in the sequence of the ATF3 gene promoter between HBEpic and NSCLC cell lines, which was validated in lung tissue samples. These findings indicated that the variants in the ATF3 gene promoter were not genetically associated with the expression of the gene and could not further alter the susceptibility to NSCLC. Furthermore, our findings regarding methylation analysis indicated that promoter regions in the ATF3 gene were completely devoid of methylation, suggesting that the increased expression of ATF3 in lung cancer was epigenetically associated with other mechanisms (such as histone deacetylation) rather than DNA methylation.[25]

Given the notion that the human ATF3 gene is mapped to chromosome 1q32.3 region, which frequently shows genetic aberration in many kinds of cancer, we consequently tried to figure out if any genetic variant lies in the CDS region of the ATF3 gene in NSCLC. After genetically screening for 60 NSCLC tissues and their corresponding paracarcinoma tissues, we found that all the samples had the same sequence of the CDS region of the ATF3 gene, suggesting the occurrence of NSCLC was not related to genetic variants in the CDS region of the ATF3 gene. To the best of our knowledge, this is the first study to detect the association of genetic variants in the ATF3 gene with lung cancer risk. Although no significant association was found in this study, we cannot rule out the possibility that genetic variants in the ATF3 gene may modify the risk of other types of cancer.

Conclusion

In summary, the present study showed that the expression of ATF3 was increased in NSCLC cells compared with that in normal bronchial epithelial cells, supporting the idea that up-regulated expression of ATF3 contributes to the tumorigenesis of NSCLC. Genetic and epigenetic analyses revealed

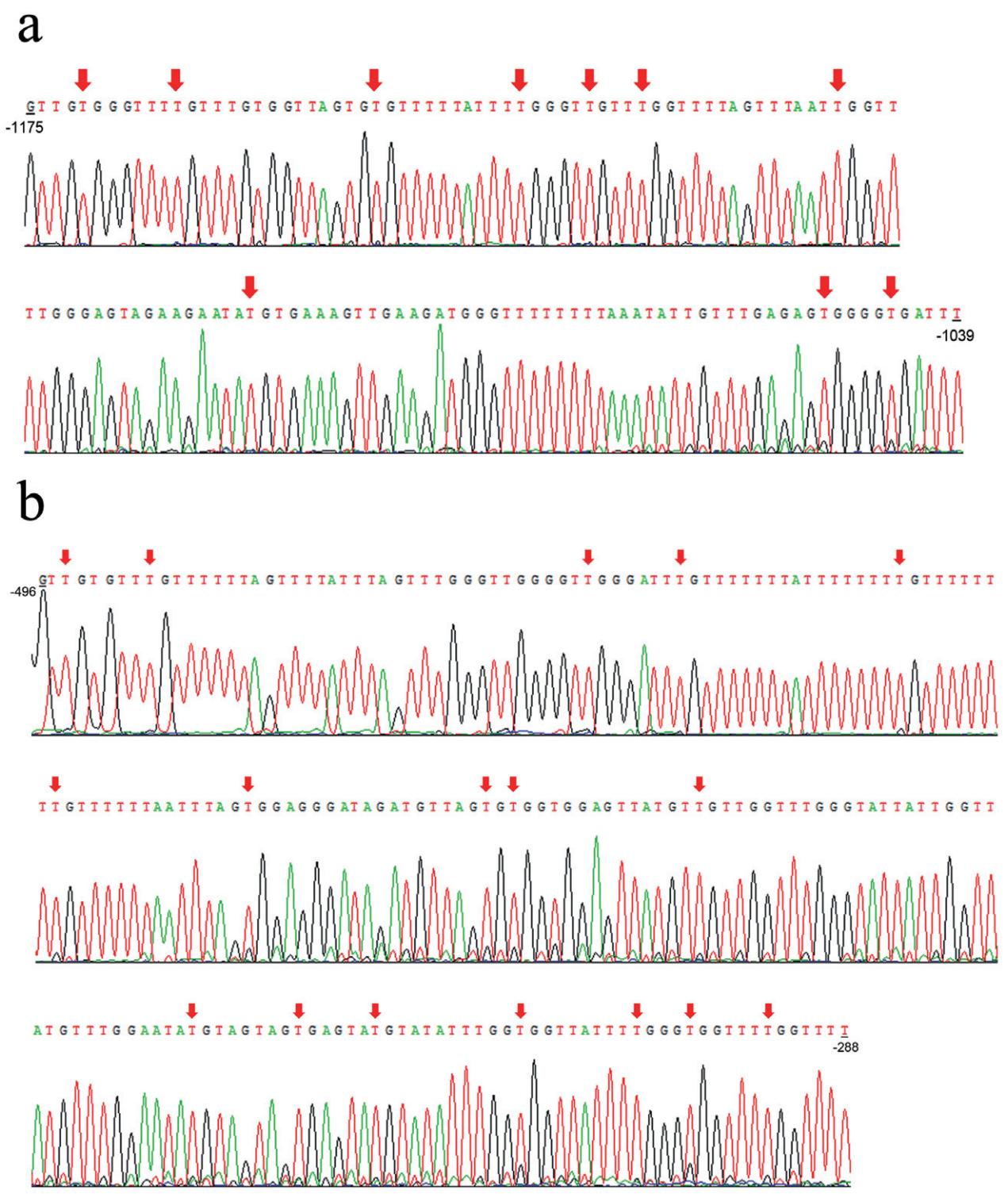

Figure 2 (a) Schematic representative of bisulfite-sequencing (BSP) for the promoter region from site -1175 to -1039bp of the ATF3 gene; (b) Schematic representative of BSP for the promoter region from site –496 to –288bp of the ATF3 gene. Red arrow: unmethylated CpG sites.

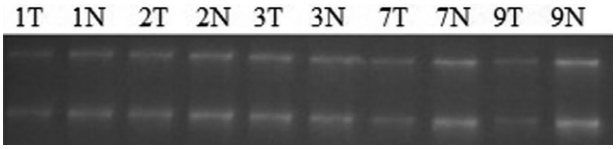

Figure 3 Schematic representative of single strand conformation polymorphism (SSCP) analysis for polymerase chain reaction (PCR) products of the coding sequence (CDS) region of the ATF3 gene. No altered pattern was found in lung cancer tissues and their corresponding paracarcinoma tissues.

that the increased ATF3 level was not attributed to aberrant methylation or variants in the promoter of the gene. In addition, our findings suggested that the genetic variant in the CDS region of the ATF3 gene was not associated with NSCLC.

Acknowledgments

We sincerely acknowledge the participation and cooperation of patients with lung cancer. This work was supported in part by the grants from National Natural Science Foundation of China (81171894 and 30973425 to H-T Zhang), Program for New Century Excellent Talents in University (NCET-09–0165 to H-T Zhang), SRF for ROCS, State Education Ministry (2008890 to H-T Zhang), Qing-Lan Project of Education Bureau of Jiangsu Province (to H-T Zhang), "333" Project of Jiangsu Province Government (to H-T Zhang), Jiangsu Province's Key Provincial Talents Program (to J Zhao) and Soochow Scholar Project of Soochow University (to H-T Zhang).

Disclosure

No authors report any conflict of interest.

References

1 Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 2004; **4**: 707–17.
2 Liu ZZJ, Chen XF, Li W *et al*. CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. *Lung Cancer* 2008; **62**: 15–22.
3 Zhang HT, Chen XF, Wang MH *et al*. Defective expression of transforming growth factor beta receptor type II is associated with CpG methylated promoter in primary non-small cell lung cancer. *Clin Cancer Res* 2004; **10**: 2359–67.
4 Zhao J, Liu Z, Li W, Liu X, Chen XF, Zhang HT. Infrequently methylated event at sites -362 to -142 in the promoter of TGF beta R1 gene in non-small cell lung cancer. *J Cancer Res Clin Oncol* 2008; **134**: 919–25.
5 Lei Z, Liu RY, Zhao J *et al*. TGFBR1 haplotypes and risk of non-small-cell lung cancer. *Cancer Res* 2009; **69**: 7046–52.
6 Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997; **390**: 465–71.
7 Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000; **19**: 1745–54.
8 Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998; **95**: 737–40.
9 Kang Y, Chen CR, Massagué J. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* 2003; **11**: 915–26.
10 Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U. ATF3 and stress responses. *Gene Expr* 1999; **7**: 321–35.
11 Chen BP, Liang G, Whelan J, Hai T. ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *J Biol Chem* 1994; **269**: 15819–26.
12 Yin X, Wolford CC, Chang YS *et al*. ATF3, an adaptive-response gene, enhances TGF{beta} signaling and cancer-initiating cell features in breast cancer cells. *J Cell Sci* 2010; **123**: 3558–65.
13 Thompson MR, Xu D, Williams BR. ATF3 transcription factor and its emerging roles in immunity and cancer. *J Mol Med (Berl)* 2009; **87**: 1053–60.
14 Bottone FG Jr, Martinez JM, Collins JB, Afshari CA, Eling TE. Gene modulation by the cyclooxygenase inhibitor, sulindac sulfide, in human colorectal carcinoma cells: possible link to apoptosis. *J Biol Chem* 2003; **278**: 25790–801.
15 Huang X, Li X, Guo B. KLF6 induces apoptosis in prostate cancer cells through up-regulation of ATF3. *J Biol Chem* 2008; **283**: 29795–801.
16 Bottone FG Jr, Moon Y, Kim JS, Alston-Mills B, Ishibashi M, Eling TE. The anti-invasive activity of cyclooxygenase inhibitors is regulated by the transcription factor ATF3 (activating transcription factor 3). *Mol Cancer Ther* 2005; **4**: 693–703.
17 Syed V, Mukherjee K, Lyons-Weiler J *et al*. Identification of ATF-3, caveolin-1, DLC-1, and NM23-H2 as putative antitumorigenic, progesterone-regulated genes for ovarian cancer cells by gene profiling. *Oncogene* 2005; **24**: 1774–87.
18 Lu F, Zhang HT. DNA methylation and nonsmall cell lung cancer. *Anat Rec (Hoboken)* 2011; **294**: 1787–95.
19 Liu Z, Zhao J, Chen XF *et al*. CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. *Lung Cancer* 2008; **62**: 15–22.
20 Pelzer AE, Bektic J, Haag P *et al*. The expression of transcription factor activating transcription factor 3 in the human prostate and its regulation by androgen in prostate cancer. *J Urol* 2006; **175**: 1517–22.
21 Janz M, Hummel M, Truss M *et al*. Classical Hodgkin lymphoma is characterized by high constitutive expression of activating transcription factor 3 (ATF3), which promotes viability of Hodgkin/Reed-Sternberg cells. *Blood* 2006; **107**: 2536–9.

22 Yin X, Dewille JW, Hai T. A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development. *Oncogene* 2008; **27**: 2118–27.
23 Wang A, Arantes S, Conti C, McArthur M, Aldaz CM, MacLeod MC. Epidermal hyperplasia and oral carcinoma in mice overexpressing the transcription factor ATF3 in basal epithelial cells. *Mol Carcinog* 2007; **46**: 476–87.
24 Wang A, Arantes S, Yan L *et al*. The transcription factor ATF3 acts as an oncogene in mouse mammary tumorigenesis. *BMC Cancer* 2008; **8**: 268-285.
25 Zhong S, Fields CR, Su N, Pan YX, Robertson KD. Pharmacologic inhibition of epigenetic modifications, coupled with gene expression profiling, reveals novel targets of aberrant DNA methylation and histone deacetylation in lung cancer. *Oncogene* 2007; **26**: 2621–34.

