Expression and Clinical Significance of TEM1 mRNA in Non-small Cell Lung Cancer*

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ABSTRACT Objective: To investigate the role of TEM1 as a therapeutic target in human endothelial cells. Methods: The mRNA expression levels of TEM1 in tumor and corresponding paratumor tissue of NSCLC in 56 cases were detected by reverse quantitative transcription polymerase chain reaction (QRT-PCR). And t tests were used to analyze statistical significances of their expression in different groups. Results: Analysis of 56 arrayed grade Ⅰ-Ⅲ NSCLC demonstrated TEM1 expression in all tissues. mRNA levels of TEM1 were higher in tumor tissue than those in corresponding paratumor tissue. TEM1 expression was correlated with lymph node metastasis and clinical stage ($P<0.05$), but was independent of histological type, patient's age and gender ($P>0.05$). Conclusion: The expression level of TEM1 is correlated to clinical stage in NSCLC. It's probable a valuable molecular marker involved in NSCLC invasion and metastasis. TEM1 expression profile in NSCLC supports efforts to therapeutically target this protein, might be potential targets for gene therapy.

Key words: Non-small cell lung cancer; Tumor endothelial marker 1(TEM1); Angiogenesis; RT-PCR

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Introduction

NSCLC is one of the most common cancers. The 5-year survival rate for all stages of NSCLC is 20-30%. Despite the advance in surgical resection coupled with systemic chemotherapy and radiation therapy, survival rates have not improved obviously in recent years. About half of newly diagnosed NSCLC patients will die of this disease duing to tumor recurrence and metastasis. The initiation, development, local invasion and distant metastasis for tumor are regulated by multiple genes, whose expression is determined by either internal or external factors. Therefore, elucidation of those factors and the pattern of their expression may help to understand the mechanism of carcinogenesis and metastasis.

A number of factors, including TEM1, have been shown to participate in cell migration and invasion. Tumor growth, metabolism, invasion, metastasis, and recurrence are closely related to tumor blood supply. Numerous clinical studies have demonstrated that the elevated expression of TEM1was strongly correlated with the density of tumor microvessels, the potential for malignancy and negative prognosis. TEM1 was identified in the tumor endothelium of human colon carcinoma [1]. TEM1 encodes a transmembrane glycoprotein and putative membrane receptor. Recent evidence suggests it may interact with extracellular matrix components including collagen I, collagen IV and fibronection, as well as Mac-2 BP/90K in promoting vascular migration and invasion [2,3]. Further analyses revealed selective TEM1 expression in tumor endothelium, pericytes and a subset of fibroblast-like cells of tumor stroma in breast carcinoma, anaplastic astrocytoma and GBM [4-9]. However, few studies to date have investigated the role of TEM1 in NSCLC. This study detected TEM1mRNA expression to investigate the possible implication in NSCLC.

1 Materials and Methods

1.1 Selection of NSCLC cases

NSCLC subjects in this study were 56 patients newly diagnosed based on the histopathology during the period of September 2009-December 2010 at the Affiliated Hospital Of Medical College Qingdao University. The average age at surgery was 52 years (range30-70 years). Tissue specimens, including tumor and corresponding paratumor tissue samples of NSCLC patients, were underwent surgical resection. Histopathologic types included adenocarcinoma (28patients, 50%) and squamous cell carcinoma (28patients, 50%). The 56 NSCLC patients all fulfilled the inclusion criteria of stage Ⅰ,II or III tumors (with no distant metastasis or M0). All the tumors were primary single NSCLC neoplasms. No patient had received antitumor treatment before surgery. The samples were frozen immediately and stored at -80 ℃ until use. Informed consent was obtained from all subjects.

1.2 Quantitative RT-PCR for TEM1

Total RNA was extracted from -80℃ freezed tumor and the relative normal samples, dissected from Surgery patients with lung cancer, using Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. Purity and quantity of the RNA was assessed by spectrophotometry (A260/280 greater than 1.8). Then

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total RNA was reverse transcripted into cDNA using the PrimeScript RT-PCR kit (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer's instructions. To assess the TEM1 gene expression, we used real-time fluorescence quantitative PCR analysis based on the TaqMan probe method. The primer sequences for the human gene were as follows: (TEM1) forward,5'-TCCTGGTGCCAACGTGTGT-3';reverse,5'-AGCAGTCAGTGATGCGCTTGT-3'; fluorescent probe, 5'-CCTGCTTGCACTGGGCATCGTGTAC-3'; And the amplicon size was 105 base pairs (bps). The GAPDH was used as the internal control with the specific primers: forward 5'-CTTAGCACCCCTGGCCAAG-3', reverse 5'-GATGTTCTGGAGAGCCCCG-3', fluorescent probe 5'-FAM-CAT-GCCATCACTGCCACCCAGAAGA-TAMRA-3', and the amplicon size was 150 bps. Primers and fluorescent probes were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai,China). The levels of TEM1 and control GAPDH mRNA transcripts were determined by the QRT-PCR in Light Cycler real time thermal cycler (Roche Diagnostics). The PCR reactions in duplicate were subjected to an initial denaturation at 95℃ for 10 seconds, followed by 40 cycles of denaturation at 95℃ for 5 seconds, annealing and extension at 60℃ for 45 seconds. The value of threshold cycle (CT) for each reaction was recorded. The relative expression level was computed using the 2-detla delta Ct analysis method, where actin was used as an internal reference [10].

1.3 Statistical Analysis

All data were shown by mean ± SE. Statistical analyses were performed using SPSS statistical software 17.0. Differences between two groups were assessed using a t test. A P value less than 0.05 was considered statistically significant.

2 Results

Following reverse transcription into cDNA, PCR product electrophoresis analysis clearly demonstrated a single TEM1 band at 105 bp, and GAPDH band at 150 bp (Figure 1), which were the expected sizes, suggesting high-specificity of the primer. Amplification curves of cDNA samples exhibited a standard S shape (Fig 2), suggesting a good amplification.

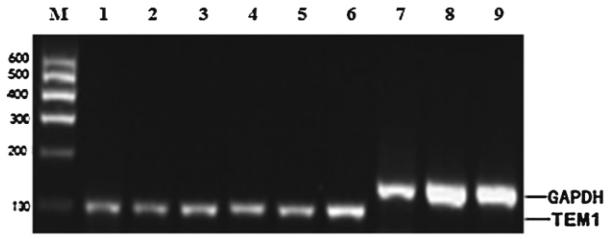
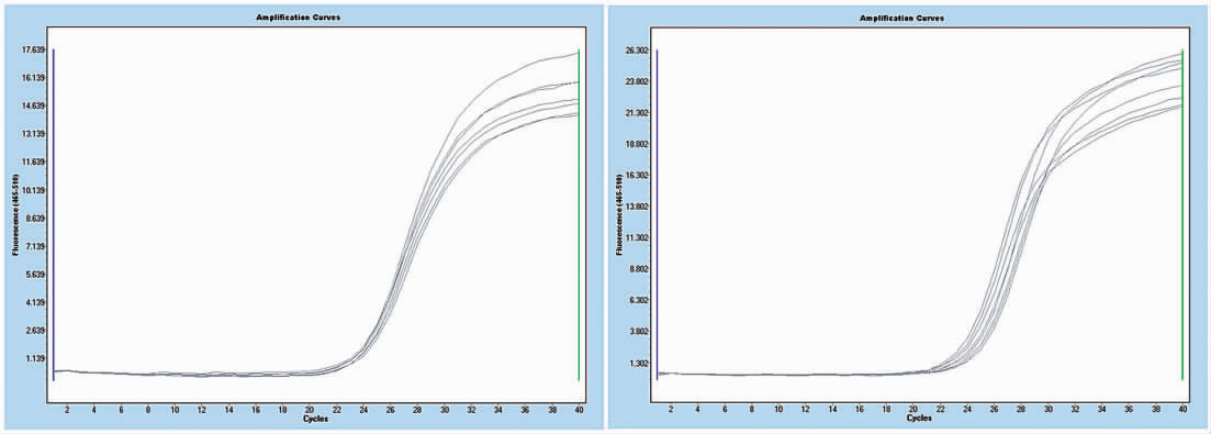


Fig.1 Gel electrophoresis of PCR products:M, size marker; 1, GAPDH; 2, TEM1.



A: GAPDH B:TEM1

Fig. 2 Amplification curve of GAPDH and TEM1.

2.1 High levels of TEM1 mRNA in tumor tissues

TEM1 mRNA was detected in all tissues. The results of QRT-PCR showed that mRNA levels of TEM1 were higher in tumor tissue than those in the corresponding paratumor tissue ($P<0.05$) (Table 1).

2.2 Correlation of TEM1 gene expression to clinical histopathological features of NSCLC

The relationship between the levels of TEM1 expression and various clinical histopathological features was analyzed, the result revealed that TEM1 expression level was correlated with lymph node metastasis and surgical pathological stage ($P<0.05$). The expression of TEM1 with lymph node metastasis was higher than that without lymph node metastasis, and the expression in stage III non-small-cell lung cancers was higher than in stage I-II. The level of TEM1 was independent of histological type, patient's age and gender(Table 2).

3 Discussion

Tumor growth, metabolism, invasion, metastasis, and recurrence are closely related to tumor blood supply. Solid tumors are dependent on angiogenesis growth when they reach a size of 1 or 2 mm^3 [11]. Tumor angiogenesis requires the participation of multiple

Table 1 Relative expression of TEM1 mRNA in different tissues.

Tissue type	Sample number(n)	TEM1 Relative to normal [a]	T	P
Tumor	56	1.3380± 0.2367	2.358	0.022
Paratumor	56	0.9012± 0.1498		

Note:[a]Data are expressed as the mean 2-△△CT(range).

Table 2 Correlation between TEM1△△CT values and clinical histopathological parameters

Pathological features	Case number	$2^{-\triangle\triangle CT}$	t	P-value
Gender			0.758	0.388
Male	29	1.3908± 0.4034		
Female	27	1.2812± 0.2396		
Tumor grade			6.148	0.016
1-2	25	0.6369± 0.1685		
3	31	1.9034± 0.3911		
Histopathology type			2.380	0.129
Squamous cell carcinoma	28	1.6378± 0.4288		
Adenocarcinoma	28	1.0381± 0.1943		
Lymph node involvement			6.571	0.013
Negative	25	0.5276± 0.1018		
Positive	31	1.9914± 0.3834		

angiogenic factors, the one important of which is TEM1. TEM1 gene mapped to chromosome 11q13. TEM1 encodes a 165 kDa single-pass transmembrane glycoprotein classified as a C-type lectin-like membrane receptor with homology to thrombomodulin and is conserved in humans and mice. Its extracellular part consists of five globular domains (N-terminal C-type lectin domain, sushi-like domain, and three epidermal growth factor (EGF)-like repeats) and a mucin-like region [12]. The antigen recognized by FB5 monoclonal antibody revealed an amino acid sequence identical to TEM1 and shown to specifically react with human tumor endothelium, pericytes and a subset of fibroblast-like cells within the tumor stroma[12, 13]. TEM1 knockout (KO) mice were fertile and appear to develop normally. However, when human HCT116 colon carcinoma cells were implanted orthotopically onto the serosal surface of the large intestine of nude TEM1 KO mice, both tumor take and growth were inhibited while the number of tumor microvessels increased [14]. St. Croix reported a series of up-regulated genes, tumor endothelial markers (TEM), differentially expressed z10-fold by tumor endothelial cells versus normal endothelial cells [1]. Little to no expression of TEM1 was noted in normal adult tissues. A recent study in breast cancer identified an association between TEM1 expression and progressive and/or recurrent disease as well as nodal involvement, all hallmarks of a more aggressive disease state [5]. Thus, TEM1 may have prognostic value for breast cancer. Recent accumulating evidences have shown that TEM1 had over-expression in a wide range of malignant cancer, such as breast cancer tissue and human brain tumors [5, 6]. In this study, TEM1mRNA was detected in all tissues. The mRNA levels of TEM1 in tumor tissue were 1.3380± 0.2367, while those in the corresponding paratumor tissue were 0.9012± 0.1498 ($P<0.05$). Expression levels of TEM1 in non-small cell lung cancer in the tumor tissue were higher than the corresponding paratumor tissue, which suggests that TEM1 may be resulted from the small cell lung cancer occurrence and development and the formation process of tumor blood essels has the important function.

Tomkowicz and colleagues have described TEM1 binding to fibronectin, as well as collagen I and IV, leading to vessel invasion, increased migration and differentiation of endosialin-expressing cells [2]. Further study has identified a C-terminal fragment of Mac-2 BP/90K as extracellular ligand of TEM1 which was weakly or no expressed in epithelial layers of most normal tissues and strongly up-regulated in the tumor cell compartment of most analyzed carcinomas [15]. The identification of Mac-2 BP/90K as a specific TEM1 ligand may provide an important functional link between the expression of TEM1 in the tumor stroma compartment and its role in metastatic spread. Recent studies have provided evidence for TEM1 expression in mesenchymal stem cells, endothelial progenitor cells, tumor pericytes, all of which contribute to tumor vasculature, and a subpopulation of tumor stromal cells [16-19]. In further study, the ubiquitous expression in vivo is in contrast to

previously reported expression limited to corpus luteum and highly angiogenic tissues such as tumors and wound tissue [20]. This may suggest that TEM1 plays not only a role in tumor-specific endothelium, but a more general role in vascular endothelium both in the adult and during embryonic development. It has become clear that there are quantitative differences between normal and tumor vasculature, evidenced by changes in the balance of angiogenesis inducers and inhibitors. We propose that it may simply be expressed at higher levels during the formation of new vessels in tumors. And it may play important roles in initiation and progression of cancers.

Expression analysis of TEM1 in NSCLC and their corresponding normal tissues has not been performed thus far. In this study, TEM1mRNA was detected in all tissues. The results of QRT-PCR showed that mRNA levels of TEM1 were higher in tumor tissue than those in the corresponding paratumor tissue ($P<0.05$). TEM1 expression level was correlated with lymph node metastasis and surgical pathological stage ($P<0.05$). The expression of TEM1 with lymph node metastasis was higher than that without lymph node metastasis, and the expression in stage III non-small-cell lung cancers was higher than in stage I-II, however, there were no significant differences in different histological types, patient's age and gender. Together, these data strongly suggest that TEM1 may contribute to the onset and development as well as invasion and metastasis of NSCLC.

This study confirmed quantitative differences of TEM1 at the mRNA level in NSCLC. TEM1 expression was associated with malignant tumor invasion and metastasis, suggesting TEM1 could be the marker for judging the metastatic potential of tumors. Since TEM1 expression is most likely elevated in tumor tissues undergoing formation and reorganization of vessels and the tumor vasculature is easily accessible for drug carriers circulating in the blood, it could provide a target for antiangiogenic therapy for malignant tumors.

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TEM1mRNA 在非小细胞肺癌中的表达及临床意义*

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摘要 目的:揭示 TEM1 其与非小细胞肺癌侵袭和转移的可能关系,为靶向治疗提供理想的药物作用靶点。方法:实时荧光定量 PCR 方法检测 56 例非小细胞肺癌肿瘤组织及癌旁组织中 TEM1 mRNA 表达水平,分析其在不同组中的表达差异。结果:TEM1 在 56 例非小细胞肺癌组织中都有表达。TEM1 表达水平在肿瘤组织中比癌旁组织表达高,并且其表达水平与淋巴结转移及肿瘤分期密切相关($P<0.05$),但与患者的病理类型,年龄及性别无关($P>0.05$)。结论:TEM1 表达水平与非小细胞肺癌分期密切相关,表明其可能是一个参与非小细胞肺癌侵袭及转移有价值的分子标记物。TEM1 可能成为潜在的基因治疗靶点。

关键词 非小细胞肺癌;TEM1;血管发生;实时荧光定量 PCR

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