

RESEARCH ARTICLE

Transmembrane Protein 166 Expression in Esophageal Squamous Cell Carcinoma in Xinjiang, China

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Abstract

Objective: Transmembrane protein 166 (TMEM166) expression in esophageal squamous cell carcinoma (ESCC) and remote normal esophageal tissues was examined to assess any role in tumour biology. **Methods:** TMEM166 mRNA expression in 36 cases with ESCC (36 tumour samples, 36 remote normal esophageal tissue samples) was detected by RT-PCR. TMEM166 protein expression was analysed in paraffin-embedded tissue samples from the same cases by immunohistochemistry. **Results:** Semi-quantitative analysis showed TMEM166 mRNA expression in ESCCs to be significantly lower than in remote normal esophageal tissues (0.759 ± 0.713 vs. 2.622 ± 1.690 , $P=0.014$). TMEM166 protein expression was also significantly reduced (69.4% vs. 94.4%, $P<0.01$). **Conclusion:** TMEM166 mRNA and protein expression demonstrated significant reduction in ESCCs compared with remote esophageal tissues, albeit with no correlation with tumour size, differentiation, stage, and lymph node metastasis, suggesting a role in regulating autophagic and apoptotic processes in the ESCC.

Keywords: Esophageal squamous cell carcinoma - transmembrane protein166 - apoptosis - autophagy - RT-PCR

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Introduction

China has a high incidence and mortality of esophageal squamous cell carcinoma (ESCC) (Guo et al., 2011). The ESCC incidence and mortality in China is half that of the world level, and is the fourth most reported malignant tumour in China (Liu et al., 2004). In 2002, there were 462,000 new ESCC samples worldwide, of which 243,854 samples were in China (Parkin et al., 2005). ESCC is the dominant type of esophageal cancer in Asia (Tomoyuki et al., 2009). Esophageal cancer could be caused by genetics acting in synergy with environmental factors (Sun et al., 2010).

The characteristic of transmembrane protein 166 (TMEM166) is a functionally unknown human gene (FLJ1339) discovered by the Peking University Center for Human Disease Genomics and the Chinese National Human Genome Center. TMEM166 shares no obvious homology to any known gene or protein in the GenBank databases. Human TMEM166 is located on chromosome 2p12, and encompasses four exons and three introns. The full-length cDNA is 1639 base pairs, and the open reading frame encodes 152 amino acids with a predicted molecular mass of 17.5 kDa and a 6.5 isoelectric point. TMEM166 contains a putative TM domain near the N-terminus (amino acid residues 34-56). TMEM166 is conserved in humans, chimpanzees, rats, mice, and dog, indicating that

it may have important functions in vertebrate animals.

RT-PCR analysis reveals that TMEM166 is expressed in various normal tissues (i.e., kidney, liver, lung, pancreas, and placenta), in all tumour tissues examined (i.e., gallbladder, colon, esophagus, liver, lung, kidney, rectum, and stomach), and in various cell lines (i.e., HeLa, MDA, HEK-293, A549, PC3, U937, Jurkat, and 293T). TMEM166-transfected HeLa and 293T cells succumbed to cell death because the TMEM166 overexpression markedly inhibited colony formation in HeLa cells. TMEM166-transfected HeLa cells showed typical morphological characteristics with autophagy at the start of cell death (20 h) and extensive autophagic vacuolisation. The cell organelle enclosure by double-membrane structures was observed by transmission electron microscopy. The overexpressed TMEM166 increased the punctate distribution of MDC staining and GFP-LC3 in HeLa cells, as well as the LC3-II/LC3-I proportion. Kinetic analysis revealed that the appearance of autophagy-related biochemical parameters preceded the nuclear typical changes of apoptosis in TMEM166-transfected HeLa cells. TMEM166 simultaneously induced programmed cell death I (apoptosis) and programmed cell death II (autophagy) (Wang et al., 2007).

Our study found that the expressed TMEM166 in remote normal esophageal tissues was significantly higher than that in ESCC by RT-PCR and IHC. The aim of this

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study was to determine whether a prognostic value exists in esophageal carcinoma.

Materials and Methods

Patients and tissue samples

Tissue specimens from 36 patients with ESCC, tumour tissues, and remote normal esophageal tissues belonging to the same inpatients, who underwent total thoracic esophagectomy in the Affiliated Tumour Hospital of Xinjiang Medical University from 2008, were used for IHC and RT-PCR analysis. The inpatients consisted of 22 males and 14 females, aged 37 to 78 years old (mean 57 years).

Semi-quantitative RT-PCR

Total RNA was extracted from homogenised specimens with TRIzol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The primer sequences and respected sizes of the PCR products of TMEM166 and β -actin genes are shown as CAAGGCCAACCGCGAGAAGATG (sense) and GTCCAGGGCGACGTAGCACAGC (antisense), with a 300 bp-long product; as well as CACCATGACAGACTGC (sense) and TCCCTAATAGTAGCGATTGAGGCTC (antisense), with a 330 bp-long . The PCR cycle numbers were both 40. The annealing temperatures were 58 °C and 57 °C. Amplified products were separated by electrophoresis with a 100 bp DNA Ladder (TaKaRa, China) in 1.5% agarose gels, and then visualised under UV light after ethidium bromide staining.

Immunohistochemistry

TMEM166 protein expression was analysed by immunohistochemistry (PV-6000). The slides were then incubated with optimally diluted monoclonal rabbit anti-human TMEM166 antibody (given by CHEN Yingyu in Peking University Center for Human Disease Genomics) as described in the manual. After three additional washes, peroxidase activity was developed with diaminobenzidine at room temperature. Finally, haematoxylin co-staining was performed.

Immunostaining positive signals were judged by second order to count scores. First, the TMEM166 expression level was graded on a scale of 1 to 4 as follows: 1, < 5% of cells positive; 2, \geq 5% to 25% of cells positive; 3, \geq 50% to 75%; 4, \geq 75% of cells positive. Second, the TMEM166 expression level was graded on a scale of 1 to 3 as follows: 1, pallid-flavens; 2, yellow and deep yellow; 3, brown and dark brown. If the first number multiplied by the second number was more than 1, the level was considered positive (Yu et al., 2005).

Immunohistostaining type

1) Diffuse type; 2) focus type; 3) mosaic (Lagergren et al., 1999).

Statistical analysis

Statistical analysis with t-test, pearson's chi-squared test, and exact probability were performed using the SPSS 13.0 software. $P < 0.05$ was considered significant.

Results

Expression pattern of TMEM166 mRNA transcript in ESCC and remote normal esophageal tissues

The percentages of positive cases for TMEM166 mRNA in 36 esophageal carcinoma tissue cases and in 36 remote normal esophageal tissue cases were 88.9% (34/36) and 100% (36/36), respectively. On the other hand, TMEM166 mRNA incidence was not statistically correlated with tumour size, differentiation, stage, and lymph node metastasis (Table 1). TMEM166 expression levels between ESCC and remote normal esophageal tissues were compared by digitalizing and normalizing the TMEM166 signals of the 36 tissue pairs against those of β -actin via semi-quantitative RT-PCR (Figure 1A) and densitometry. The average TMEM166/ β -actin ratio of tumour tissues was calculated to be 0.759 ± 0.713 , which is significantly lower than that of remote normal esophageal tissues (2.622 ± 1.690 ; $P < 0.01$; Figure 1B).

Expression of TMEM166 protein in ESCC detected by IHC

Among the 36 paired samples, the percentages of positive cases for TMEM166 protein detected by IHC were 69.4% (25/36) in esophageal carcinoma tissues and 94.4% (34/36) in remote normal esophageal tissues. The TMEM166 protein expression level was significantly lower in the tumour specimens than that in remote tissues

Table 1. Immunohistochemical Analysis of Human Esophageal Carcinomas

Clinicopathological parameters	case	TMEM166 +~+++	-	Positive rate (%)	P
Tumor size					
< 5cm	14	11	3	78.6	>0.05
\geq 5cm	22	14	8	63.4	
Differentiated degree					
Poor	12	8	4	75	>0.05
Well, Middle	24	17	7	70.8	
TNM stage					
I, II	13	8	5	61.5	>0.05
III	23	17	6	73.9	
Lymph node metastasis(N)					
N ₀	11	8	3	72.7	>0.05
N ₁₋₃	25	16	9	64	

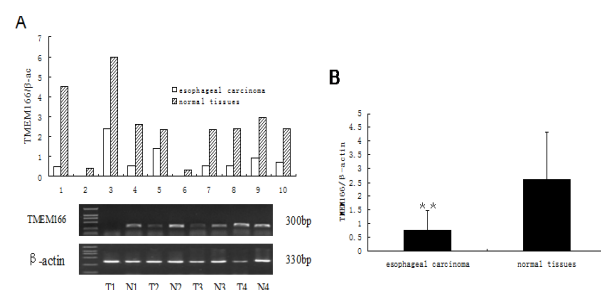


Figure 1. Expression of TMEM166 mRNA in Human Esophageal Carcinoma. (A) The expression levels of TMEM166mRNA in human ESCC (T1-T4) and in four representative of remote normal esophageal tissues (N1-N4) were detected by RT-PCR. β -actin was used as the internal loading control. (B) TMEM166 mRNA levels in 36 esophageal carcinoma samples and 36 remote normal esophageal tissues were analyzed. The difference is statistically significant (** $P < 0.01$)

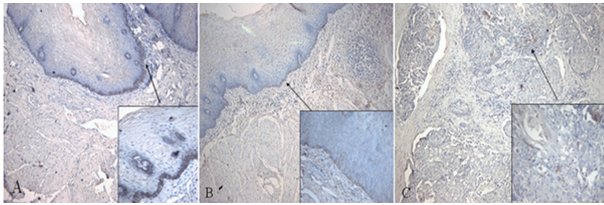


Figure 2. Representative Images of TMEM166 Immunohistochemical Staining in Remote Normal Esophageal Tissues and Esophageal Carcinoma Tissues (10 \times , 40 \times in the right corner). (A) Positive staining of TMEM166 protein was strong in the cellular membrane of normal tissues basal layer cells. (B) Weak staining was seen in the cytoplasm of intermedium cells and ecderson cells (C) weaker or no staining in carcinoma

by χ^2 test ($P < 0.01$). The TMEM166 expression is mainly located in the cytoplasm in stratum intermedium, normal esophagus epithelial tissue surface layer, cancer cells, and the cellular membrane in the esophagus epithelial tissue basal layer (Figure 2).

Discussion

TMEM166, a novel transmembrane protein that regulates cell autophagy and apoptosis, was detected by the Peking University Center for Human Disease Genomics, which focuses on the research of apoptosis-related gene and novel cytokines (Wang et al., 2007). As of this writing, studies on TMEM166 are limited to the primary stage, with the expression of TMEM166 in ESCC still unknown. Using RT-PCR and IHC, researchers can understand the relationships between the TMEM166 expression in ESCC and the histology grade and clinicopathological parameters. Using RT-PCR and IHC also identifies the characteristic of TMEM166 expression in ESCC, and determines the therapeutic value in esophageal carcinoma. Apoptosis or programmed cell death (PCD) is a genetically controlled program of cellular self-destruction, which is of central importance to the development and homeostasis of virtually all animals. PCD includes programmed cell death I (apoptosis) and programmed cell death II (autophagy) (Gozuacik and Kimchi, 2004). Autophagy in eukaryotic cells constitutes a degradative mechanism for the removal and turnover of bulk cytoplasmic constituents via the endosomal-lysosomal system. Autophagy is an adaptive cell response to nutrient deprivation to ensure the upkeep of minimal housekeeping functions. It is involved in physiological processes as diverse as biosynthesis, metabolism regulation through specific enzyme elimination, morphogenesis, cellular differentiation, tissue remodelling, aging, and cellular defence (Gozuacik and Kimchi, 2004; Lum et al., 2005; Mizushima, 2005). The biochemical mechanisms involved in autophagy remain largely unexplored in type II cell death. Complex interrelationships exist between the autophagic and apoptotic cell death pathways (Levine and Yuan, 2005). Several apoptosis regulators also play a role in autophagy (Arico et al., 2001; Yamada and Araki, 2001; Inbal et al., 2002; Shohat et al., 2002). Autophagy activation can either prevent or lead to apoptosis (Boya et al., 2005), which suggests that autophagy acts upstream of

the signalling transduction events leading to apoptotic cell death. Autophagy plays an essential role during starvation, cellular differentiation, cell death, and aging, as well as in preventing several types of cancer and disease (Klionsky et al., 2000). A novel anti-tumour and gene therapy was based on autophagy, so that TMEM166 can be used as a potential theoretical gene to regulate cell autophagy and apoptosis.

Cancers development and progress through the accumulation of various genetic alterations, and the inactive apoptosis mechanism is an important mechanism of ESCC development. RT-PCR was used to detect the mRNA of TMEM166 in ESCC and remote normal esophageal tissues. In this study, the TMEM166 mRNA expression was found in all tissues, except for one tumour tissue that was negative for TMEM166 immunostaining. However, the expressed TMEM166 strap brilliance in remote normal esophageal tissues was significantly higher than that in esophageal carcinoma. Semi-quantitative analysis showed that the TMEM166 mRNA expressions in esophageal carcinoma and remote normal esophageal tissues were 0.759 ± 0.713 and 2.622 ± 1.690 , respectively, with statistical significance ($P < 0.01$). RT-PCR analysis revealed a progressive decrease in the TMEM166 expression with increasing severity of esophageal lesions. TMEM166 expression was significantly higher in remote normal esophageal tissues compared with ESCC tissues. The TMEM166 protein expression levels in primary tumours and remote normal esophageal tissues were compared by staining tissues with 36 paired samples of ESCC and remote normal esophageal tissues using the anti-TMEM166 antibodies. The TMEM166 protein expression in esophageal carcinoma and remote normal esophageal tissues were 69.4% (25/36) and 94.4% (34/36), respectively ($P < 0.01$). The TMEM166 expression was mainly located in the cytoplasm of remote normal esophageal tissues stratum intermedium, surface layer, and in the basal layer cellular membrane. Immunostaining showed a stronger tendency in the cellular membrane of the basal layer cells. Weak staining was seen in the cytoplasm of intermedium cells and ecderson cells, whereas weaker or no staining was seen in the cytoplasm of ESCC. However, some tumour tissues were positive for TMEM166 immunostaining, which is not surprising. Tumour cell lines are immortalised and activated for proliferation, but tumour tissues consist of multiple cells in different cell cycle phases. Thus, tumour cell lines are usually different from tissues in numerous aspects. Protein translocation is relevant to the functional performances. TMEM166 was originally observed close to the inside surface of the cellular membrane, but was then transferred to the cytoplasm of normal esophageal epithelium tissue stratum intermedium and surface layer cells while being localised to the cytoplasm in tumour cells. TMEM166 induces autophagy and apoptosis, which play a significant role in cell death following MCAO injury, with mediation through the Bcl-2 crosstalk (Li et al., 2012). The cytoplasm translocation of TMEM166 protein results in enhanced autophagy and apoptosis. Thus, the TMEM166 expression incidence is not statistically correlated with tumour size, differentiation, stage, and lymph node metastasis. In

this study, we demonstrated that the TMEM166 mRNA or protein expression decreased significantly in ESCC compared with remote normal esophageal tissues. A low TMEM166 expression in tumours affects the tumour growth, including ESCC, by interfering with PCD and whether or not polymorphisms of TMEM166 playing a role like methylenetetrahydrofolate reductase (MTHFR) in pathogenesis of esophageal cancer in the Chinese population is still unknown (Fang et al., 2011). However, additional studies are needed to clarify this issue.

In conclusion, the functional polymorphism in TMEM166 gene plays a role in developing ESCC. Our results demonstrate a significantly lower TMEM166 expression in the ESCC sequence compared with remote normal esophageal tissue. The simultaneous down-regulation of TMEM166 mRNA and down-regulation of TMEM166 protein strongly suggest TMEM166 association with esophageal tumorigenesis, particularly the specific role of TMEM166 in the biology of ESCC. Although the TMEM166 expression did not influence the clinical outcome in our study, TMEM166 may still serve as a novel therapeutic target for cancer treatment. Further studies with a larger sample size, including all representative cancer patients, are needed to verify our observation.

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