Alternative splicing isoform of T cell factor 4K suppresses the proliferation and metastasis of non-small cell lung cancer cells

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Genet. Mol. Res. 14 (4): 14009-14018 (2015)
Received May 31, 2015
Accepted August 19, 2015
Published October 29, 2015
DOI http://dx.doi.org/10.4238/2015.October.29.20

ABSTRACT. The Wnt pathway has been implicated in the initiation, progression, and metastasis of lung cancer. T cell factor 4, a member of TCF/LEF family, acts as a transcriptional factor for Wnt pathways in lung cancer. Increasing amounts of evidence have shown that TCF-4 has multiple alternative splicing isoforms with transactivation or transrepression activity toward the Wnt pathway. Here, we found the presence of multiple TCF-4 isoforms in lung cancer cell lines and in normal bronchial epithelial cells. TCF-4K isoform expression was significantly decreased in lung cancer cells compared with normal bronchial epithelial cells and was identified as a transcriptional suppressor of the Wnt pathway in non-small cell lung carcinoma (NSCLC). Overexpression of TCF-4K significantly inhibited the proliferation and migration of NSCLC cells. Collectively, our data indicate that TCF-4K functions as a tumor suppressor in NSCLC by down-regulating the Wnt pathway.

Key words: Wnt pathway; TCF-4; Lung neoplasm; Splicing isoforms

INTRODUCTION

The Wnt pathway is an essential embryonic pathway that regulates cell fate determination, proliferation, and polarity (Stewart, 2014). The canonical Wnt pathway leads to the stabilization of β-catenin, which binds with the transcriptional factors T cell factor/lymphatic enhancer factor (TCF/LEF) to activate targets genes. The TCF/LEF family is comprised of *TCF1*, *TCF3*, *TCF4*, and *LEF*, which encode proteins that have the essential roles of transduction of Wnt pathway signaling and transactivation of target genes. Genetic and functional stabilization of TCF/LEF transcriptional factors seems to be a major determinant of Wnt pathway function. Wnt pathway dysregulation is associated with cancer development as a result of its impact on downstream target genes that are key regulators of the cell cycle and apoptosis, such as *cyclinD1* and *c-myc* (Logan and Nusse, 2004). It has been proposed that the Wnt pathway is associated with lung cancer development. Elevated Wnt ligands and decreased Wnt pathway inhibitors have been revealed in non-small cell lung cancer (NSCLS) specimens and cell lines (Van Scoyk et al., 2008; Liu et al., 2011); however, the study of TCF/LEF abnormity in NSCLC is not well established.

T cell factor 4 (TCF-4) has been reported to function as a main transcriptional factor in NSCLC (Yue et al., 2008; Nguyen et al., 2009). The several splicing isoforms of TCF-4 might function as bipartite factors that act as either transcriptional activators or repressors. *TCF4* isoforms have been cloned and sequenced in hepatocarcinoma cell (HCC) lines, and have revealed 14 different TCF-4 isoforms that have been named alphabetically. It has been validated that HCC cells expressed TCF-4K isoform displayed low TCF transcriptional activity; cell proliferation rate, and colony formation (Tsedensodnom et al., 2011).

In this study, we identified the presence of multiple TCF-4 isoforms in lung cancer cells lines and in normal bronchial epithelium cells. TCF-4K isoform expression was significantly decreased in NSCLC cell lines compared with normal bronchial epithelium cells. The TCF-4K isoform was then cloned and was identified as an inhibitory transcriptional factor of the Wnt pathway in lung cancer. Furthermore, overexpression of TCF-4K inhibited proliferation and migration of NSCLC cells *in vitro*.

MATERIAL AND METHODS

Cell culture and transfection

The NSCLC cell lines A549, H460, SKand the normal bronchial epithelial cell line 16HBE were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in 1640 (Gibco, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Melbourne, Australia) in a humid atmosphere containing 5% CO_2 at 37°C. Transfection was performed using the X-tremeGENE HP DNA Transfection Reagent (Roche, Madison, WI, USA). To establish A549 cells stably expressing TCF-4K, we transfected pEGFP-N1/TCF-4K (see below) or empty vector pEGFP-N1 as a negative control, followed by selection of colonies using 300 μg/mL kanamycin and expansion for further characterization, and A549 cells routine cultured were set as the blank control.

Screening and cloning of TCF-4K

Total cellular RNA was extracted from three human NSCLC cell lines (A549, SK, and H460 cell lines) and the normal bronchial epithelial cell line (16HBE) using RNAiso reagent (TaKaRa,

Dalian, China). First-strand cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) according to manufacturer instruction, using 1 µg total RNA. For identifying total TCF-4 transcription products, polymerase chain reaction (PCR) was carried out with a primer pair 1F (forward, 5'-CCG CTC GAG CGG ATG CCG CAG CTG AAC GGC GG-3') and 17R (reverse, 5'-CGC GGA TCC GCG CTA TTC TAA AGA CTT GGT GAC GAG CGA CAG CGG CT-3') that spans from the beginning of exon 1 to the end of exon 17 (Tsedensodnom et al., 2011). The PCR product of approximately 1700 bp from the 16HBE cell line was cloned into the pEGFP-N1vector (Sangon, Shanghai, China) using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the individual clones were DNA sequenced at Sangon Biotech Co., Ltd. (Shanghai, China).

Nested reverse transcription (RT)-PCR

The mRNA levels of the TCF-4K isoform were measured by nested RT-PCR to avoid the amplification of other TCF4 isoforms. The sequence of the TCF-4K mRNA (GenBank ID: HM352839) obtained from the National Center for Biotechnology Information database was used to design primer pairs for the nested RT-PCR reaction. The outer primers were used in the first reaction and 2 µL of this product was re-amplified using the nested primers (Table 1).

Table 1. Primer sequences for nested RT-PCR.

Primer	Sequence (5'-3')
Outer forward	CCC GAA CCT ATC TCC AGA TG
Outer reverse	TTT TCT CCT GCA CGG TTT G
Nested forward	AGC TTT CTG TCT TCT AGG TTC C
Nested reverse	TCA GTC TGT GAC TTG GCG TC

RT-PCR = reverse transcription-polymerase chain reaction.

Western blot

Proteins were lysed and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% bovine serum albumin (BSA) for 30 min. Antibodies against TCF4 (Cell Signaling Technology, Danvers, MA, USA), GAPDH (Abcam, Cambridge, UK), and C-myc (Proteintech, Wuhan, China) were also used. Horse radish peroxidase (HRP)-labeled secondary antibody was used. Blots were assessed by an ECL system (Amersham, Piscataway, NJ, USA).

Wnt/TCF transcriptional activity assay

For analysis of TCF transcriptional activity, A549-stable clones over-expressing TCF-4K were co-transfected with TOPFlash or FOPFlash (Millipore, Bedford, MA,USA) and PRL-TK (Promega, Madison, Wisconsin, USA). The PRL-TK was acted as internal reference, its activity was used for normalization of transfection efficiency. At 48 h post-transfection, the luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) and the relative transcriptional activity was determined by the ratio of the TOP value to FOP basal activity.

Cell proliferation assay

Cell proliferation rates were measured using a Cell Counting Kit (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). A549-stable clones over-expressing TCF-4K or pEGFP-N1 empty vector as a control were plated in 96-well plates at a density of 2000 cells/well and incubated for 24, 48, 72, or 96 h. To each well, 10CCK-8 reagent was added 2 h prior to detection. The OD 450 nm value in each well was determined by a microplate reader (Biotek ELX800, Winooski, Vermont, USA).

Cell cycle assay

The effects of TCF-4K on lung cancer cell cycle progression were assessed using propidium iodide flow cytometry. A549-stable clones over-expressing TCF-4K or pEGFP-N1 empty vector as a control were plated in 6-well plates at 3 x 10^5 cells per well. After 24 h, the cells were washed with phosphate buffered saline (PBS), pH 7.4, harvested, and fixed in 70% ethanol. Cells were treated with DNase-free RNase and stained with propidium iodide. Cell samples were analyzed on a FACSCalibur (BD Biosciences, Bedford, MA, USA) and all cell cycle phase fractions were determined. The concentration of cell samples was 1 x 10^6/ml,the number of cell samples was 2 x 10^6.

Transwell assay

A549-stable clones over-expressing TCF-4K or pEGFP-N1 empty vector as a control (4 x 10^5) were suspended in 200 µL serum-free medium and seeded into the upper chamber of a transwell insert with an 8 µm pore size membrane (Corning Costar Corp., Cambridge, MA, USA). RPMI 1640 containing 10% FBS was placed in the lower chamber as a chemoattractant. After incubation for 12 h, the migrated cells on the underside of the filter membrane were fixed and stained with 0.1% crystal violet. The number of migrated cells was counted in 8 randomly selected microscopic fields and photographed. The protocol used for the invasion assay was the same as that used for the migration assay, except that the transwell insert was coated with Matrigel (BD Biosciences, Heidelberg, Germany).

Statistical analysis

SPSS 15.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were expressed as the means ± SEM, using a two-tail Student's *t*-test to carry out comparisons of two independent groups. A P value < 0.05 was considered statistically significant. All experiments were performed in triplicate.

RESULTS

Expression of TCF-4 isoforms in lung cancer cell lines and in normal bronchial epithelial cells.

To investigate the existence of multiple TCF4 isoforms in lung cancer cell lines and in normal bronchial epithelial cells, three lung cancer cells lines (A549, H460, and SK) and normal bronchial epithelial cells (16HBE) were used. RT-PCR was carried out using a primer pair that

spanned from the beginning of exon 1 to the end of exon 17. The PCR products were separated on 2% agarose gels. Multiple bands were detected in the 3 lung cancer cell lines and in normal bronchial epithelial cells. The band around 1700 bp was detected in 16HBE but was not present in the three lung cancer cell lines. We hypothesize that this band might represent a potential tumor suppressor isoform. The PCR product from the normal bronchial epithelial cell line 16HBE was cloned into a pEGFP-N1 vector plasmid for sequencing to confirm the specific TCF-4K isoform, and was used for TCF-4K the functional assay (Figure 1).

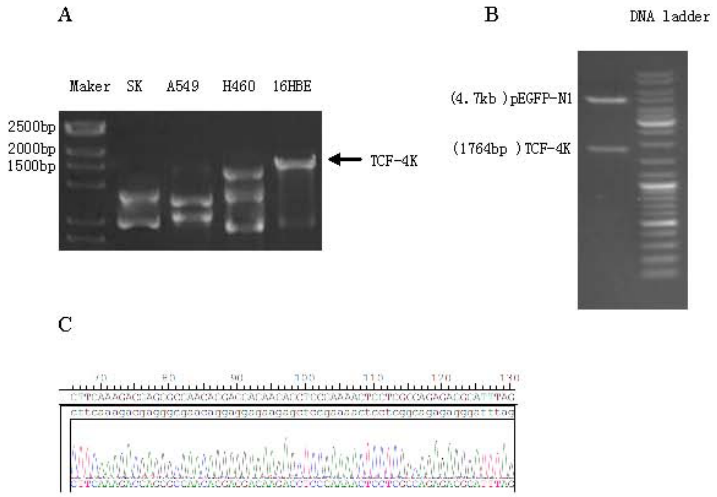


Figure 1. Isolation and identification of TCF-4K. **A.** RT-PCR analysis of the TCF-4K isoform in SK, A549, H460, and 16HBE cell lines using the indicated specific primers. **B.** Identification of recombinant plasmid double enzyme. **C.** A portion of the sequence map of TCF-4K. RT-PCR = reverse transcription-polymerase chain reaction.

Over-expression of TCF-4K in A549 cells and the effects of TCF-4K on TCF transcriptional activation of the Wnt pathway

Nested RT-PCR and western blot were performed to confirm the expression of TCF-4K in A549-stable clones over-expressing TCF-4K. As shown in Figure 2A and 2B, we found that TCF-4K could be detected in the A549-stable clones over-expressing TCF-4K (TCF-4K group) while lacked in negative control group (NC) and blank control group (BC). To determine the effect of TCF-4K on the transcriptional activity of Wnt pathway, A549-stable clones over-expressing TCF-4K were co-transfected with TOP Flash or FOP Flash and PRL-TK acted as internal reference. Transfection of TCF-4K, but not that of the control vector, significantly inhibited the activity of the luciferase reporter gene in A549 cells (Figure 2C). Furthermore, as shown in Figure 2D, the expression of c-Myc, a well-known TCF-4 downstream target (Pajic et al., 2000), was significantly suppressed by TCF-4K. The above data indicate that over-expression of TCF-4K down-regulates the transcriptional activity of the Wnt pathway.

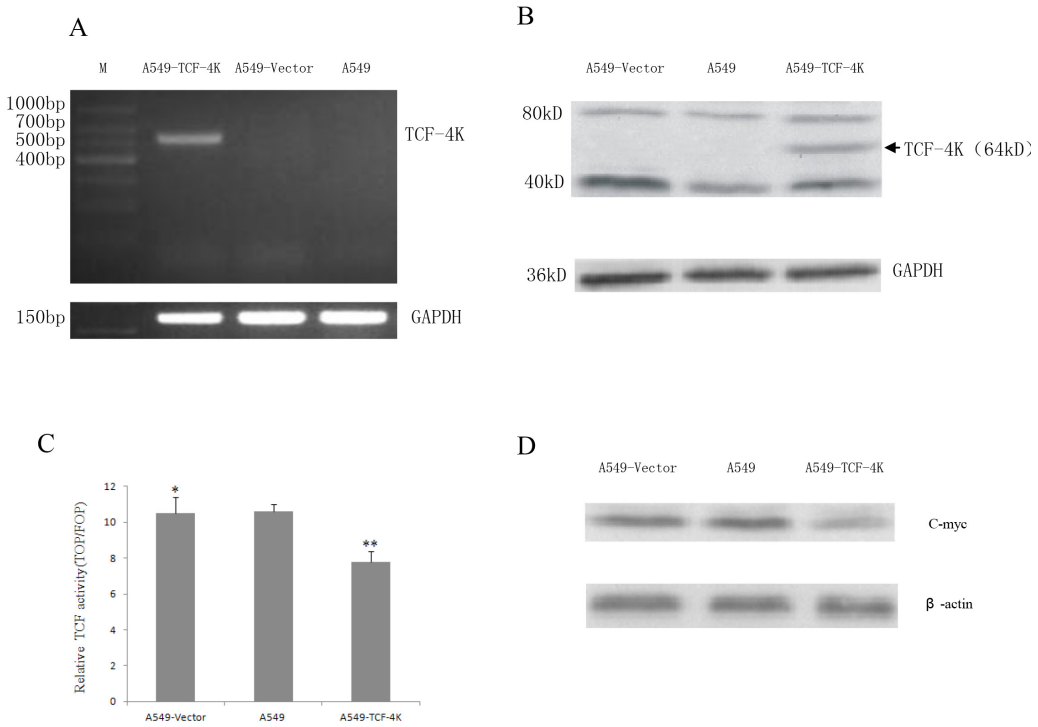
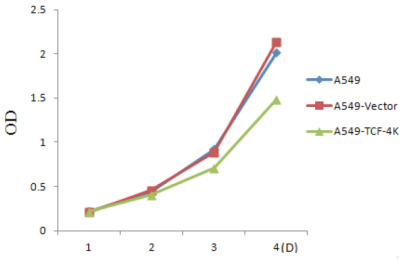


Figure 2. Effect of TCF-4K on TCF transcriptional activation of the Wnt pathway. **A.** mRNA levels of the TCF-4K isoform were measured by nested RT-PCR in the A549-Vector, A549, and A549-TCF-4K groups. **B.** Western-blot analyses of TCF-4K over-expression using an anti-TCF4 antibody. **C.** For analysis of TCF transcriptional activity, luciferase activity was measured at 48 h post-transfection, and the relative transcriptional activity was determined by the ratio of the TOP value to FOP basal luciferase activity; *P > 0.05, **P < 0.01. **D.** Western-blot analyses of C-myc protein in the 3 groups using an anti-C-myc antibody. RT-PCR = reverse transcription-polymerase chain reaction.

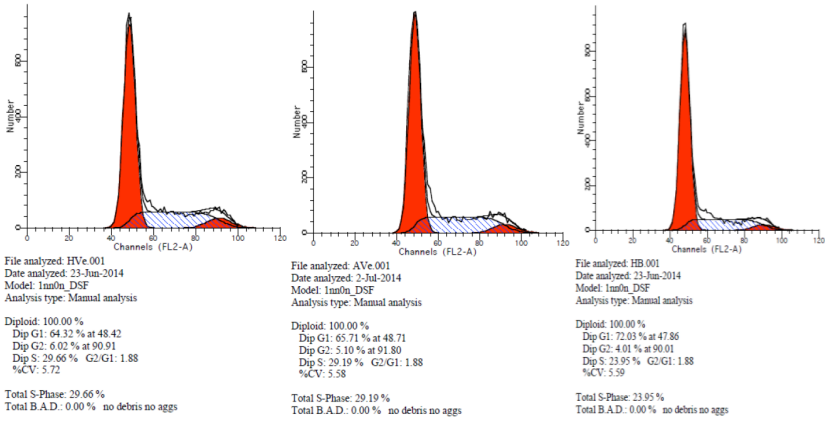
Effects of TCF-4K on the proliferation and migration of A549 cells

A CCK-8 assay was used to examine the proliferation of A549 cells, and determined that overexpression of TCF-4K significantly inhibited the proliferation of A549 cells (Figure 3A). The effect of TCF-4K on the cell cycle was detected by flow cytometry. The percentage of cells at G0/G1 phases in TCF-4K group (72.03%) was significantly higher than that of the blank control group (BC) (64.32%, $P < 0.05$) or negative control group(NC) (65.71%, $P < 0.05$) and S phase in TCF-4K group (23.95%) was significantly lower than that of the blank control group (BC) (29.19%, $P < 0.05$) or negative control group (NC) (29.66%, $P < 0.05$) (Figure 3B). Therefore, we concluded that transfection with TCF-4K induced cell arrest at G0/G1 phases. A transwell assay indicated that the numbers of cells that migrated through the microporous membrane were decreased in the TCF-4K group compared to the control groups. The result showed that overexpression of TCF-4K inhibited the migration of A549 cells (Figure 3C). These data suggest that TCF-4K inhibited the proliferation and migration of NSCLC cells.

A

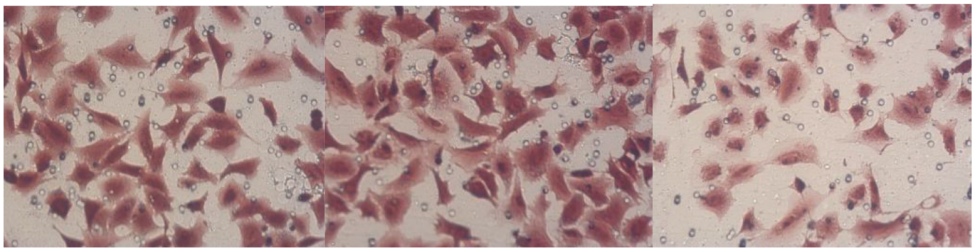


B



A549-Vector group A549 group A549-TCF-4K group

C



A549-Vector A549 A549-TCF-4K

Figure 3. Effects of TCF-4K on the proliferation and migration of A549 cells. **A.** The proliferation of A549 cells was detected by CCK-8. **B.** Flow cytometry cell cycle analysis. **C.** A transwell assay was used to test A549 cell migration in the three groups.

DISCUSSION

Alternative splicing plays a pivotal role in development as well as in the pathologic process of human disease. The vast majority of protein-coding genes have alternative splicing isoforms, which display diverse or even opposite function and might lead to cell dysfunction (Tang et al., 2013). It is now evident that the unbalanced expression of splicing variants or the failure to properly express the correct isoforms is part of the biology of cancer cells (Pajares et al., 2007). For example, abnormal splicing profiles of the oncogenes *BRCA1/2* and *APC* and tumor suppressive gene *TP53* have been identified in breast, colorectal, and ovarian cancer cells (Brandão et al., 2011; Cheah et al., 2014; Marcel et al., 2014).

The Wnt/β-catenin pathway is a conserved signaling pathway implicated in embryonic development and postnatal tissue homeostasis. It has been revealed that abnormal activation of the Wnt/β-catenin pathway is associated with tumorigenesis of the lung and poor prognosis and resistance to chemotherapy (Van Scoyk et al., 2008). Over-expression of Wnt ligands such as Wnt1, Wnt2 and Wnt5 and down-regulation of Wnt inhibitors such as WIF1 have been revealed in lung cancer cells. However, the downstream mediators of the Wnt pathway, the T cell factor/ Lymphoid enhancer factors, have not been fully studied. TCF/LEF constitutes a family of highly conserved transcriptional regulators that is comprised of four members in humans: *TCF1*, *LEF1*, *TCF3*, and *TCF4*. In a commonly accepted canonical model, TCFs/LEF bind Groucho/TLE co-repressor proteins and inhibit target genes in the absence of a Wnt signal but associate with β-catenin and convert into activators after cell stimulation by Wnt proteins. The human *TCF4* gene is composed of 17 exons with several alternative splicing sites, leading to the possibility of multiple splicing isoforms. To investigate the expression pattern of TCF-4 isoforms in lung cancer, cDNA from three lung cancer cell lines (A549, H460, and SK) and normal bronchial epithelial cells (16HBE) were used as template. RT-PCR was carried out using a primer pair that spans from the beginning of exon 1 to the end of exon 17. As expected, the PCR products showed a divergence of bands with distinct molecular weights.

Upon analysis, it was noted that the PCR products from lung cancer cells lacked bands with molecular weight around 1700 bp compared with normal bronchial epithelial cell products. This band was cut, cloned, and sent for sequencing. Sequence analysis was performed using the NCBI nucleotide blast web service, and validated that the 1700 bp band that was missing in lung cancer cells represented an alternative splicing isoform of TCF-4. This isoform had also been previously identified as an inhibitory transcriptional factor of the Wnt pathway in hepatocellular carcinoma cell lines(Tsedensodnom et al., 2011). The TCF4 gene includes five essential domains for transcription function: an N-terminal β-catenin-binding domain (BCBD, exon 1), a Groucho-related-gene (Grg)/ transducin-like enhancer of split (TLE) transcriptional corepressor binding site (exons 9 and 10), a high mobility-group-box (HMG-box) DNA-binding domain (exons 10 and 11), an adjacent nuclear localization signal (NLS, exon 12), and a C-terminal binding protein binding site (CtBP, exon 17). Furthermore, Exon 4, the C-terminal tail (exons 12-16), two highly conserved LVPQ motifs (end of exon 7), and the SxxSS motif (beginning of exon 9) are alternative splicing sites for the generation of multiple isoforms (A. Duval et al., 2000; T. Pukrop et al., 2001).

We further investigated the effect of TCF-4K on the transcriptional activation of the Wnt pathway in lung cancer cell lines. The result showed that TCF-4K inhibited reporter activity as well as c-Myc expression in A549 cells. A CCK8 assay showed the relative proliferation of cells in the pEGFP-N1/TCF-4K group compared to cells in A549 group was inhibited. To identify the mechanism by which TCF-4K affected the proliferation of A549 cells, we examined the cell cycle using flow

cytometry. The result showed that over-expression of TCF-4K increased the percentage of G0/G1 phase cells whereas the proportion of cells in G2 and S phases was decreased. Furthermore, a transwell assay showed that over-expression of TCF-4K could significantly inhibit the migration, and therefore, presumably the metastasis ability, of A549 cells.

In conclusion, we demonstrated that TCF-4K was markedly expressed in 16HBE cells while lacking in lung cancer cells. Overexpression of TCF-4K might decrease TCF transcriptional activity, which would lead to the observed reduction in expression of C-myc in A549 cells. Research (Pajic A et al., 2000) has demonstrated C-myc overexpression in human malignant cells and has shown that this results in promotion of cell growth by regulating the cell cycle and cell apoptosis. Therefore, we supposed that TCF-4K might regulate relevant target genes like C-myc to affect cell proliferation. In contrast to the conclusions of Tsedensodnom et al. (2011) from their research on hepatoma carcinoma cells, we found that over-expression of TCF-4K could significantly inhibit the migration of A549 cells, which might be caused by the down-regulation of migration-relevant target genes. Therefore, our data indicate that TCF-4K might function as tumor suppressor in NSCLC by down-regulating the Wnt pathway. The substantial roles of TCF-4K in cancer cell biology suggest its potential application as a molecular therapeutic target for NSCLC treatment. The expression of TCF-4K in lung cancer cells might have been below our threshold of detection. Further experiments will be required to detect the expression of TCF-4K in NSCLCs of different pathologies and differentiation states.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by grants from the National Nature Science Foundation of China (NSFC #81201684 and #81201841) and the National Key Clinical Specialist Construction Programs of China [(2012)#649].

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