Thrombospondin 2 Gene Expression Is Correlated with Decreased Vascularity in Non-Small Cell Lung Cancer

Yoshiro Oshika, Keiko Masuda, Tetsuji Tokunaga, Hiroyuki Hatanaka, Takashi Kamiya, Yoshiyuki Abe, Yuichi Ozeki, Hiroshi Kijima, Hitoshi Yamazaki, Norikazu Tamaoki, Yoshito Ueyama, and Masato Nakamura

Department of Pathology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193 [Y. Os., K. M., T. T., H. H., Y. A., H. K., H. Y., N. T., Y. U., M. N.], and Department of Surgery II, National Defense Medical College, Saitama 359-8513 [T. K., Y. O.], Japan

ABSTRACT
Stromal vascularity is thought to be a major factor involved in the progression of carcinoma. However, the crucial mechanisms of vascularization in the stroma are not well understood. Vascularity could be regulated by various cytokines produced by neoplastic or stromal cells in carcinoma. Thrombospondin (TSP) has an inhibitory role against vascularization in vitro, although the biological significance of TSP has not been characterized in vivo. We examined expression of TSP1 and TSP2 genes in 78 non-small cell lung cancers (NSCLCs) and 33 extraneoplastic lung tissue samples by reverse transcription-PCR. TSP1 expression was detected in 66.7% (52 of 78) of NSCLCs and in 69.7% (23 of 33) of extraneoplastic lung tissue specimens. TSP2 expression was seen in 48.7% (38 of 78) of NSCLCs, whereas 72.7% (24 of 33) of extraneoplastic lung tissue samples showed TSP2 gene expression. TSP2 expression was significantly decreased in NSCLC as compared with extraneoplastic lung tissue (χ² test, P = 0.019). Vascularity in the NSCLC was inversely correlated with TSP2 gene expression (Mann-Whitney U test, P = 0.009). Patients with adenocarcinoma positive for TSP2 gene expression (22 of 49) showed significantly better prognosis than those without TSP2 (27 of 49; Cox-Mantel test, P = 0.034). TSP1 expression showed no apparent correlation with these factors. These results suggested that TSP2 had an inhibitory role against vascularization and progression of NSCLC.

INTRODUCTION
TSP is a family of glycoproteins that induces platelet aggregation and inhibits angiogenesis (1–3). TSP is secreted by platelets, fibroblasts, smooth muscle cells, monocytes, macrophages, and various neoplastic cells (4–7). Five members of the TSP family (TSP1–5) are encoded by independent genes (8, 9) and can be structurally divided into two groups. TSP1 and TSP2 are similar homotrimetric molecules, each containing a procollagen homology region and three properdin-like type I repeats (8), whereas TSP3, TSP4, and TSP5 lack these regions (10). TSP1 (11) and TSP2 (12) were shown to be inhibitory factors for angiogenesis due to the procollagen homology region and the type I repeats (13) in vitro. TSP1 shows inhibitory effects on angiogenesis and progression of bladder cancer (14), breast carcinoma (3), and glioblastoma (15), whereas the in vivo function of TSP2 is not well understood. In the present study, we examined gene expression of TSP1 and TSP2 in NSCLC and extraneoplastic lung tissues by RT-PCR and discuss their clinicopathological significance.

MATERIALS AND METHODS
Tumor Specimens. Seventy-eight NSCLC specimens and 33 extra-neoplastic lung tissue samples were obtained from surgical specimens. We prepared frozen sections to determine the neoplastic region in the surgical specimens and specifically took viable cancer materials and noncancer regions, avoiding necrotic or degenerative foci. Tissues were rapidly frozen and stored at −80°C until analyses. Total cellular RNA was prepared from the frozen specimens by standard procedures. Surgical specimens were also processed for routine histopathological analysis. The histopathology and number of the specimens were as follows: adenocarcinoma, 49; squamous cell carcinoma, 23; large cell carcinoma, 5; adenosquamous carcinoma, 1; stage I, 44; stage II, 8; stage IIIa, 23; and stage IIIb, 3.

TSP1 and TSP2 Gene Expression. We evaluated TSP1 and TSP2 expression by RT-PCR. Primers used were 5′-ACCGATTCCAGAGTCTGCC-3′ and 5′-ATGGGACGTCACAATCAGC-3′ for TSP1 (GenBank X04665, 131–623), and 5′-TCCTTCTCATGGTACAACCG-3′ and 5′-CTGTGTCAACAATCGCAGTGTC-3′ for TSP2 (GenBank L12350, 2066–2500). PCR consisted of 30 rounds of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min (Gene Amp PCR System 9600; Perkin-Elmer). Probes used for hybridization were prepared by PCR amplifi-

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2 To whom requests for reprints should be addressed, at Department of Pathology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan. Phone: 81-463-93-1121; Fax: 81-463-91-1370.

The abbreviations used are: TSP, thrombospondin; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR.

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cation with each primer set. Blots of products (Zeta-Probe; Bio-Rad) were hybridized with photochemically labeled cDNA probes. We confirmed the absence of the cross-hybridization with each probe. RT-PCR products of the housekeeping gene β2-m were examined as a control (Figs. 1C and 2C).

Analysis of Vascularization. Vascularization of cancer stroma was determined immunohistochemically. Tumor sections (formalin-fixed, paraffin-embedded) were deparaffinized and incubated with anti-CD34 antibody (NCL-end; Novo Castra, 1:20). Then, sections were incubated with biotin-labeled anti-mouse IgG (Nichirei, Tokyo, Japan) for 10 min and horseradish peroxidase-conjugated streptavidin (Nichirei) for 5 min. Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride. A computer image analysis system (Video Analyzing System, VIDAS; Carl Zeiss, Jena, Germany) was used for quantitative evaluation of vascularity in cancer stroma. The microvessel counts were evaluated at five distinct visual fields (0.739 mm²) with ×200 magnification.

Statistical Analysis. Differences in survival between subgroups of patients were compared with the Cox-Mantel test, and survival curves were plotted according to the method of Kaplan-Meier. The χ² test was applied for comparisons of frequency between two groups, and the Mann-Whitney test was used for analysis of vessel counts.

RESULTS

TSP Gene Expression. TSP1 gene expression was detected in 52 of 78 NSCLCs (66.7%; Fig. 1A) and 23 of 33 extraneoplastic lung tissue specimens (69.7%; Fig. 2A). TSP2 gene expression was detected in 38 NSCLCs (48.7%; Fig. 1B) and 24 extraneoplastic lung tissue specimens (72.7%; Fig. 2B). TSP2 expression was decreased in NSCLCs as compared with extraneoplastic lung tissue (χ² test, P = 0.019; Table 1). Expression of these genes was not apparently correlated with mRNA expression level evaluated by Northern blotting analysis (data not shown), and Southern blotting analyses showed neither amplification nor rearrangement of the TSP1 or TSP2 gene (data not shown).

Vascularization and TSP2 Gene Expression. Immunoreaction products for CD34 were detected on the walls of blood vessels, and vascularization in cancer stroma was quantified using the VIDAS system (Fig. 3, A and B; Carl Zeiss). Vessel count was significantly higher in TSP2-negative (78.0 ± 10.2/X200) than in TSP2-positive (28.2 ± 14.0/X200; P = 0.009, Mann-Whitney U test) NSCLCs. TSP1 gene expression showed no correlation with vessel count in cancer stroma.

Prognosis of TSP2-positive NSCLC. Patients with NSCLC positive for the TSP2 gene expression showed no significant difference in prognosis as compared with those without TSP2 expression. Adenocarcinomas positive for TSP2 expression (n = 22) showed a significantly better prognosis than those without TSP2 expression (n = 27; P = 0.034, Cox-Mantel; Fig. 4). TSP1 expression did not show any prognostic relevance. TSP1 and TSP2 gene expression were not correlated with differentiation or pathological stage of NSCLC.
DISCUSSION

Thrombospondin was first described as a glycoprotein secreted from α-granules of platelets. Five subtypes were identified and shown to be encoded by independent genes. TSP1 (11) and TSP2 (12) showed inhibitory activity against angiogenesis in vitro (13). TSP1 had inhibitory effects on angiogenesis and progression in various types of cancer (3, 14, 15), whereas the in vivo function of TSP2 is not well understood. In our study, TSP2 was detected more frequently in extraneoplastic lung tissues than in NSCLC. NSCLC cell lines also showed TSP2 expression at low frequency (two of eight cell lines, data not shown). The decreased TSP2 gene expression in stromal cells may contribute to progression of NSCLC.

TSP2 gene expression exhibited an inverse correlation with stromal vessel count and overall survival in adenocarcinoma of NSCLC. Vascularity in the cancer stroma is regulated by the balance between various angiogenic and angioinhibitory factors (16). It is supposed that TSP2 acts as an angioinhibitory factor in the NSCLC stroma, and we hypothesize that TSP2 gene expression in stromal cells may be decreased during the process of NSCLC development, resulting in skewing of the balance toward angiogenesis.

TSP1 and TSP2 gene expression were detected in a small number of normal colon mucosa and normal renal tissue.4 In contrast, extraneoplastic lung tissue frequently showed expression of TSP1 and TSP2 genes (69.7 and 72.7%, respectively). In our previous study, vascular endothelial growth factor was expressed in most extraneoplastic lung tissue specimens (17). We consider angiogenesis to be regulated by complicated mechanisms and the balance between levels of various molecules, including vascular endothelial growth factor and TSP2 in the lung.

We found no significant relationship between TSP1 gene expression and clinical or pathological features of NSCLC. Previous studies demonstrated inhibitory effects of TSP1 against angiogenesis (11, 13). On the other hand, TSP1 has been shown to promote angiogenesis under certain conditions in vascular smooth muscle cell lines (18, 19). The function of TSP1 in vivo is still unclear. It is unlikely, however, that TSP1 gene expression has a clinically significant role in NSCLC progression.

A recent study showed that the type I repeat of TSP1 induced apoptosis of endothelial cells (20). TSP2 also has a type I repeat and structurally similar domains to TSP1 (8) and can be secreted from the same cells (21). These molecules, however, have distinct biological functions (22) and are differently regulated (23). TSP2 may have important roles in the lung, especially as an angio-inhibitory factor preventing progression of NSCLC. Additional studies are necessary to clarify how TSP2 is involved in the development and progression of NSCLC.

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Fig. 3 Vascularity in NSCLC. Blood vessels were immunohistochemically demonstrated with anti-CD34 ($\times110$). A, NSCLC without TSP2 gene expression; B, NSCLC with TSP2 gene expression.

Fig. 4 Survival curve of adenocarcinoma according to TSP2 gene expression. Solid line, NSCLC with TSP2 gene expression (n = 22); broken line, NSCLC without TSP2 gene expression (n = 27).

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