CLINICAL PATHOLOGICAL SIGNIFICANCE OF EXPRESSION OF TSPAN-1, KI67 AND CD34 IN HUMAN LUNG CANCER

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ABSTRACT

Introduction: The overexpression of Tspan-1 has been found in human lung cancer (LC), however, its effects on LC was unclear. The aim of this study was to investigate the expression of Tspan-1, Ki67 and CD34 in LC and their clinical pathological significance.

Materials and methods: The expression of Tspan-1, Ki67 and CD34 was tested by immunohistochemistry on Paraffin-embedded sections of LC tissues; the correlation test among Tspan-1, Ki67, and CD34 expression was performed by Spearman test; the association of Tspan-1, Ki67 and CD34 expression with clinical pathological characteristics and prognosis of LC was analyzed by Kaplan-Meier survival analysis and Cox’s proportional hazards model.

Results: All cases were followed up 60 months. The overexpression of Tspan-1, Ki67 and CD34 was found in LC tissues, positively associated with each other (P <0.001) and correlated with clinical stage (P <0.05), however, negatively correlated with survival rate (P <0.05). Five-year survival rate was significantly lower in Tspan-1, Ki67, and CD34 overexpression group (log rank=10.877 p=0.001; log rank=6.62 p=0.010, log rank=9.306 p=0.002, respectively). All three markers were independent prognostic indicators for patients’ overall survival (P =0.015, P =0.002, P =0.004, respectively).

Conclusion: The expression of Tspan-1, Ki67 and CD34 is significantly associated with development of LC. Overexpression of Tspan-1, Ki67 and CD34 suggests poor prognosis.

Key words: lung cancer; Tspan-1; Ki67; CD34; Prognosis.

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Introduction

Lung cancer (LC) is one of the most common malignant cancers¹¹, the resection rate of which was about 20%. One of the principal reasons was delayed diagnosis. Most of them were confirmed in their advanced stage and the overall 5-year survival rate was very low about 10% or 14%¹². Most of the patients with LC, including the postoperative LC patients, need other therapeutic approaches such as radiotherapy and chemotherapy, but some disadvantages, such as side effects, which limited its applications. Gene diagnosis and gene therapy were emerging, as the times require.

Tspan-1 (Tetraspanins-1, Gene ID: 10103) is a new member of the tetraspanins group. Sequence analysis of Tspan-1 revealed a structure typical for tetraspanins, with the presence of four transmembrane domains delimiting two extracellular regions as well as conserved amino acid residues⁶. Tspan-1 had been found to be overexpressed in some tumors⁴. Studies reported that Tspan-1 expression might be associated with tumor cells proliferation, invasion and motility⁴, ⁹. All of these reports suggest that Tspan-1 may play a critical role in the progression of tumor growth and metastasis in numerous human tumors.
The antibody of Ki67, which was firstly prepared by Gerdes et al in 1983 to recognize the nuclear antigen Ki67(11), has been widely used to estimate growth fraction in different cancer lesions(12). Ki67, which is a non-Histone protein constituting part of the nuclear matrix during interphase and of the chromosome scaffold during mitosis, is expressed by cells in active phases of the cell cycle(10). Despite a large number of studies performed in lung cancer patients, the prognostic value of Ki-67 remains controversial(13, 14).

Angiogenesis, an essential process in progression of malignant tumors, is closely correlated with tumor growth and postoperative prognosis(15). Endothelial antigen CD34, which is also called Human hematopoietic progenitor cell antigen, is a direct marker of the degree of neoangiogenesis expressed mainly on hematopoietic precursor cells and vascular endothelial cells.

The aim of the present study is to determine the correlation of Tspan-1, Ki67 and CD34 expression, immunohistochemically detected in lung cancer tissue, with clinical pathological factors and survival of patients with lung cancers.

Material and methods

Clinical data of the patients

Sixty-three specimens were obtained from the patients with LC undergoing surgery of the lung in our hospital during 2005-2007. Of them, 47 patients were male and the other 16 were female, and the mean age was 58 year old (from 41 to 76). Histologically, 26 specimens were squamous cancer cell, 35 specimens were adenocarcinoma, only 2 specimens were small cell lung cancer; 10 cases were well differentiated, 38 cases were intermediate differentiated, and 15 cases were poorly differentiated. The follow-up period of the patients exceeded 60 months. Informed consent was obtained before each specimen was taken. The study protocol was approved by the Ethics Committee of the second affiliated hospital of Nantong University.

Antibodies used in immunohistochemistry

The antibodies used for immunohistochemistry in this study were: Tspan-1 rabbit anti-human polyclonal antibody (1:100; gifted by professor Li Chen, Department of Pathology, Nantong University, China, devised by professor Li Chen and prepared under the cooperation of the American San Francisco Gene Biological Company, San Francisco, CA, USA), mouse monoclonal antibody against human Ki67 (1:100, Lot:4081001, American San Francisco Gene Biological Company, San Francisco, CA, USA), mouse monoclonal antibody against human Primitive hematopoietic cells CD34 (1:100, Lot:50405117, American San Francisco Gene Biological Company, San Francisco, CA, USA), HRP-goat anti-rabbit IgG (1:200; Sigma-Aldrich, St. Louis, MO, USA), HRP-goat anti-mouse IgG (1:200; Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemical examination of expression of Tspan-1, Ki67, and CD34

Two-step immunohistochemical method was performed on formalin-fixed, paraffin-embedded 5-μm sections from all patients to detect the expression of Tspan-1, Ki67, and CD34 in LC. Five consecutive slides were prepared from each tissue. The slides were deparaffinized by dimethyl benzene, dehydrated by gradient ethanol. For antigen retrieval, they were heated at 95°C for 10 min in sodium citrate buffer (10 mM sodium-citrate mono-hydrate, pH 6.0). Slides were allowed to cool for 20 min at room temperature, incubated in 0.3% H2O2 at room temperature for 15 min to inhibit endogenous peroxidase, and then incubated with primary antibodies overnight at 4°C. Two-step reagent kit (HRP-anti-mouse/rabbit IgG) was then applied to detect the immunoreactivity. Slides were stained by DAB (diaminobenzine), and counter-stained by hematoxylin. Rabbit IgG was used to replace the primary antibody as control staining. Hepatocellular cancer tissue was used as positive control. For microscopy, five random high-magnification fields (×400) were selected from each slide to count 1,500 tumor cells.

Judgment and record of immunohistochemistry results

Cells with distinct brown cytoplasmic or membrane staining were judged to be Tspan-1 positive samples. For each sample, 10 random microscopic fields (×400), which correspond to approximately 2000 cells, were inspected. The samples were initially graded based on the percentage of positively stained cells: 0, positively stained cells <5%; 1, 6% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, >75%. Here the Tspan-1 expression was divided into two groups: low expression ≤50% and high expression ≥50%. Ki67 was expressed in nucleus of LC cell: low expression ≤25% and high expression ≥25%. The average number of CD34-positive capillaries and small venules was carefully counted in the
three areas of maximal vascularization under × 400 magnification. The counting standards for CD34-positive capillaries and small venules were that vascular lumen or modeling formed by CD34-positive endothelial cells clusters was counted as a single microvessel, excluding the vascular luminal area > 8 red blood cells in diameter, or the vessel wall had thick muscular layer, or a single CD34-positive cell. Average Microvessel density (MVD) was obtained by computer image analysis system in 3 microvascular intensive areas under High Power-view (HPV). MVD < 15/HPV was defined as CD34 low expression and MVD > 15/HPV as CD34 high expression. Capillaries in areas of lung parenchyma were counted and taken as the contrast to the estimated staining.

Statistical analysis
The data of the expression of the proteins are presented as means ± SD. Spearman test was applied to detect the correlation among Tspan-1, Ki67, and CD34 expression. Fisher’s exact test was used to compare the expression of all proteins as groups (positive vs. negative) with various clinical pathological parameters. Survival analysis was undertaken using Kaplan-Meier method and group differences in survival time were investigated by log-rank test. The multiple factor survival analysis was evaluated using Cox’s proportional hazards model. P values less than 0.05 was considered statistically significant. SPSS (Statistic Package for Social Science) for Windows (version 13.0, SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results
Expression of Tspan-1, Ki67, and CD34 in LC
The expression of Tspan-1 in LC cells varies from cytoplasm to membrane, or mixed. (Figure 1. Showed by arrow) High expression of Tspan-1 was detected in 40/63 (63.5%) of all cases. Ki67 were mainly expressed in nucleus. (Figure 1. Showed by arrow) High expression of Ki67 was detected in 45/63 (71.4%) of all cases. CD34 was expressed in microvessel of tumor stroma. (Figure 1. Showed by arrow) High expression of CD34 was detected in 41/63 (65.1%) of all cases. The expression levels of three proteins in lung cancer tissues were shown in Table 1.

Correlation of Tspan-1, Ki67, and CD34 expressed in LC
The correlation of expressions of Tspan-1, Ki67, and CD34 in LC was examined by Spearman test. Statistical analysis showed that Tspan-1 was positively associated with Ki67 (r=0.4692, P =0.0001< 0.001), and CD34 (r=0.5511, P=0.0000 <0.001). It also indicated that Ki67 was positively associated with CD34 (r= 0.4212, P =0.0006 <0.001). Correlations of the expression of Tspan-1, Ki67, and CD34 with clinical pathological factors in LC were listed in Table 1.

Table 1: The relationship between Tspan-1, Ki67, and CD34 expression and clinicopathological factors.

<table>
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<tr>
<th>Protein</th>
<th>Low</th>
<th>High</th>
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| Age     | 25  | 50   | 0.003 | 0.009 | 0.007
| Gender  | Male | 47  | 17   | 0.002 | 0.002
| Tumor size | 100 | 80< 15 | 7< 17 | 13< 11< 0.027 |
| Tumor location | 200 | 200> 12 | 5< 11< 0.116
| Necrosis | 0.449 | 0.003 | 0.250
| SCC     | 26  | 11   | 0.15 | 0.20
| AC      | 15  | 12   | 0.26 | 0.20
| SCLC    | 2   | 0    | 1.1  | 1.1
| Differentiation | 0.001< 0.001 | 0.390
| Histological type | 0.027< 0.001 | 0.887
| LN metastasis | 0.001< 0.001 | 0.687
| Yes     | 29  | 4    | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006
| No      | 4   | 15   | 11   | 21   | 17   | 17   | 17   | 17   | 17   |
The overexpression of Tspan-1 was negatively related to LC cell differentiation, clinical staging and lymph node metastasis (P < 0.05). The overexpression of Ki67 was negatively related to cancer cell differentiation and clinical staging (P < 0.05), and the overexpression of CD34 was negatively related to the size of lung cancer, clinical staging and lymph node metastasis (P < 0.05). There was no significantly difference between low expression and over expression of the three proteins in terms of age, gender, and the histology of lung cancer (P > 0.05) (Table 1).

**Correlation of the expression of Tspan-1, Ki67, and CD34 with the patients’ survival**

Survival analysis was done in 63 patients in whom follow-up data and results for the expression of Tspan-1, Ki67, and CD34 were available. By using the Kaplan-Meier analysis, the patients with Tspan-1, Ki67, and CD34 overexpression were significantly associated with short overall survival (log rank=10.877 p=0.001, log rank=6.62 p=0.010, log rank=9.306 p=0.002, respectively) (Figure 2A, B, C). 5-year survival in patients with Tspan-1, Ki67, and CD34 all high expression was significantly lower than patients with Tspan-1, Ki67, and CD34 not all high expression. (log rank=16.287, p <0.001)

**Discussion**

Tspan-1 was found to be over-expressed in some tumors, such as gastric cancer[4, 7], cervical carcinoma[9], colorectal adenocarcinoma[8,16], ovarian carcinomas[8], hepatocellular carcinoma[5] and skin squamous cell carcinoma[17], and associated with prognosis of these tumors. Latterly Chen et al[5] found that Tspan-1 protein expressed in normal lung tissue and LC tissue. In the present study, we found that the high expression of Tspan-1 was detected in 63.5% LC tissues. There is also a strong positive correlation between the level of Tspan-1 expression and degree of LC cell differentiation and clinical stages. The overexpression of Tspan-1 increased the risk of death. 5-year survival rate in patients with over-expression of Tspan-1 was significantly lower than those patients with low expression of Tspan-1. So Tspan-1 was an independent factor affecting prognosis of LC.

The nuclear antigen Ki67, which is expressed only in proliferating cells and has a marked role in maintaining the fidelity of DNA replication, is commonly used as a marker to evaluate proliferation of tumor cells. Scagliotti et al.[18] firstly reported that patients with higher Ki67 expression (>25% positive cells) had a significantly lower disease-free survival. Later, more studies investigated the role of Ki67 overexpression in NSCLC (non-small-cell lung cancer)[19, 20] showing that it was correlated with poor clinical prognosis.

In the present study, we have chosen the similar “cut-off point”: a percentage of stained tumor cells greater than 25%, and the results showed that the percentage of patients with high expression of Ki67 reached to 71.4%. A significant correlation between overexpression of Ki67 and lung cancer cell differentiation and clinical staging was found (P <0.05). 5-year survival rate in patients with overexpression of Ki67 was significantly lower than those patients with low expression of Ki67.

Tumor angiogenesis play a key role in progression, invasion, and metastasis of tumor[21]. High MVD and tumor vessel invasion have been shown to be closely related to poor survival[22]. MVD, as an index of the intensity of tumor angiogenesis, is
commonly marked by CD34. In this study, MVD ≥ 15 /HPV was defined as CD34 overexpression. In this cohort, the rate of CD34 overexpression in lung cancer was 65.1%. The overexpression of CD34 was negatively related to the size of lung cancer, clinical staging and lymph node metastasis (P <0.05). The survival curve revealed that 5-year survival rate in patients with CD34 overexpression was significantly lower than the low expressed group. So CD34 can objectively reflect the MVD in tumor, but also it can be an important marker for metastasis and prognosis of lung cancer.

Ki67 and CD34 have already been used as biomarker to estimate the prognosis of lung cancer for several years, however, Tspan-1 was report to correlate with the prognosis of some other tumors[4, 16, 17], and the association between expression of Tspan-1 and prognosis of lung cancer was reported rarely. The results of this study not only revealed that the overexpression of Tspan-1 was associated with the poorer prognosis of LC, but also its overexpression was positively associated with Ki67 (r=0.4692, P =0.0001< 0.0001), and CD34 (r=0.5511, P=0.0000 <0.001). The five-year survival rate of patients who suffered overexpression of Tspan-1, Ki67, and CD34 was the lowest among the groups (log rank=16.287, p<0.001). Combined with Ki67 and CD34, Tspan-1 can serve as predictive factor to estimate the postoperative prognosis of patients with lung cancer. Tspan-1, Ki67 and CD34 all overexpression suggests poor prognosis.

The limitations of our study are the small sample size and the length of the minimum follow up required. As far as the size of the study group is concerned, most of the patients suffered from NSCLC (non-small-cell lung cancer) except only 2 patients with SCLC (small cell lung cancer). Surgery was not recommended as the first choice for SCLC, so the specimen of SCLC was not so much as NSCLC. In the further research, more SCLC tissues, such as biopsy by punctured or under bronchoscopy need to be collected. Nevertheless, despite the relatively short follow up period, we were able to demonstrate that a significant impact of the overexpression of Tspan-1, Ki67, and CD34 could be the analyzed factors on 5-year survival for patients with lung cancer.

References

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