Thrombospondin-1 and -2 Messenger RNA Expression in Invasive Cervical Cancer: Correlation with Angiogenesis and Prognosis

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ABSTRACT

Purpose: TSP association with clinicopathological features, including microvessel count, regarding prognostic significance was examined in patients presenting with invasive cervical cancer.

Experimental Design: Gene expression of TSP-1 and TSP-2 was assessed by reverse transcription-PCR in 10 normal cervix and 78 invasive cervical cancer samples.

Results: TSP-1 and TSP-2 mRNA expression was detected in seven (70.0%) of the normal cervical specimens. TSP-2 mRNA expression in normal cervix was significantly higher than that in cases involving cervical cancer (P = 0.032). TSP-1 mRNA expression was significantly lower in tumors characterized by advanced stage (P = 0.047). Fifty-three patients displaying stage Ib-IIb cervical cancer underwent radical hysterectomy and pelvic lymphadenectomy. Expression of TSP-1 and TSP-2 mRNA was significantly lower in tumors exhibiting parametrial invasion (P = 0.016 and P = 0.049, respectively). Microvessel counts were significantly higher when decreased TSP-1 expression was evident (P = 0.029). The microvessel count in patients lacking TSP-2 mRNA expression was higher than that observed in patients displaying TSP-2 mRNA expression, although it was not statistically significant (P = 0.062). Subjects demonstrating TSP-1 mRNA expression exhibited significantly better prognosis than those lacking TSP-1 mRNA expression (P = 0.0038). Furthermore, TSP-1 mRNA expression was an independent prognostic factor in the multivariate analysis.

Conclusions: These findings provide evidence that TSP-1 expression is of value as a prognostic factor in cervical cancer. The inverse correlation between TSP expression and microvessel count also indicates that decreased TSP expression may be associated with an angiogenic phenotype in this class of neoplasm.

INTRODUCTION

Disease stage is the most significant prognostic criterion in individuals presenting with invasive cervical cancer; however, patient survival after radical hysterectomy and pelvic lymphadenectomy is dependent on several factors. These factors include lymph node status, tumor size, paracervical involvement, depth of stromal invasion, and lymph vascular space invasion.

Recent studies, including our previous investigation, have shown that quantification of angiogenesis as measured by microvessel counts can be used as a prognostic factor in cervical cancer (2). Tumors are believed to secrete many angiogenic factors (3). Angiogenesis is regulated by the balance of a variety of angiogenic stimulators and inhibitors. We previously demonstrated that expression of VEGF and platelet-derived endothelial cell growth factor is involved in the promotion of angiogenesis in cervical cancer (2, 4).

TSP-1 is a high-molecular-weight, multifunctional glycoprotein, which was first described as a product of thrombin-stimulated platelets (5). TSP-1 is synthesized and secreted by various cell types, including fibroblasts, smooth muscle cells, monocytes, macrophages, osteoblasts, and neoplastic cells (6–12). Experimental evidence indicates that TSP-1 can exhibit adhesive as well as antiadhesive behavior (13). TSP-1 is also capable of fostering and retarding metastatic spread. In addition, TSP-1 can stimulate and inhibit angiogenesis as well as stimulate and reduce proteolytic activity and fibrinolysis. Presently, the role of TSP-1 in tumor angiogenesis and progression is controversial. The TSP family currently consists of five members, TSP-1, -2, -3, -4, and -5/COMP (cartilage oligomeric matrix protein). Homotrigenic TSP-1 and TSP-2 are structurally similar; however, they differ from pentameric TSP-3, -4, and -5 (14–16). In contrast, the role of TSP-2 in tumor growth and angiogenesis has scarcely been examined.

The biological role and expression pattern of TSP have not been characterized in cervical cancer. Therefore, we investigated the expression of TSP-1 and TSP-2 mRNAs in 10 normal cervix and 78 invasive cervical cancer specimens using semiquantitative RT-PCR. Moreover, the association of TSP-1 mRNA and TSP-2 mRNA expression with respect to clinicopathological features, including microvessel count, was determined. Additionally, we determined their prognostic significance for cervical cancer.

PATIENTS AND METHODS

Patients and Tissue Samples. The patient population for this study consisted of 78 individuals presenting with invasive cervical cancer (Department of Obstetrics and Gynecology of...
Okayama University Medical School, Okayama, Japan). Biopsy specimens were obtained before the initiation of treatment. Each specimen was snap-frozen and stored at $-80^\circ$C until required for RNA extraction. The second sample was fixed in 10% formaldehyde solution for histopathological examination.

Histological cell types of the tumors were assigned according to the WHO classification: 42 were classified as squamous cell carcinoma, 23 as adenocarcinoma, and 13 as adenosquamous carcinoma. Staging was reviewed based on International Federation of Obstetrics and Gynecology (FIGO) staging system: 23, 40, 11, and 4 were categorized as stage Ib, stage II, stage III, and stage IV, respectively. The median age at the time of treatment was 53 years (range, 26–90 years). Radical hysterectomy and pelvic lymphadenectomy were performed on 54 subjects demonstrating stage Ib-IIb disease but otherwise exhibiting good physical condition. Patients displaying lymph node metastasis, parametrial involvement, deep stromal invasion, or marked lymph-vascular space involvement were treated with adjuvant external whole pelvic irradiation (50 Gy) or adjuvant combination chemotherapy. The remaining 24 patients were treated primarily by radiotherapy or concurrent chemoradiotherapy. Disease-free survival was defined as the interval from the initial therapy to the recurrence or to November 30, 2000. The median duration of follow-up was 28 months (range, 3–59 months). Disease recurred in 25 (32.1%) of 78 patients. Additionally, 10 normal cervical specimens were obtained from patients demonstrating benign gynecological disease.

**RNA Preparation of Sample and RT-PCR.** Total RNA was prepared from each specimen with an RNeasy Total RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Tissues exhibiting RNA characterized by high quality 18S and 28S bands on ethidium bromide-stained gels were preferentially selected.

RT was conducted according to the Thermoscript RT-PCR System (Life Technologies, Inc., Rockville, MD) protocol for RT of 3 µg of total RNA. Transcribed products were subjected to PCR for TSP-1 (sense primer, 5'-ACCGCATTCAGAGTCTGGC-3'; antisense primer, 5'-ATGGGACCTGCAACACTACG-3'), TSP-2 (sense primer, 5'-CTGTGTAACACACTGCTGGC-3'; antisense primer, 5'-CCTCTTCTATCGGTACACC-3') and β-actin (sense primer, 5'-CTTACCATGGATGATGATAT-3'; antisense primer, 5'-TTGGCTCATCTTCGCGGTT-3' (17). TSP cDNA amplification was initiated with denaturation for 3 min at 94°C followed by 30 cycles of 1-min denaturation at 94°C. Annealing was then conducted at 60°C for 1 min, followed by a 1-min extension at 72°C. The PCR profile for β-actin consisted of an initial denaturation of 3 min at 94°C, followed by 30 cycles of 1-min denaturation at 94°C, 1-min annealing at 55°C, and a 1-min extension at 72°C. The PCR mixture was maintained at 72°C for 15 min for final extension. The details of PCR reaction mixture have been described elsewhere (18). Final PCR products were then electrophoresed on a 2% agarose gel and stained with ethidium bromide. UV-generated bands were photographed using Polaroid Type 667 films. Photographs were quantitated with an image scanner GT-9500 (EPSON, Suwa, Japan) and analyzed with Basic Quantifier software (Bio Image, Ann Arbor, MI).

To obtain the semiquantification of TSP mRNA levels, cDNA amounts were corrected by β-actin as an internal standard. For this reason, a technique based on a competitive PCR approach employing a nonhomologous internal standard was used (COMPETITOR; Competitive DNA Construction Kit; Takara, Kyoto, Japan). cDNAs derived from samples were coamplified in the presence of serial dilutions of β-actin COMPETITOR. The point of equal intensity between the bands of β-actin COMPETITOR and the cDNA template was evaluated. cDNAs in the presence of $1\times10^7$ copies of β-actin were subsequently used in the amplification of TSP genes. PCR products derived from TSP-1 genes were assigned to the strongly positive (+), positive (+), or negative (−) TSP-1 expressing groups. PCR products derived from TSP-2 genes were assigned to the positive (+), or negative (−) TSP-2 expressing groups as a result of few cases of strongly positive TSP-2 expression.

**Immunohistochemical Staining for Microvessels.** Expression of factor VIII-related antigen was assessed in formalin-fixed, paraffin-embedded sections via the ABC procedure. Briefly, anti-factor VIII-related monoclonal antibody (DAKO-PATTS, Copenhagen, Denmark) was used as a primary antibody. The entire tumorous lesion was scanned under low-power magnification to select regions displaying the most intense vascularization. Microvessel number was recorded by counting any positively stained endothelial cell or endothelial cell cluster as a single, countable microvessel in a $\times100$ microscopic field. The 10 most neovascularized regions were selected as a minimum. The mean of the top three counts was used as the microvessel count for each case. Microvessel number was determined by an investigator (N. S.) with no knowledge of the TSP mRNA levels.

**Statistical Analyses.** Univariate analysis included the Mann-Whitney $U$ and the Spearman rank correlation tests. Survival rates were calculated by the Kaplan-Meier method, and differences were examined by the log rank test. Factors found to be significant were then chosen for stepwise Cox’s multivariate proportional hazard model to ascertain their prognostic values. These analyses were performed using the Stat-View 5.0 software (Abacus Concepts, Berkeley, CA). $P$s less than 0.05 were considered statistically significant.

<table>
<thead>
<tr>
<th>Case</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tr>
<td><strong>TSP-1</strong> expression</td>
<td>2a</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2a</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>TSP-2</strong> expression</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

Fig. 1 Detection of mRNA for TSP-1 and TSP-2 in normal cervix and invasive cervical cancer. Total RNAs from tissues were extracted, transcribed to cDNAs, and subjected to PCR for TSP-1 (492 bp) and -2 (433 bp). Cases 1–3, normal cervix; cases 4–10, invasive cervical cancer.
TSP-1 and TSP-2 Expression in Cervical Cancer

RESULTS

Expression of TSP mRNA in Normal Cervix and Cervical Cancer. Fig. 1 displays representative photographs of the final RT-PCR products for TSP-1 and TSP-2 in normal cervix (cases 1–3) and invasive cervical cancer (cases 4–10). Expression of TSP-1 and TSP-2 mRNA was detected in 7 (70.0%) of 10 normal cervical specimens. TSP-2 mRNA expression of normal cervix was markedly higher than that of invasive cervical cancer (P = 0.032; Table 1). TSP-1 mRNA expression was significantly lower in advanced stage tumors (P = 0.047; Table 2). No meaningful differences in TSP-2 mRNA expression with respect to age, histological cell type, or clinical stage were observed.

Expression of TSP mRNA and Clinicopathological Features in Cervical Cancer Treated with Radical Hysterectomy and Pelvic Lymphadenectomy. TSP-1 and TSP-2 mRNA expression were significantly lower in tumors exhibiting parametrial invasion (P = 0.016 and P = 0.049, respectively; Tables 3 and 4). No correlation was present between TSP mRNA expression and other clinicopathological factors. Microvessel counts were significantly higher when decreased TSP-1 expression was evident (P = 0.029; Table 5). The microvessel count in patients lacking TSP-2 mRNA expression was higher than that observed in patients displaying TSP-1 mRNA expression; however, the result was not statistically significant (P = 0.062).

Association of TSP mRNA Expression with Survival. Fig. 2 presents the disease-free survival curves of 78 patients displaying invasive cervical cancer according to the TSP-1 mRNA expression status. Patients exhibiting TSP-1 mRNA expression demonstrated a markedly better prognosis than those lacking TSP-1 mRNA expression (P = 0.0038). There was no association between TSP-2 mRNA expression and patient outcome (Fig. 3). Furthermore, TSP-1 mRNA expression was an independent prognostic factor in the multivariate proportional hazard model (Table 6). Patient disease-free survival in 54 cases of cervical cancer treated with radical hysterectomy and pelvic lymphadenectomy was poorer in instances characterized by the absence of TSP mRNA expression. This finding, however, was not statistically significant (data not shown).

DISCUSSION

In the current study, we demonstrated higher TSP mRNA expression in normal cervical tissues in contrast to the lower levels observed in invasive cervical cancer specimens. This finding suggests that down-regulation of TSP expression may contribute to the progression of cervical cancer. TSP-1 expression has been shown to be linked to the p53 tumor suppressor gene (19). Wild-type p53 protein results in increased expression of p53 target genes (20) reported that the majority of tumors exhibiting low TSP expression were found to express altered p53 protein in bladder cancer. However, very low prevalence of p53 mutations has been documented in cervical cancer (21). In contrast, HPV DNA is identified as the causative agent in at least 90% of cervical cancer cases (22). These HPV oncoproteins, E6 and E7. Moreover, E6 protein stimulates rapid degradation of p53 (23). Therefore, we can hypothesize that HPV DNA might be involved in TSP down-regulation in invasive cervical cancer. In fact, Bequet-Romero and Lopez-Ocejo (24) recently showed lower levels of TSP-1 in...
and TSP-2 transcripts in HPV positive cells as compared to controls.

TSP mRNA expression was further examined so as to determine the correlation with clinicopathological features and prognosis in invasive cervical cancer. TSP-1 mRNA expression was shown to be significantly decreased in advanced cases. Because clinical stage strongly associates with prognosis, we surmise that the absence of TSP-1 mRNA expression may contribute to the progression of these tumors. However, conflicting results exist between TSP expression and tumor progression (20, 25–30). Bornstein (13) stated that resolution of these apparently conflicting conclusions can be found in differences in the nature and number of TSP receptors characteristic of the various tumors. The utility of TSP expression as a prognostic indicator also remains controversial. TSP-1 protein expression appears to be inversely correlated with prognosis in colorectal, oral, and bladder cancers (20, 25, 31). This finding was consistent with our study regarding the association of TSP-1 expression with favorable prognosis. We are able to use TSP-1 expression as a useful prognostic factor in patients presenting with invasive cervical cancer. However, a large cohort of patients are needed to confirm it.

### Table 4 Association between TSP-2 mRNA expression and clinicopathological factors in cervical cancer treated with radical hysterectomy and pelvic lymphadenectomy

<table>
<thead>
<tr>
<th>Variables</th>
<th>TSP-2 expression</th>
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<tr>
<td>Age</td>
<td>(−)</td>
<td>22/13</td>
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<tr>
<td>≥50</td>
<td>(+)</td>
<td>13/6</td>
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<td>Histological cell type</td>
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<tr>
<td>SCC</td>
<td>(−)</td>
<td></td>
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<tr>
<td>Non-SCC</td>
<td>(+)</td>
<td></td>
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<tr>
<td>Tumor size</td>
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<td></td>
</tr>
<tr>
<td>≤4</td>
<td>(−)</td>
<td></td>
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<tr>
<td>&gt;4</td>
<td>(+)</td>
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<tr>
<td>Stromal invasion</td>
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<td></td>
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<tr>
<td>≤2/3</td>
<td>(−)</td>
<td></td>
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<tr>
<td>&gt;2/3</td>
<td>(+)</td>
<td></td>
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<tr>
<td>Parametrial invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>(+)</td>
<td></td>
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<tr>
<td>Vaginal invasion</td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>(−)</td>
<td></td>
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<tr>
<td>Positive</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
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</tr>
<tr>
<td>Negative</td>
<td>(−)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>(+)</td>
<td></td>
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<tr>
<td>LVS involvement</td>
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<tr>
<td>Negative</td>
<td>(−)</td>
<td></td>
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<tr>
<td>Positive</td>
<td>(+)</td>
<td></td>
</tr>
</tbody>
</table>

*NS, not significant; SCC, squamous cell carcinoma; LVS, lymph-vascular space.

### Table 5 Association between microvessel count (MVC) and TSP mRNA expression in cervical cancer treated with radical hysterectomy and pelvic lymphadenectomy

<table>
<thead>
<tr>
<th>TSP mRNA expression</th>
<th>MVC</th>
<th>P</th>
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<tbody>
<tr>
<td>TSP-1 (−)</td>
<td>22</td>
<td>43.7 ± 17.7</td>
</tr>
<tr>
<td>(+)</td>
<td>23</td>
<td>33.4 ± 10.3</td>
</tr>
<tr>
<td>TSP-2 (−)</td>
<td>10</td>
<td>22.3 ± 10.6</td>
</tr>
<tr>
<td>(+)</td>
<td>40</td>
<td>40.5 ± 15.7</td>
</tr>
</tbody>
</table>

*NS, not significant; MVC, mean ± standard deviation.

### Table 6 Risk factors affecting disease-free survival determined by multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison</th>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th>P</th>
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<tr>
<td>Stage</td>
<td>I+II:III+IV</td>
<td>3.52</td>
<td>1.49–8.34</td>
<td>0.004</td>
</tr>
<tr>
<td>TSP-1 mRNA Positive:Negative</td>
<td>3.16</td>
<td>1.25–7.98</td>
<td>0.015</td>
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</table>
quently, this information could afford novel opportunities for therapeutic intervention in this disease. It has been proposed that TSP-1 functions as both a stimulator and an angiogenic inhibitor. TSP-1 possesses ligand-binding sites for CD36. CD36 was reported to be an essential mediator of antiangiogenic action on endothelial cells (32). TSP-1 also has ligand-binding sites for transforming growth factor β and α,β integrin, which are thought to be proangiogenic (13, 33). The effect of TSP-1 on angiogenesis has been reported to be concentration dependent. That is, at low concentrations, TSP-1 inhibits angiogenesis, whereas at high concentrations, TSP-1 stimulates angiogenesis (12). Recently, Taraboletti et al. (34) demonstrated the formation of two fragments (M, 25,000 and 140,000) exerting opposing actions on vascular endothelial cells following enzymatic proteolysis of the TSP-1 molecule. The heparin binding 25 kDa fragment is the angiogenic domain of TSP-1. On the contrary, the 140 kDa fragment retains the angiogenic inhibitory effect of TSP-1. In contrast, the role of TSP-2 in angiogenesis has been scarcely examined.

TSP gene or protein expression is reported to be significantly correlated with decreased microvessel counts in oral cancer, colorectal cancer, non-small cell lung cancer, glioma and bladder cancer (20, 25, 30, 31, 35–37). On the contrary, Bertin et al. (38) noted that TSP-1 and TSP-2 mRNA expression in desmoplastic-rich invasive breast ductal carcinoma coincided with a high microvessel density. Axelrod et al. (39) also reported that elevated TSP-1 expression was positively associated with higher microvessel counts in epithelial ovarian cancer. The effects of TSP in tumor angiogenesis may be dependent on tumor type and environmental setting. The present study demonstrated that loss of TSP-1 and TSP-2 is closely correlated with increased tumor vascularity in invasive cervical cancer. Streit et al. (40) reported that combined expression of TSP-1 and TSP-2 completely suppressed squamous cell carcinoma development. Therefore, a combination of these angiogenesis inhibitors may provide a new antiangiogenesis therapy in cervical cancer. Although the relationship between TSP and VEGF expression has been scarcely examined, we found that TSP-1 expression groups were significantly correlated with low VEGF expression levels in our previous investigation (4).

In conclusion, our findings provide evidence that TSP-1 expression can serve as an indicator representing less aggressive potential and favorable prognosis in cervical cancer. The inverse correlation between TSP expression and microvessel count also indicates that decreased TSP expression may be associated with an angiogenic phenotype in this class of neoplasm. As antiangiogenic molecules, TSP-1 and -2 will be novel candidates for therapeutic intervention in this disease.

REFERENCES


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