

Functional studies of a novel oncogene TGM3 in human esophageal squamous cell carcinoma

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INTRODUCTION

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^[1]. Chen *et al* observed the down-regulation of TGM3 gene in human ESCC using polymerase chain reaction (RT-PCR) and Northern blot techniques^[2]. To our best knowledge, this is the first time to investigate the protein expression of TGM3 in ESCC. The expression levels of TGM3 gene decreased in ESCC tissues compared with their normal counterparts. The results are consistent with that from RT-PCR and Northern blot. Therefore, TGM3 gene might be related to human ESCC and further study may provide insight into the mechanisms of carcinogenesis of esophagus. TGM3 is a member of a family of Ca²⁺-dependent enzymes, which can catalyze the substrates to form a complex that is related to the differentiation of the stratified squamous epithelia^[3]. The gene is localized on chromosome 20q11.2^[4]. The full-length cDNA of TGM3 is about 2.6 kb, encoding 692 amino acids. TGM3 as a proenzyme, whose molecular weight is about 77 ku, becomes highly active upon proteolytic cleavage into 50 ku (amino-terminal) and 27 ku species^[2].

Tissue microarray was first introduced in 1998^[5], which allows simultaneous analysis of many tumors using small-diameter cores sampled from larger blocks of tissue, but may be limited by tumor heterogeneity^[6]. This study used TMA for the study of four molecules of interest as prognostic factors in ESCC, including methods for assessing immunocytochemical scoring of microarrays. TMA blocks were constructed from 99 cases of ESCC with corresponding normal tissues, taking four cores from different areas of each tumor and adjacent esophageal epithelia. Immunocytochemical labelling was performed for TGM3. The extent and intensity of scoring was determined for each core and the degree of agreement was determined for results from the assessment of two, three or four cores for each carcinoma. In a subset of 99 representative cases, the labelling in the TMA was compared with that in whole-tissue sections of the

Abstract

AIM: To investigate the role of transglutaminase 3 (TGM3) gene in human esophageal squamous cell carcinoma (ESCC), and analyze its relationship with clinicopathological parameters.

METHODS: Gene expression of TGM3 in fresh esophageal cancer tissues and their corresponding normal mucosas was detected immunohistochemically(IHC) by means of tissue microarray(TMA). Its correlation with clinical characteristics was evaluated and analyzed by univariate analysis. All statistical analyses were performed by SPSS version 10.0.

RESULTS: Expression rate of TGM3 was reduced to 81.8% in ESCC. Expression of TGM3 was significantly inversely correlated with histological grade of esophageal carcinoma (in grade I, II and III tumors, the reduced expression was 4/7, 57/71, and 20/21, respectively, $P < 0.05$), whereas it had no obvious correlations with lymph node metastasis and depth of invasion.

CONCLUSION: Reduced expression of TGM3 may play an important role in esophageal carcinogenesis.

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Key words: Esophageal squamous cell carcinoma; Immunohistochemically; Tissue microarray

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same carcinomas. An adequate sample of carcinoma was obtained in more than 90% of the 396 cores. TMA is a reliable tool to demonstrate cellular and molecular alterations in ESCC. We recommend using the mean results from four cores for biological studies, with analysis of categorical data based on quartile groups.

MATERIALS AND METHODS

Materials

Specimens of cancer tissues and matched adjacent normal mucosa were taken from 99 consecutive patients with squamous cell carcinoma of the thoracic esophagus who had undergone esophagectomy with regional lymph node dissected from Oct 2004 to Sept 2005 at the Department of Thoracic Surgery, the First Affiliated Hospital of Anhui Medical University. None of them received irradiation or chemotherapy preoperatively. The patients included 87 men and 12 women with a mean age of 54 (range 36-79) years. Six tumors were located in the upper thorax, 61 in the middle thorax and 32 in the lower thorax (Table 1). The removed specimens were examined histologically with hematoxylin and eosin staining, and then the clinicopathologic stage was determined according to TNM classification.

Construction of TMA

Archival paraffin-embedded, formalin-fixed tissues are collected. Two pathologists selected representative areas from each donor tumor block, and then punched cores 0.6 mm in diameter, from the donor block. Sections of the resultant tissue microarray were sliced and transferred to glass slides for processing of TGM3 by immunohistochemistry (Pictured is a 120-specimen TMA with 0.6 mm spots).

Immunohistochemical staining

Immunohistochemical analysis was done retrospectively. Resected esophageal specimens, including both tumor and normal mucosae, were fixed in a 40 g/L formaldehyde solution and embedded in paraffin. The following antibody was used in this study: goat polyclonal anti-human TGM3 antibody (Santa Cruz Biotech Co, USA) diluted 1:100 in PBS. Four μm thick sections of formalin-fixed paraffin-embedded tissue blocks of esophageal tumors were sliced. TMAs were deparaffinized, dehydrated and blocked to remove endogenous peroxidase activity by 3 mL/L H_2O_2 in methanol for 30 min. TMAs were treated with microwave in 0.1 mol/L citrate buffer pH 6.0 at 96°C for 15 min. After incubation with 100 mL/L normal goat serum to block non-specific binding, they were then incubated with the primary antibodies overnight at 4°C. After antibody was washed with PBS, TMAs were incubated with the secondary antibody and the third antibody (Streptavidin/HRP) according to the manufacturer's instructions, and finally DAB was visualized. Tissues were counterstained with hematoxylin. Negative control was designed by using PBS instead of primary antibody.

Evaluation of staining

The cytoplasmic expression of TGM3 in the ESCC was

Table 1 Clinical and histopathological characteristics of patients

Characteristics	n = 99 (%)
Sex	
Male	87 (87.9)
Female	12 (12.1)
Location of tumor	
Upper thoracic	6 (6.1)
Middle thoracic	61 (61.6)
Lower thoracic	32 (32.3)
Histological grade	
I	7 (7.1)
II	71 (71.7)
III	21 (21.2)
Depth of invasion	
T1	5 (5.1)
T2	35 (35.4)
T3	59 (59.6)
T4	0 (0)
Lymph node metastasis	
Positive	32 (32.3)
Negative	67 (67.7)

evaluated as described previously and scored as follows: (a) 0, < 5% of the epithelial cells in the respective lesions; (b) 1, 5%-25% of the epithelial cells in the respective lesions; (c) 2, 26%-50% of the epithelial cells in the respective lesions; (d) 3, 51%-75% of the epithelial cells in the respective lesions; and (e) 4, >75% of the epithelial cells in the respective lesions. The intensity was graded as follows: (a) 0, negative; (b) 1+, weak; (c) 2+, moderate; and (d) 3+, strong (as intense as normal mucosa). A final score between 0 and 12 was achieved by multiplication of the extent of positivity and intensity^[7,8]. Scores of 8-12 were defined as "strong expression", scores of 3-7 were defined as "reduced expression", and scores of 0-3 were defined as "markedly reduced expression". Positive staining more than 5% in cell cytoplasm was defined as positive staining; less than 50% in cell cytoplasm was defined as reduced expression; more than 50% in cell cytoplasm was defined as preserved expression^[9].

Statistical analysis

χ^2 test or Fisher's exact probability test and Spearman rank correlation coefficient analysis were used to assess the association between immunohistochemical features and clinicopathological characteristics. A *P* value less than 0.05 was considered statistically significant. All the statistical analyses were performed using the SPSS 10.0 V for Windows.

RESULTS

Expression of TGM3 in esophageal squamous cell carcinoma

TGM3 positive expression showed brown stained signals in the normal mucosa cytoplasm and reduced or positive expression in ESCC, with only a small number of expressions in cell membranes. No nuclear expression of TGM3 was observed in cells. The positive expression rate of TGM3 in 99 esophageal cancer patients was 60.6% (60/99). Significant positive correlation was found in TGM3 expression of the cases between paired normal

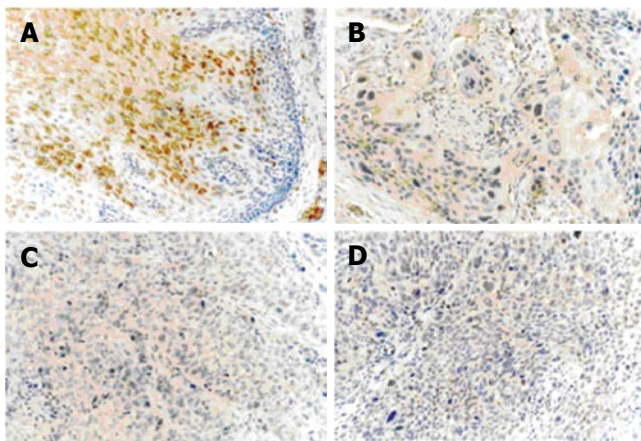


Figure 1 Immunohistochemical analysis of TGM3 in paired ESCC tissue samples using anti- TGM3 antibody (1:100). Diffuse and strong staining was detected in the cytoplasm of the normal epithelial cells (A), while sporadic and weak staining was observed in the cytoplasm of matched esophageal cancer epithelial cells (B: well-differentiated, C: moderately-differentiated, D: poorly-differentiated) (original magnification $\times 200$).

and cancerous tissue of esophageal carcinoma ($P < 0.05$). The reduced expression rate of TGM3 was 81.8% (81/99) (Figure 1).

Relationship between TGM3 expression and clinicopathologic variables in esophageal squamous cell carcinoma

Expression of TGM3 correlated significantly only with histological grade of esophageal squamous cell carcinoma. Significant inverse correlation existed between the intensity of TGM3 expression and histological grade ($P < 0.05$). No significant correlation was found between abnormal expression of TGM3 and lymph node metastasis and depth of invasion (Table 2).

DISCUSSION

Transglutaminase(TGM) enzymes are widespread in both plants and animals^[10,11]. They catalyze the formation of anisodipeptide cross-linking between the ϵ -NH₂ side chain of a protein-bound lysine residue and the γ -amide side chain of a protein-bound glutamine residue, thereby forming an insoluble macromolecular aggregate that is used for a variety of cellular functions. To date, there are nine known TGM enzymes encoded in the human genome^[12], and interestingly, three of them are active in the epidermis and its appendages. These include: the TGM1 enzyme^[13] which can function as membrane-associated^[14], soluble full-length, and soluble proteolytically activated processed forms in the epidermis^[15]; the soluble, "tissue" TGM2 enzyme^[16], and the soluble TGM3 proenzyme, which also requires proteolytic activation^[17,18]. The TGM3 enzyme is expressed during the late stages of the terminal differentiation of the epidermis and in certain cell types of the hair follicle^[19]. The enzyme is thought to be critically involved in the cross-linking of structural proteins and in the formation of the cornified cell envelope, thereby contributing to rigid structures that play vital roles in shape determination and/or barrier functions^[20-22]. Although TGM3

Table 2 Relationship between clinicopathological parameters and expression of TGM3

Type	Cases	TGM3		P
		Preserved	Reduced	
Histological grade				
I	7	3	4	< 0.05
II	71	14	57	
III	21	1	20	
Depth of invasion				
Mucous layer	5	0	5	> 0.05
Muscular layer	35	5	30	
Full-thickness	59	13	46	
Lymph node metastases				
Positive	32	6	26	> 0.05
Negative	67	12	55	

mRNA represents less than 2% of the TGM transcripts, the activated TGM3 accounts for up to 75% of the total TGM activity in mammalian epidermis^[15].

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. Therefore it is important to demonstrate what these oncogenes are and how they work in carcinogenesis. Identifying the genetic differences between normal and tumor cells or tissues will help discover the genes that directly cause tumor or are associated with tumorigenesis and provide novel markers for early detection and appropriate therapy.

Although the role of TGM3 has been well established in skin keratinocytes, little information is available concerning its involvement in esophageal epithelia. In previous study, TGM3 gene showed down-regulation in human ESCC tissues^[2,23]. To verify this differential expression, we first investigated immunohistochemically the expression of TGM3 protein in paired ESCC by means of TMA. The results of IHC revealed that TGM3 reduced expression in 81.8% (81/99) examined tumor tissues relative to the corresponding normal tissues. Among 99 esophageal tumors examined, which were histologically squamous-cell carcinomas, seven tumors were in grade I, 71 were in grade II and 21 were in grade III. Our data showed that decreased TGM3 expression was uncommon in 3 phases of primary esophageal cancer. This is in agreement with previous studies on a variety of cancers, such as laryngeal carcinoma^[24], head and neck squamous cell carcinoma^[25], oral squamous cell carcinoma from leukoplakia^[26]. In all these studies, reduction or loss of TGM3 expression was significantly associated with dedifferentiation, increased invasiveness, and high incidence of lymph node metastasis, hematogenous recurrence and poor prognosis in a number of human carcinomas. In our investigation, 81.8% of ESCC showed reduced expression of TGM3. As a marker associated with squamous cell histological grade, the level of TGM3 expression had an inverse correlation with histological grade, but not with lymphatic metastases and depth of tumor invasion. TGM3, therefore, is one of the most important adhesion molecules expressed by epithelial cells and is regarded as an invasion suppressor molecule. Further investigations are needed to predict the prognostic value of TGM3 for esophageal tumor patients.

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