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• ESOPHAGEAL CANCER •

Overexpression of ETS2 in human esophageal squamous cell carcinoma

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Abstract

AIM: To study the expression pattern of ETS2 (erythroblastosis virus oncogene homolog 2) in human esophageal squamous cell carcinoma (ESCC).

METHODS: Reverse transcription polymerase chain reaction (RT-PCR) and Northern blot were performed to examine the expression level of ETS2 mRNA in 37 pairs of ESCC tissue samples. Western blot and immunohistochemistry were carried out to check the expression level of ETS2 protein in 30 pairs of ESCC tissue specimens.

RESULTS: RT-PCR and Northern blot analysis showed that ETS2 mRNA upregulated in 75.7 % (28/37) examined ESCC tissues relative to matched normal tissues. From those 37 cases, 14 cases were randomly selected to perform Western blot and the results revealed that ETS2 protein overexpressed in 71.4 % (10/14) checked ESCC tissues compared with the corresponding normal tissues. Moreover, the expression patterns of ETS2 protein in those 14 cases were identical to those of ETS2 mRNA displayed by RT-PCR or Northern Blot. Immunohistochemistry analysis showed that the expression level of ETS2 protein rose in 75 % (12/16) tumor epithelial cells contrasted to the normal cells. Altogether the expression level of ETS2 protein increased in 73.3 % (22/30) checked ESCC tissue samples contrary to their normal counterparts.

CONCLUSION: The results suggested that ETS2 overexpressed in paired human ESCC tissue samples at both mRNA and protein levels and may be associated with the tumorigenesis of esophagus.

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INTRODUCTION

Erythroblastosis virus oncogene homolog 2 (ETS2) is a proto-oncogene, which is located in human chromosomal region 21q22.3 and encodes a 56 kD protein that is phosphorylated by a Ca^{2+}-dependent mitogenic signal process[1,2]. ETS2 gene expresses in various tissues, including blood, breast and prostate. ETS2 may be involved in the regulation of cellular proliferation and differentiation and may play a critical role in T-cell activation and cytokines production[3-6].

As a member of ETS oncogene family, ETS2 gene has the oncogenic potential. It is similarly transposed as a consequence of nonrandom chromosomal translocations; especially, the *t* (8;21)(q22;q22) translocation is the most frequently noted breakpoint involving chromosome 21 for acute myelogenous leukemia[7]. An acute non-lymphoblastic leukemia with a complex *t* (6;18;21) chromosomal translocation, has so far been associated with higher expression level of ETS2[8]. It had been reported that ETS2 gene was associated with the growth and invasion of breast carcinoma cells and was required to maintain the transformed state for human prostate cancer cells[9,10].

Esophageal cancer ranks among the 10 most frequent cancers in the world, with a predominant distribution in developing countries. Our previous study showed that genetic susceptibility to esophageal cancer was one of the important causes for the high prevalence and familial aggregation of this disease in some areas of northern China[11]. We observed the upregulation of ETS2 gene in human esophageal squamous cell carcinoma (ESCC) using cDNA microarray technique[12]. To our best knowledge, this study first investigated the expression patterns of ETS2 gene and ETS2 protein in ESCC by reverse transcription polymerase chain reaction (RT-PCR), Northern blot, Western blot and immunohistochemistry. Both the expression levels of ETS2 gene and ETS2 protein increased in ESCC tissues contrary to their normal counterparts. The results were consistent with the microarray results. Therefore, it indicated that ETS2 gene might be related to the formation of human ESCC and its further study may provide the insight into the mechanisms of carcinogenesis of esophagus.

MATERIALS AND METHODS

Human tissues

Samples of ESCC and matched normal esophagus tissues were collected from 37 patients who had not receive chemotherapy or radiotherapy before surgery. The tissues were immediately stored in liquid nitrogen until analysis and each sample was confirmed by histological examination. Total RNA and total protein of the samples were extracted using Trizol solution (Life Technologies, Rochville, ML) per manufacturer's protocol. Total RNA was quantitated and assessed for purity by means of UV spectrophotometry and electrophoresis in denaturing formaldehyde gel. The standard curve of protein was prepared to determine the protein concentration of tissue samples with Bicinchoninic Acid Protein Assay Kit (SIGMA, St. Louis, MO). Sixteen pairs of ESCC paraffin slides were obtained from Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College.

RT-PCR

Before reverse-transcription, 5 μg total RNA of each sample was treated with 20 units DNase I (Promega, Madison, WI), 40 units RNasin (Promega, Madison, WI) at 37 ℃ for 15 min to remove contaminated genomic DNA. Then first strand cDNA was synthesized with SuperScript Preamplification System For First Strand cDNA Synthesis kit (Life Technologies, Rochville, ML). Two microliters of reverse-transcription product were used as the template to amplify specific fragment of ETS2 gene. PCR conditions were as follows: initial denaturation at 94 ℃ for 3 min, followed by 28 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 1 min. The expression of housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to normalize the template input. The sequences of the PCR primer pairs of ETS2 and GAPDH were as follows: ETS2, 5'-GTGGACCTATTCAGCTGTGG-3', 5'-TTCCCCGACGTCTTGTGGAT-3'; GAPDH, 5'-ACCACAGTCCATGCCATCAC-3', 5'-TCCACCACCCTGTTGCTGTA-3'.

Northern blot

Briefly, 30 μg total RNA of each sample was dissolved in loading buffer containing formamide and formaldehyde, heated at 70 ℃ for 10 min, electrophoresed on the 2 % formaldehyde agarose gel and transferred to a positively charged nylon membrane. The membrane was prehybridized in 5 ml hybridization solution (6×SSC, 2×Denhart's solution, 0.1 % SDS, 100 μg/ml denatured salmon sperm DNA) at 68 ℃ for 2 h. Then ^{32}P-labelled probe (bases 1202-1952) was added and hybridization was performed at 68 ℃ for 18 h. The membrane was washed twice at room temperature in 2×SSC, 0.1% SDS for 20 min, once at 65 ℃ in 0.5×SSC, 0.1 % SDS for 20 min and exposed to X-ray films at -70 ℃ for 72 h.

Western blot

Anti-ETS2 antibody was purchased from Santa Cruz biotechnology, Inc (Santa Cruz, CA). Thirty-microgram total protein of each sample was mixed with 20 μl sample buffer (100 mM Tris· Cl, 200 mM DTT, 4 % SDS, 20 % glycerol and 0.2 % bromphenol blue) before separation by SDS-PAGE (10 %) electrophoresis system. Samples were then transferred to PVDF membrane and nonspecific binding was blocked with 5 % nonfat dry milk for 1 h at room temperature. Then the filter was incubated with anti-ETS2 antibody (1:500) for 2 h at room temperature. An enhanced chemiluminescence system (Santa Cruz, Santa Cruz, CA) was used for signal detection.

Immunohistochemistry

Four-micron paraffin-embedded slides were dewaxed in xylene, rehydrated in ethanol and treated with H_2O_2 to block the endogenous peroxidase activity. Antigen retrieval was achieved by microwaving in a citrate buffer (pH 6.0) for 15 min. The slides were incubated with anti-ETS2 antibody at a 1:50 dilution for 2 h at 37 ℃. Biotinylated secondary antibody and peroxidase-conjugated streptavidin steps were performed using UltraSensitive™ S-P kit (Maxim Biotech, Fujian, China) according to the manufacturer's protocol. DAB was used as the chromogen and hematoxylin as the counterstain. Negative control was performed by substituting PBS for the primary antibody.

RESULTS

Overexpression of ETS2 in paired ESCC tissue samples at mRNA level

RT-PCR and Northern blot were performed to confirm the differential expression of ETS2 in ESCC at transcriptional level. RT-PCR analysis showed that ETS2 gene overexpressed in 24 pairs of tumor versus normal tissues among a total of 31 tested cases (Figure 1). Northern blot analysis showed that the expression level of ETS2 gene increased in 4 of 6 cases of tumor tissues relative to the matched normal tissues (Figure 2).

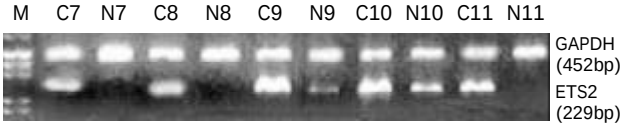

Figure 1 RT-PCR analysis of ETS2 expression in ESCC tissues (Lanes C) and matched normal esophagus tissues (Lanes N). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR product sizes were 229bp for ETS2 and 452bp for GAPDH.

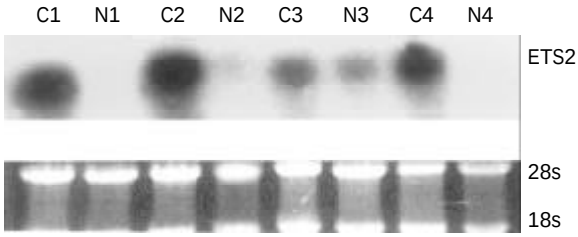

Figure 2 Northern blot results of ETS2 gene in ESCC tissues (designated as C) and matched normal esophagus tissues (designated as N). Thirty-microgram total RNA of each sample was resolved by formaldehyde gel electrophoresis, transferred to a nylon membrane, and hybridized with ^{32}P-labelled ETS2 probe.

Upregulation of ETS2 in paired ESCC tissue samples at protein level

Western blot and immunohistochemistry were conducted to verify the differential expression of ETS2 in ESCC at translational level. From the above mentioned 37 cases, 14 pairs of ESCC tissue samples were randomly selected to carry out Western blot and the results showed that ETS2 protein was upregulated in 71.4 % (10/14) examined ESCC tissues relative to the corresponding normal tissues (Figure 3). The expression patterns of ETS2 protein in those 14 cases were identical to those of ETS2 mRNA revealed by RT-PCR or Northern Blot.

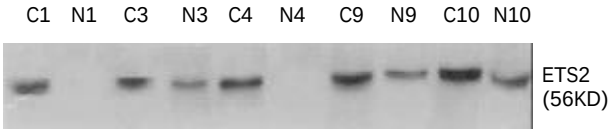

Figure 3 Elevation of ETS2 protein expression in paired ESCC tissue samples detected by Western blot. Total protein was extracted from the tissues and equal amounts of total protein of each sample (30 μg) were loaded. Blots were hybridized with anti-ETS2 antibody (1:500). An enhanced chemiluminescence system (ECL) was used for signal detection. The molecular weight of ETS2 protein is 56 kD.

Immunohistochemistry results displayed that 75 % (12/16) checked ESCC cases existed diffuse and strong staining in the nucleus of tumor cells, while sporadic and weak staining was observed in the nucleus of the matched normal esophageal epithelial cells (Figure 4).

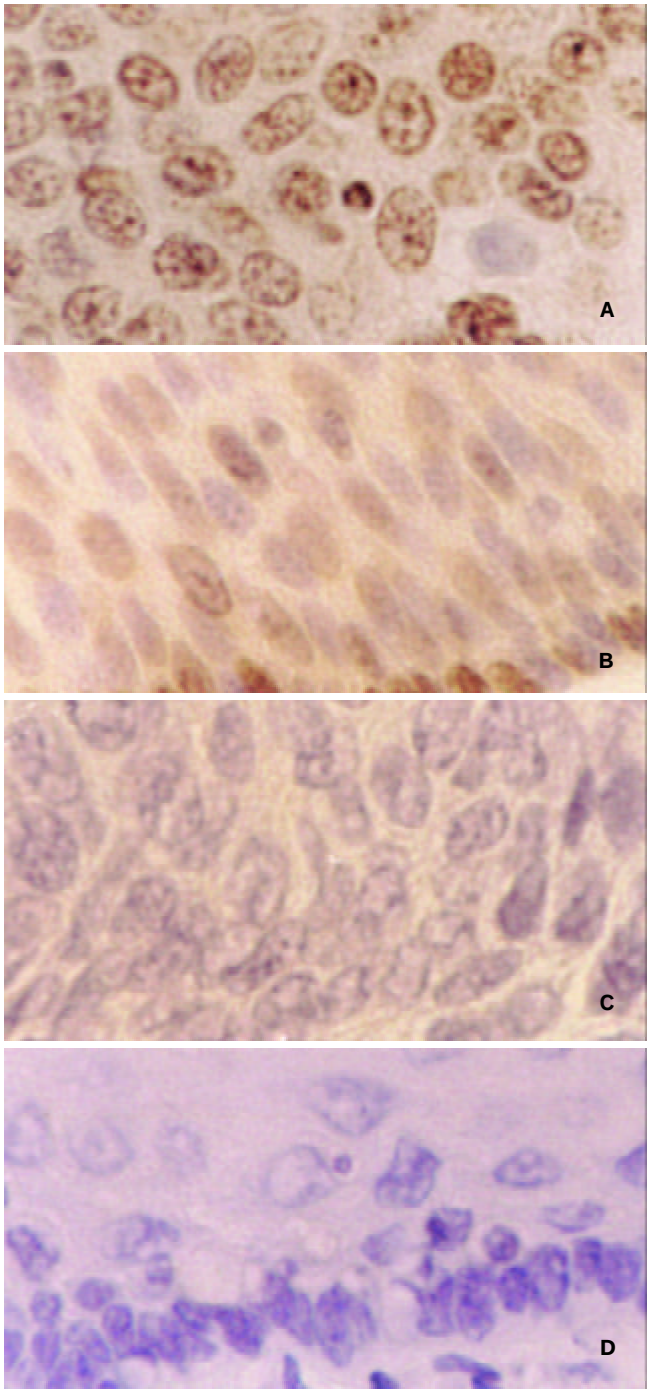

Figure 4 Immunohistochemistry analysis for ETS2 protein in paired ESCC tissue samples using anti-ETS2 antibody (1:50). Diffuse and strong staining was detected in the nucleus of the ESCC epithelial cells (A), while sporadic and weak staining was observed in the nucleus of matched normal esophageal epithelial cells (B). No positive staining was observed in negative controls (C, D). (original magnification ×400).

DISCUSSION

The understanding of the molecular basis of tumor development has progressed dramatically in the last two decades. It is well known that tumor is essentially a genetic disease. So it is important to demonstrate what genes are and how they work in carcinogenesis. Identifying the genetic differences between normal and tumor cells or tissues will discover the genes that directly cause tumor or be associated with tumorigenesis and provide novel markers for early detection and appropriate therapy.

In our previous study, ETS2 gene showed upregulation in human ESCC tissue[12]. To verify this differential expression, we explored the expression pattern of ETS2 in paired ESCC tissue samples at both mRNA and protein levels. The results of RT-PCR and Northern blot revealed that ETS2 overexpressed in 75.7 % (28/37) examined tumor tissues relative to the corresponding normal tissues. From those 37 cases, 14 cases were randomly selected to perform Western blot and the results showed that the expression level of ETS2 protein elevated in 71.4 % (10/14) checked ESCC tissues compared with the corresponding normal tissues. Moreover, the expression patterns of ETS2 protein in those 14 cases were identical to those of ETS2 mRNA displayed by RT-PCR or Northern Blot. Immunohistochemistry analysis showed that the expression level of ETS2 protein raised in 75 % (12/16) examined tumor epithelial cells contrasted to the normal counterparts. Altogether ETS2 protein overexpressed in 73.3 % (22/30) tested ESCC tissues relative to the matched normal tissues. Therefore, the data suggested that ETS2 abnormally expressed not only in the transcriptional level but also in the translational level for human ESCC and the increasing transcription of ETS2 mRNA in ESCC may result in increasing translation of ETS2 protein.

As a transcription factor, ETS2 protein controls the transcription of some important genes participating in a number of biological processes including cell growth and apoptosis. Although many studies have been done, the accurate function of ETS2 in biological and pathophysiologic state is still unclear. Previous studies showed that the overexpression of ETS2 led to different results in different cells. Some promoted tumorigenesis[13,14], while others were arresting[15]. So it indicated that ETS2 could mediate multiple different signal pathways and might be involved in carcinogenesis with some unlike ways. In the present study, we found the overexpression of ETS2 in ESCC at both mRNA and protein levels. It suggested that ETS2 might be associated with the formation of ESCC. But how ETS2 acts during the neoplasia of esophagus and whether its function is tissue-specific or organ-specific are still unknown. Although a lot of work was focused on the chromosomal aberrations of human ESCC[16-18] and even in several studies frequent loss of 21q was observed[19,20], 21q had not yet been investigated in detail. Hence the variation of 21q22.3 in human ESCC, and which ETS2 gene is located in remain unclear. Further investigations will help us to make clear whether the overexpression of ETS2 is caused by the gain of 21q22.3 in human ESCC. As mentioned above, the higher expression level of ETS2 was found in an acute non-lymphoblastic leukemia with a complex *t* (6;18;21) chromosomal translocation[8]. Another speculation is that the upregulation of ETS2 in human ESCC may be associated with the chromosomal translocation of 21q22.3. More studies about chromosomal aberrations will be helpful to demonstrate the precise function of ETS2 and its molecular mechanisms in tumorigenesis of esophagus.

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