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Alterations in tumour suppressor gene p53 correlate with inhibition of thrombospondin-1 gene expression in colon cancer cells

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Abstract If activation of the *p53* gene is involved in the progression or metastasis of colon cancer, it may affect the angiogenic phenotype *in vivo*. To verify this hypothesis, we studied the correlation between *p53* accumulation and expression of thrombospondin-1 (*TSP1*) in colon cancer specimens. Levels of *TSP1* gene expression were estimated by Northern blotting in 65 colon cancers. Accumulation of *p53* and the distribution of *TSP1* protein were evaluated immunohistochemically. Various levels of *TSP1* gene expression were seen in colon cancers, while *p53* accumulation was confirmed in 42 of the 65 colon cancers. The level of *TSP1* gene expression demonstrated a significant inverse correlation with *p53* accumulation in colon cancer. Colon cancer cells expressed *TSP1* protein and *p53* accumulation reciprocally in the same nests. These results suggest that alterations in the tumour suppressor gene *p53* may inhibit *TSP1* expression in colon cancer.

Key words Thrombospondin-1 · *p53* · Colon cancer

Introduction

The *p53* gene is the most frequently altered tumour suppressor gene in solid human malignancies, which suggests a major role for *p53* protein in human carcinogenesis [8, 12]. Alterations of *p53* are also common genetic events in colon cancer [2, 36] and are frequently accompanied by alleic loss of 17p, a chromosome region containing multifunctional transcriptional factors. Both *p53* mutations and alleic deletion of 17p occur in association with tumour progression and are observed in 50–70% of these cancers [11, 29]. Activation or alteration of the *p53* gene may be involved in progression or metastasis in carcinoma. However, the precise association between altered *p53* and progression in colon cancer has not been determined [2, 11, 29].

The *p53* protein has been shown to change the phenotype of angiogenesis *in vitro*. Cultured fibroblasts with loss of the wild-type allele of the *p53* gene switched to an angiogenic phenotype coincident with a decrease in *TSP1* expression [4]. *TSP1* expression was also shown to be inversely associated with *p53* alteration in bladder cancer [9, 10]. *TSP1* is one of the components of the extracellular matrix in a wide variety of tissues [22, 23, 27]. It is a multifunctional glycoprotein, which is secreted from platelets [1, 17] and modulates platelet aggregation, wound healing, protease activity and cellular function [6, 15, 16, 18]. *TSP* is also synthesized and secreted by various types of cells: fibroblasts [15], smooth muscle cells [18], monocytes, macrophages [16], osteoblasts [6] and various neoplastic cells. *TSP1* has also been shown to modulate angiogenesis [7, 13, 20, 21, 38] and tumour progression [24, 34]. The *TSP1* induces the attachment and spreading of squamous carcinoma cells, melanoma cells and breast carcinoma cells [3, 26, 31, 32, 35]. Decreased *TSP1* gene expression is correlated with metastatic potential in melanoma, non-small-cell lung cancer, and breast carcinoma cell lines [39]. Transfection of *TSP1* cDNA into a human breast carcinoma cell line reduced primary tumour growth, metastatic potential, and angiogenesis [38]. Although the roles of *TSP1* in proliferation and angiogenesis in various cancers are controversial, recent studies have suggested that alterations of *p53* affect *TSP1* expression [4, 10].

It has been reported that loss of tumour suppressor gene expression and oncogene activation are both associated with the enhancement of angiogenesis [30]. Wild-type *p53* down-regulated endogenous vascular endothelial growth factor (VEGF) mRNA as well as VEGF promoter activity [19]. Activated *p53* may change the angiogenic phenotype by regulation of other angiogenic fac-

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tors. The purpose of this study was to investigate the correlation between *p53* status and *TSP1* expression in colon cancer.

Materials and methods

Specimens from 65 patients (28 female and 37 male, mean age 60.7) with colonic adenocarcinoma who underwent surgical resection between October 1989 and February 1992 at Tokai University Hospital were examined in the study. Samples from the surgical specimens were rapidly frozen and stored at –80°C until they were analyzed.

Levels of *TSP1* gene expression were evaluated by Northern blotting analysis. *TSP1*-specific cDNA probes (493 bp) were prepared by PCR amplification with primers Th1-S (X04665, 131–150), 5-ACCGCATTCCAGAGTCTGGC-3 and Th1-A (604–623), 5-ATGGGGACGTCCAACTCAGC-3, and their sequences were confirmed with a automated sequencer (ABI PRISM 310, Perkin Elmer, Calif.). Blots of total cellular RNA (15 µg, GeneScreen Plus, New England Nuclear) were hybridized with ^{32}P-labelled probes, and levels of *TSP1* gene expression were estimated by densitometry (Interactive Build Analysis System, Zeiss). We evaluated expression of the *TSP1* gene-specific transcript (4.4 kb) by autoradiography and also examined housekeeping gene expression by stripping and rehybridization of the blots with a β_2-microglobulin cDNA probe to control for the amount of RNA loaded in each lane. The β_2-microglobulin cDNA probes were prepared by PCR amplification with primers β_2-S, 5-ACCCCCACTGAAAAAG-ATGA-3 and β_2-A, 5-ATCTTCAAACCTCCATGATG-3, and their sequences were also confirmed with an automated sequencer (ABI PRISM 310, Perkin Elmer, Calif.). The levels of *TSP1* gene expression were estimated by densitometry (Interactive Build Analysis System, Zeiss) [14].

Formalin-fixed (10%), paraffin-embedded sections of the colon cancer were examined immunohistochemically to evaluate of *p53* and *TSP1*. Antigenicity of *p53* was retrieved by autoclaving at 120°C for 5 min. After blocking of endogenous peroxidase activity (methyl alcohol, 3% H_2O_2) and nonspecific binding (with 10% normal goat serum), specimens were incubated with mouse anti-human *p53* monoclonal antibody (DO-7, Novo Castra; 1:20), mouse anti-human *TSP1* monoclonal antibody (thrombospondin-Ab-1, Chemicon; 1:20) at room temperature. The sections were incubated with biotin-labelled anti-mouse IgG (Nichirei, Tokyo, Japan) and horseradish peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan). Reaction products were visualized with 3,3'-diaminobenzidine with H_2O_2. Accumulation of *p53* was determined by the degree of nuclear accumulation [5].

Double immunostaining was also performed to examine the histological distribution of *TSP1* expression and *p53* accumulation directly on the same sections. *TSP1* was detected by peroxidase reaction with amino-ethylcarbazole, after which *p53* was detected with the alkaline phosphate reaction with fast blue (Fig. 2).

Histological sections were cut from the centre of each colonic tumour, and stained with haematoxylin and eosin (H&E) and with Victoria blue to define the vascular wall. Histological examination was performed independently by two pathologists. The degrees of venous and lymphatic invasion were classified according to *a* previous study [33]. The degree of venous invasion (v-factor) was classified into four groups: v0, no venous invasion; v1+, minimal venous invasion, i.e. one or two foci of venous invasion in the histological sections; v2+, moderate venous invasion, i.e. three or four foci of venous invasion; and v3+, severe venous invasion with more than five invasion foci. The degree of lymphatic invasion (ly-factor) was also recorded: ly1+, mild lymphatic invasion; ly2+, moderate lymphatic invasion; and ly3+, severe lymphatic invasion.

Differences between the groups in the density of signals on Northern blots were analyzed by the Mann-Whitney U test.

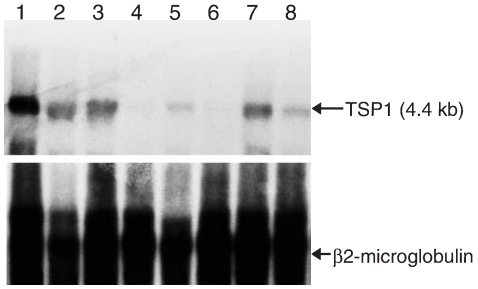

Fig. 1 Northern blotting analysis of *TSP1* gene expression in colon cancer specimens: *TSP1*-specific transcripts were detected (4.4 kb). Blots rehybridized with a β_2-microglobin probe as internal controls are also shown

Table 1 Associations between *TSP1* expression level and tumour characteristics (*v-factor;* degree of venous invasion, *ly-factor;* degree of lymphatic invasion)

	TSP1 expression level	
v-factor		*P*=0.187
v0, v1+	4.18±5.9	
v1+ <	2.25±4.6	
ly-factor		*P*=0.482
ly0, ly1+	3.67±5.7	
ly1+ <	1.43±2.7	
T grading		*P*=0.068
T0, T1, T2	6.89±7.0	
T3, T4	2.48±4.9	
N grading		*P*=0.063
N0	4.66±6.0	
N1, N2, N3	2.19±4.7	
p53 Accumulation		*P*=0.002*
Yes	1.95±4.4	
No	6.09±6.1	
K-ras Mutation		*P*=0.830
Yes	2.36±3.6	
No	3.83±6.3	
Liver metastasis		*P*=0.217
Yes	1.55±3.7	
No	3.91±5.7	

* Statistically significant (Mann-Whitney U test)

Results

We classified tumours as positive for *p53* overexpression when more than 5% of the tumour nuclei were positively stained [5]. Accumulation of *p53* was immunohistochemically confirmed in 42 of the 65 colon cancers. The average levels of *TSP1* gene expression (ratio to β_2-microglobin gene, *TSP1*/β_2 m) were $3.4\pm5.4\times10^{-3}$ (Fig. 1). The mean density of *TSP1* gene in colon cancers showing *p53* accumulation was $2.0\pm4.4\times10^{-3}$, while that in those without *p53* accumulation was $6.1\pm6.1\times10^{-3}$. Thus, *TSP1* gene expression level demonstrated significant inverse correlation with *p53* accumulation (*P*=0.0022, Mann-Whitney U test, Table 1).

TSP1 protein was detected in the colon cancer cells, while *TSP1* protein was predominantly distributed in the

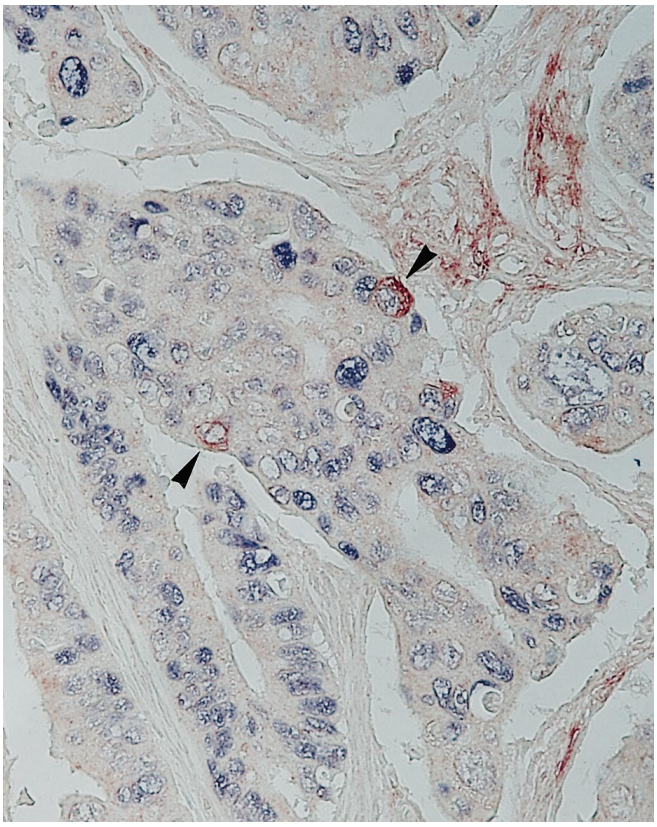

Fig. 2 Double-immunostaining analysis of *TSP1* and *p53*. Colon cancer tissues expressing the *TSP1* gene were double-immunostained with anti-*TSP1* and anti-*p53* antibodies. Colon cancer cells expressed *TSP1* protein (*red*, *arrowhead*) reciprocally in cells without *p53* accumulation (*blue*). ×300

stroma. Double-immunostaining indicated that colon cancer cells expressed *TSP1* protein and *p53* accumulation reciprocally in the same nests (Fig. 2).

TSP1 expression level was not correlated with histological type, venous involvement or lymphatic invasion. The *TSP1* expression level showed a trend towards lower grades (T0–2,N0) in the case of higher *TSP1* expression, with borderline significance. There were no correlations between *TSP1* and any TNM grade in these colon cancer patients (Table 1).

Discussion

Alterations of *p53* have been reported to be associated with poor prognosis in colon cancer patients in several studies, but contradictory findings have also been reported [2, 11, 29]. Alterations of *p53* are extremely rare in adenomas without severe dysplasia, but become more frequent with advancing progression to carcinoma. Recently, it has been reported that activated oncogenes and loss of expression of tumour suppressor genes such as *p53* might regulate some cytokines that are capable of modulating angiogenesis, including VEGF or *TSP1* [25, 30]. Tumour suppressor genes, including *p53* genes, were reported to affect angiogenesis through VEGF expression [28, 37].

Experiments on cultured fibroblasts from Li-Fraumeni patients have demonstrated that tumour cells switch to an angiogenic phenotype coincident with loss of the wild-type *p53* allele and a concomitant decrease in *TSP1* expression [4]. An inverse association was also reported between *TSP1* expression and *p53* alterations in bladder cancer [10]. In this study, we demonstrated that *TSP1* expression was inversely associated with *p53* accumulation in colon cancer. Moreover, we did not find the inverse association between the expression of *TSP2* gene, which is known to be the molecule most closely resembling *TSP1*, and *p53* in colon cancer (data not shown). This supports the earlier observation that *TSP1* expression was down-regulated by *p53* gene aberration [4, 10]. It has been suggested that *p53* accumulation is closely associated with angiogenic phenotype through *TSP1* expression in colon cancer.

The *TSP1* gene expression level was not significantly correlated with activated *c-K-ras* oncogene in colon cancers (data not shown). We also found that the level of *TSP1* expression determined by Northern blotting was more intense in the carcinoma than in extraneoplastic tissue. *TSP1* expression was reported to be increased in the stroma within and immediately adjacent to malignant tumours relative to that in normal mucosa [3, 9].

Decreased *TSP1* expression was correlated with higher T and N grades with borderline significance, which is consistent with the observation that reduced *TSP1* expression induced aggressive tumourigenicity *in vitro* [39].

The results of this study strongly support the concept that tumour suppressor genes such as *p53* affect the angiogenic phenotype by regulating the production of *TSP1*. This modulates cancer progression and/or angiogenesis.

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