The *FUS1* gene inhibits EC109 cell growth mediated by a lentivirus vector

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Accepted: 15 January 2013

ABSTRACT

The effects of the *FUS1* gene on the oesophageal carcinoma cell line EC109 are investigated. The messenger RNA (mRNA) expression level of the *FUS1* gene was detected by a reverse transcription polymerase chain reaction (RT-PCR) technique in the cell lines SHEE, SHEEC and EC109. The full length of the *FUS1* gene was amplified using a PCR technique from the total RNA of umbilical mesenchymal stem cells. The *FUS1* gene was cloned into a pSL6-IRES-EGFP vector and identified by PCR, digestion and sequencing. The recombinant pSL6-FUS1-IRES-EGFP plasmid was transfected into 293FT cells and the resulting lentivirus was collected. The growth of EC109 cells after transfection with lentivirus containing the *FUS1* gene was determined by MTT assay and plate colony formation. Expression of the *FUS1* gene in EC109 cells was weaker than that in SHEE, SHEEC cells and human umbilical vein endothelial cells (HUVEE; used as a control). Transfection efficiency was more than 80% after 48 h. Cell growth assessed by MTT assay was inhibited by about 40% compared with the control group; a finding that was in accordance with the plate colony formation results. The results suggest that the *FUS1* gene might be a candidate tumour suppressor gene for the treatment of oesophageal carcinoma; however, these results require confirmation in *in vivo* studies.

KEY WORDS: Esophageal neoplasms.
FUS1 gene.
Genes, tumor suppressor.

Introduction

Oesophageal carcinoma is one of the most malignant gastrointestinal cancers and ranks the sixth most frequent cause of cancer death worldwide.[1] Research suggests that the disease is associated with chemical agents, bacterial or viral infection and genetic abnormality.

Some progress has been made on the molecular mechanism underlying oesophageal carcinoma. Deletion, mutation, augmentation and promoter methylation of many genes (e.g., *p53*, *cyclinD1*, *p16*, *RASFF1*) have been reported to be associated with the carcinogenesis, development and prognosis of oesophageal cancer.[2–5] Cytogenetic evidence indicates that the loss of 3p21 is a frequent occurrence in patients who have oesophageal cancer.[6–8] In this region, the association of many tumour suppressor genes (e.g., *RASSF1*, *VHL*) with cancer have been confirmed.[9] In additional to these genes, *FUS1*, *TUSC4* and *BLU* are considered strong candidates as tumour suppressor genes that could inhibit the growth of lung cancer cells.[10–13]

It is unclear whether or not the *FUS1* gene is related to oesophageal cancer, and therefore this study aims to investigate the function of this gene and the possibility that it may inhibit the growth of the EC109 oesophageal cancer cell line.

Materials and methods

Cell lines and reagents

The SHEEC, SHEE and EC109 cell lines were a gift kindly donated by Dr. Shen Zhongying. All cells were cultured in RPMI 1640 medium (Invitrogen, USA) with 10% fetal bovine serum (FBS) and 100 µg/mL penicillin and streptomycin. All primers were synthesised by Invitrogen (Shanghai, China). *Thermophilus aquaticus* (*Taq*) DNA polymerase was obtained from Takara (Japan). The plasmids related to the lentivirus package were generously donated by Dr. Chen Yangchao (Chinese University of Hong Kong).

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Lentivirus construction and package containing the **FUS1** *gene*

The *FUS1* gene was cloned using the following primers: (sense) 5′-CGG GAATTC GCCGCC ATGGGCGCCAGCGGGTC-3′ and (antisense) 5′-CC GTCGAC TCACTTGTCGTCG ***TCATCCTTGTAATCCACCTCATAGAGGATCAC***-3′. The EcoR I and Sal I sites, respectively, were introduced into the primers. The antisense primer was linked with the FLAG tag sequence (indicated in bold above), which expressed the peptide sequence DYKDDDDKG. After the polymerase chain reaction (PCR) product was digested with EcoR I and Sal I, it was ligated into the pSL6 vector and the sequence was verified by DNA sequencing (Fig. 1). pSL6-FUS1, together with other plasmids, was co-transfected into 293FT cells to produce the lentivirus, using a method described previously.[14]

Genetic background and detection of **FUS1** *expression*

The genomic DNA was extracted from the cell lines by PCR using the primers listed in Table 1. The PCR method was performed in a GeneAmp 9700 (ABI). Amplification

condition were as follows: one cycle at 94˚C for 3 min, then 28 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, and finally a temperature reduction to 4˚C to end the reaction. The messenger RNA (mRNA) obtained from the cell lines was reverse transcribed into complementary DNA (cDNA), and used as the template for the expression assay. The PCR products obtained were subjected to electrophoresis and the results were observed and photographed in a UVP mini darkroom.

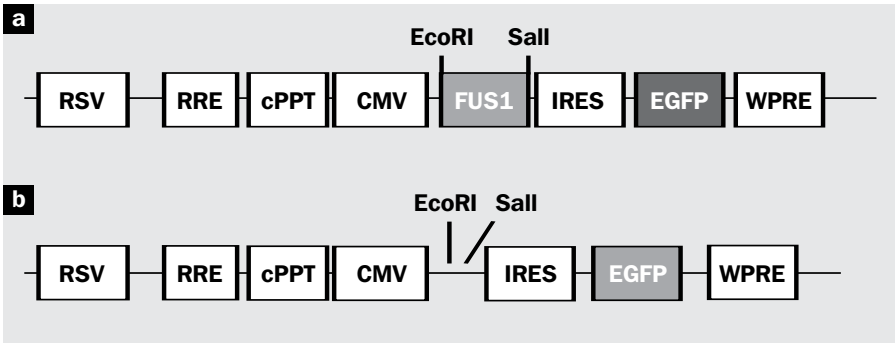

Fig. 1. Schematic of lentivirus vector: **a)** with *FUS1* gene; **b)** control.

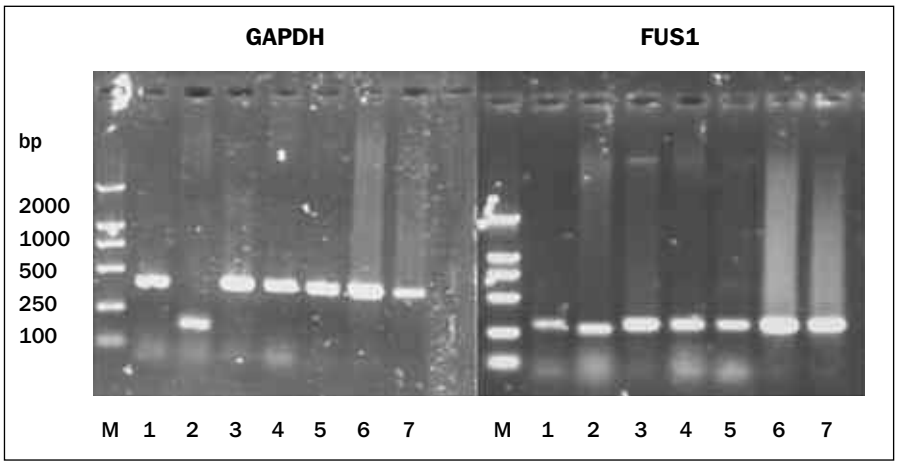

Fig. 2. Genetic testing of the *FUS1* gene. M: DNA marker; Lanes 1–5: genomic DNA from the cell lines HUVEE, C666-1, SHEE, SHEEC and EC109, respectively. Lanes 6 and 7: genomic DNA from single cells of two normal human peripheral blood samples.

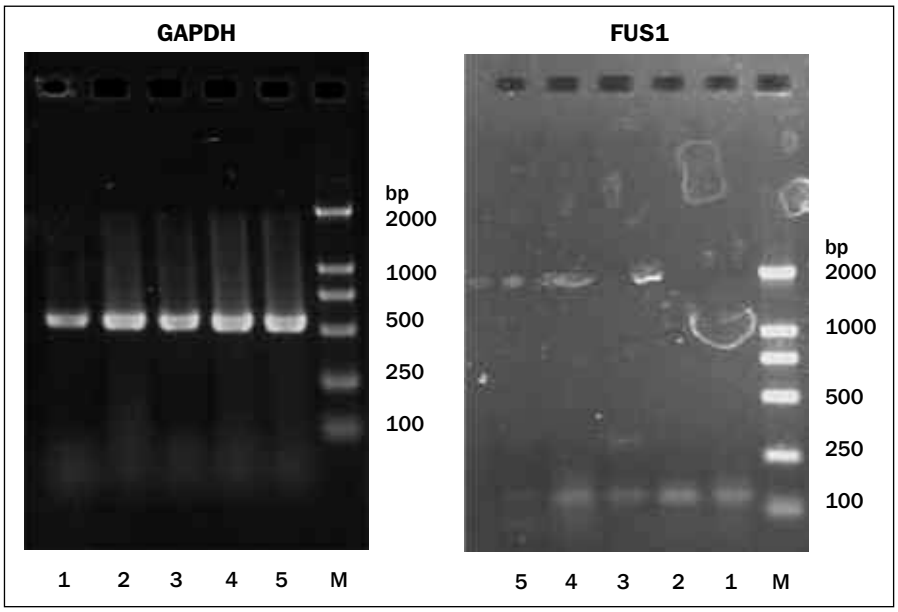

Fig. 3. mRNA level of the *FUS1* gene in the cell lines studied. M: DNA marker. Lanes 1–5 represent the cDNA amplified from the cell lines HUVEE, C666-1, SHEE, SHEEC and EC109, respectively. GAPDH was used as the reference gene.

MTT assay

Cells (10^4 per well) were seeded in a 96-well culture plate. The cells were incubated for 12 hours prior to transfection with 10^5 pSL6-FUS1 virus and the pSL6 control. After the cells had been transfected with the virus for 48 hours, cell growth was detected using an MTT assay.

Plate colony formation test

The transfected cells were diluted by approximately 50% and then 200 cells per well were placed in a six-well plate. After 14 days the cell clones were observed and counted using fluorescence and brightfield microscopy, the latter employing Giemsa staining as described previously.[11]

Western blotting

Protein was purified using RIPA buffer and loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted. Expression of β-actin and FUS1 was determined using β-actin and FLAG antibodies (M2, Sigma).

Results

Genetic background and expression of FUS1 *gene*

The presence of the *FUS1* gene in the cell lines was assessed by a PCR method, using mononuclear cell DNA from human peripheral blood cells as a control, and the GAPDH as the reference gene. Results showed there was no loss of 3p21 in the genome. However, the GAPDH gene in the C666-1 cell line (a nasopharyngeal carcinoma cell line) might be a short fragment deletion. Reverse transcription PCR (RT-PCR) results showed that expression of the *FUS1* gene was significantly down-regulated in the SHEE, SHEEC and EC109 cell lines (Fig. 3).

Lentivirus transfection into EC109 cells

EC109 cells were transfected with the purified lentivirus of pSL6-FUS1 and pSL6, and high infection efficiency of up to 80% was achieved (data not

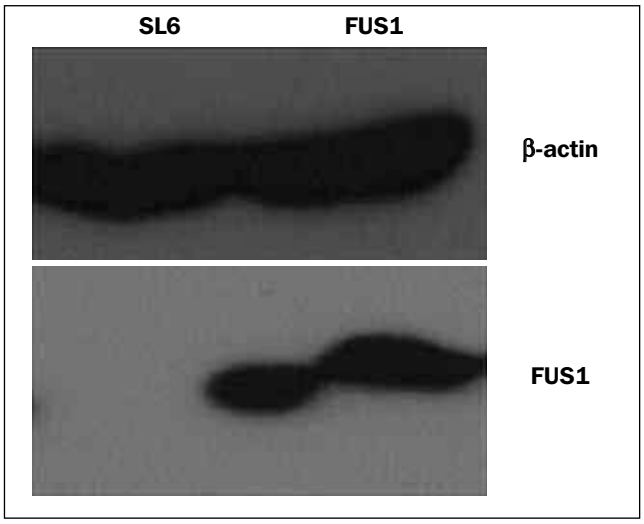

Fig. 4. FUS1 protein expression. The transfected cell protein was detected using antibody to β-actin and FLAG. SL6 and FUS1 represent cells infected with lentivirus pSL6 and pSL6-FUS1.

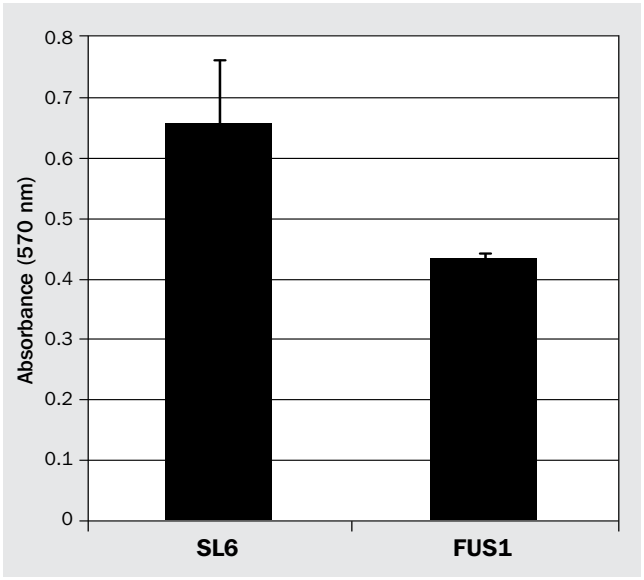

Fig. 5. Result of the MTT assay of cells infected with lentivirus pSL6 and pSL6-FUS1.

shown). Following extraction of the protein product, strong expression was observed using an antibody detection method (Fig. 4).

MTT assay

An MTT assay was performed after EC109 cells had been infected with the lentivirus for two days. Results showed that the *FUS1* gene inhibited cell growth by approximately 40% (Fig. 5).

Plate colony formation test

The transfected single cells were cultured for 14 days and the cell accumulations were observed. Fewer cell clones were observed in the *FUS1*-positive group than in the control group; a result consistent with that of the MTT assay. However, the ratio of enhanced green fluorescent protein (EGFP)-positive cell collections was markedly lower in the pSL6-FUS1 group (Fig. 6).

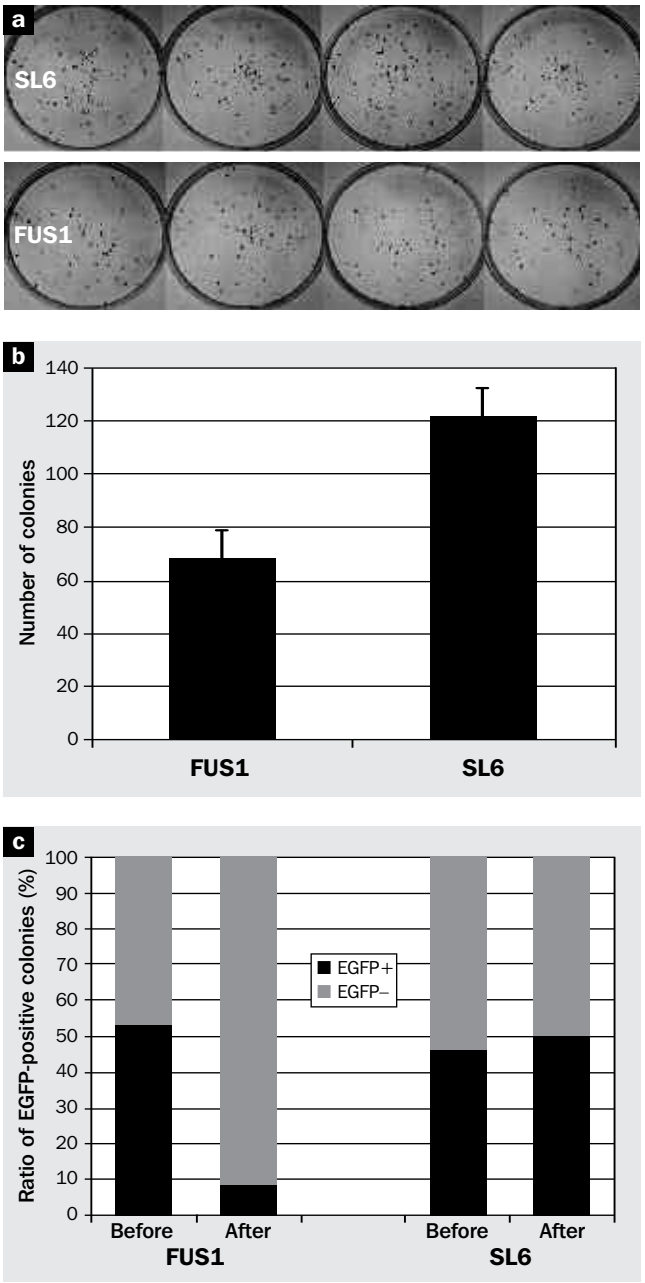

Fig. 6. Effect of the *FUS1* gene on plate clone formation. **a)** Plate clone formation in the six-well plate stained with Giemsa. **b)** Number of colonies in the two groups. **c)** Ratio of EGFP-positive colonies. SL6 and FUS1 represent cells infected with lentivirus pSL6 and pSL6-FUS1.

Discussion

The *FUS1* gene is located in a critical chromosomal region (3p21) and is a known tumour suppressor gene, strongly inhibiting the growth of lung cancer cells through the p53 pathway, and has been shown to have significant effect in preclinical trials.[12,14–19] In lung cancer patients, FUS1 protein is down-regulated while genetic mutations are not frequently observed. While study of tumour inhibition by the *FUS1* gene has focused mainly on lung cancer, it is possible that the *FUS1* gene is also involved in the pathogenesis of oesophageal carcinoma.

Table 1. Primers used to test genetic background and gene expression.

Gene	Primer sequence	cDNA product length (bp)	DNA product length (bp)
GAPDHg	5'TACAAGCGTTTTCTCCCTAAA3'		396
	5'GCCCAATACGACCAAATCTAA3'		
FUS1	5'AGACAATCGTCACCAAGAAC3'	131	300
	5'TCATAGAGGATCACAGGGAA3'		
GAPDHc	5'CAACGGATTTGGTCGTATT3'	541	
	5'CACAGTCTTCTGGGTGGC3'		

GAPDHg and *GAPDHc* primers were applied to test gene expression level.

The genetic background of EC109 cells (an oesophageal carcinoma cell line), SHEE cells (immortalised cell line by human papillomavirus [HPV]) and SHEEC cells (immortalised cell line by HPV, but conferring tumourigenesis)[20,21] was detected by a PCR technique. The results showed that there was no deletion in 3p21; however, mRNA expression level was down-regulated in all the cell lines studied. This appearance is different to that seen in lung cancer; however, recent reports provided evidence that miRNA and the structure of the untranslated region of *FUS1* mRNA might be involved in the down-regulation of FUS1 protein.[22,23]

Owing to the presence of the IRES element in the lentivirus vector, FUS1-positive cells also expressed EGFP. This made cells visible under fluorescence microscopy. However, as transfection efficiency failed to reach 100%, the true inhibitory effect of the *FUS1* gene detected by MTT assay and colony formation might have been masked.

In this study, *FUS1* gene inhibition of the growth of the EC109 oesophageal cancer cell line is reported. This suggests that the *FUS1* gene may be involved in the pathogenesis of oesophageal carcinoma. The molecular mechanism behind this may be similar to that in lung cancer, upregulating Apaf-1 protein expression that is a critical step in the p53 pathway.[19] However, any association of the *FUS1* gene with oesophageal carcinoma must be confirmed by *in vivo* tests and unequivocal clinical data. □

The authors wish to thank Professor Z. Y. Shen for his support and the supply of SHEE, SHEEC and EC109 cell lines for this research. Funded by grants from the Natural Science Foundation of China (No. 30600733) and Guangdong Provincial Science and Technology (2010A040302003).

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