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Tenascin XB Is a Novel Diagnostic Marker for Malignant Mesothelioma

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Abstract. *Background/Aim: Malignant mesothelioma (MM) is an aggressive tumor with poor prognosis. The establishment of a new diagnostic and therapeutic approach for MM is expected. This study investigated the diagnostic significance of tenascin XB (TNXB) for MM. Materials and Methods: TNXB gene expression was found to be significantly higher in MM tumor tissues compared to paired normal tissues, as assessed by the Gene Expression Omnibus database. The inhibition of TNXB using small interfering RNAs suppressed the proliferation and colony formation of MM cells. Expression of TNXB and calretinin, a current diagnostic marker of MM, was evaluated by immunohistochemistry. Results: The sensitivity and specificity of TNXB for MM were 80.0% and 69.5%, respectively. When the detection of TNXB was combined with that of calretinin, 83.3% of MM cases were detected. Conclusion: These findings suggest that TNXB is a novel diagnostic biomarker for MM. A combination of detecting TNXB and calretinin may be useful for the differential diagnosis of MM from lung adenocarcinoma.*

Malignant mesothelioma (MM) is a highly aggressive and fatal neoplasm. The prognosis remains unfavorable and the median survival after diagnosis is 9-12 months (1, 2). MM is typically associated with exposure to asbestos (3). The latency period of MM development is some decades from asbestos exposure. The number of patients with MM is forecasted to steadily increase because of past occupational asbestos exposure. In early stages of MM, surgical resection should be considered. However, it is difficult to completely remove MM because of invasion and metastasis. Standard chemotherapy with cisplatin and pemetrexed is recommended as first-line chemotherapy (4). The immune-check point inhibitor nivolumab is also approved for use as second-line therapy (5). However, the prognosis of MM is still unsatisfactory. Furthermore, the pathological diagnosis of MM is difficult, especially its differentiation from lung adenocarcinoma. Therefore, novel diagnostic and therapeutic strategies need to be established for MM.

We previously identified 18 transcripts, including osteopontin, that correlated with susceptibility to pemetrexed (6). Three of these 18 transcripts were identified as deriving from tenascin X-A and -B (*TNXA*/*TNXB*) genes. Tenascin is an extracellular matrix protein glycoprotein. TNX was reported as a differential diagnostic marker between MM and metastatic carcinoma, as well as between MM and ovarian carcinoma or primary peritoneal carcinoma (7). *TNXA* is a duplicated section of *TNXB* and is probably a pseudogene; however, the function and clinical significance of *TNXB* in MM has not been clarified yet. In this study, we investigated the potential diagnostic significance and therapeutic use of TNXB in MM.

Materials and Methods

TNXB gene expression analysis. Gene expression data in the GSE51024 study from the Gene Expression Omnibus (GEO) database was used (8). *TNXB* gene expression of malignant pleural mesothelioma (MPM) and paired normal tissues by Affymetrix U133 plus 2.0 chips was statistically analyzed (8).

Cell culture. Five human MM cell lines were used in this study. The normal mesothelium cell line Met5A was used as a standard control. NCI-H28, NCI-H2452, NCI-H2052, NCI-H226 and Met5A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). ACC-MESO4 was obtained from the Riken Cell Bank (Tsukuba, Japan) (9). Five human lung adenocarcinoma cell lines: A549, NCI-H441, HCC-827 with epidermal growth factor receptor (*EGFR*) exon 19 deletion and NCI-H1975 with *EGFR* exon 20 T790M mutation were acquired from the ATCC. LC2/ad was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK). These cells were maintained in RPMI-

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1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37˚C in an incubator with 5% carbon dioxide. All cell lines were routinely screened for the presence of mycoplasma by MycoAlert™ Mycoplasma Detection Kit (Lonza, Geneva, Switzerland).

RNA isolation and quantitative real-time polymerase chain reaction (PCR) analysis. Total messenger RNA was extracted from cells with TRIzol reagent (Thermo Fisher Scientific (Waltham, MA, USA) or ISOGEN (Nippongene, Tokyo, Japan) as previously described (10, 11). cDNA was synthesized from 1 μg of total RNA using Superscript Reverse Transcriptase (Thermo Fisher Scientific). Quantitative real-time PCR assay was performed using the TaqMan probe Human Gene Expression Assay (Thermo Fisher Scientific). The *TNXB* probe was Hs00372889_g1. Glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) was used as a normal control (Hs02786624_g1). Gene expression was quantified as the $2^{-\Delta\Delta Ct}$ value (12).

Oligonucleotide transfection. Gene silencing was performed by small interfering RNAs (siRNAs) purchased from Thermo Fisher Scientific. The predesigned siRNA targeting *TNXB* was s14302. Silencer Select Negative Control #1 siRNA was used as a negative control (Thermo Fisher Scientific). H2452 and H226 cells were seeded in a 6-well plate or 100 mm dish and transfected with siRNA using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) for 74 hours. The final concentration of siRNA was 25 nM.

Proliferation assay. Overall, 2,000 H2452 and H226 cells per well were plated in a 96-well plate. Cell proliferation was quantified by MTS assay using an absorbance reader. The absorbance measurement was performed every day. Cultures were grown for 3 or 4 days. Cell growth was assessed by the absorbance rate relative to the initial day's level.

Colony formation assay. Overall, 350 H2452 and H226 cells were seeded in a 6-well plate and incubated for 2 weeks and 3 weeks, respectively. Then the medium was aspirated. The wells were washed with phosphate-buffered saline and fixed with 99.8% methanol for 15 min. After staining with Giemsa for 15 minutes, the number of colonies was counted. A colony was defined as consisting of more than 50 cells.

Immunohistochemistry. TNXB and calretinin protein expression was evaluated by immunohistochemistry (IHC) using MM and lung adenocarcinoma tissue microarrays (TMA), MS801a and HLugA150CS02, respectively (US Biomax, Rockville, MD, USA). MS801a was composed of 30 MM cases and HLugA150CS02 included 75 lung adenocarcinoma tissues. Six bronchioalveolar carcinoma samples were excluded. Overall, 30 MM samples and 69 lung adenocarcinoma samples were finally evaluated. Slides were stained overnight at 4˚C with rabbit polyclonal antibody to human TNXB (ab198871; Abcam, Cambridge, MA, USA) at a final dilution of 1:50. Rabbit monoclonal antibody to human calretinin (MA5-14540) was used at a final dilution of 1:100 (Thermo Fisher Scientific). The slides were incubated with anti-rabbit IgG (BA-1000, Vector Labs, Burlingame, CA, USA) as a secondary antibody and the avidin-biotin complex method was performed as previously described (13). Images were captured by AX80 optical microscope (Olympus, Tokyo, Japan). Stained areas were measured by the LuminaVision program (MITANI Corporation, Tokyo, Japan) using three independent views of every sample. The cut-off point for the proportion of stained area was determined using the mean of three measurements by receiver operating characteristic (ROC) curves.

Table I. *Numbers of tenascin XB (TNXB)- and calretinin-positive and -negative cases by immunohistochemistry.*

		Calretinin, n	
	TNXB	Positive	Negative
Mesothelioma	Positive	5	19
	Negative	1	5
Adenocarcinoma	Positive	3	18
	Negative	1	47

Table II. *Sensitivity and specificity when using tenascin XB (TNXB), calretinin, and their combination as diagnostic markers for malignant mesothelioma.*

Marker	Sensitivity	Specificity
TNXB	80.0%	69.5%
Calretinin	20.0%	94.2%
TNXB+Calretinin	83.3%	68.1%

Statistical analysis. Continuous variable data are expressed by the mean and standard error (SE). Paired samples of MM and normal lung parenchyma in the dataset were compared by the Wilcoxon signed-rank test. Other data between two independent groups was evaluated by the Mann–Whitney *U*-test. All *p*-values were two-sided, and the significance level was set at less than 0.05. All statistical analyses were performed by JMP software (SAS Institute Japan, Tokyo, Japan)

Results

We evaluated the *TNXB* gene expression of malignant pleural mesothelioma (MPM) patients in the GSE51024 study from the Gene Expression Omnibus database (8). Figure 1A shows the *TNXB* gene expression level in MPM and paired normal tissues (n=41). *TNXB* gene expression levels were found to be significantly higher in MPM tissues than in paired normal tissues ($p<0.001$) (8). In the present study, we also evaluated *TNXB* expression using MM and lung adenocarcinoma cell lines. High *TNXB* expression was observed in three out of five MM cell lines. In contrast, five lung adenocarcinoma cell lines showed lower expression than Met5A (Figure 1B). These results suggest that high *TNXB* expression may contribute to the carcinogenesis of MM.

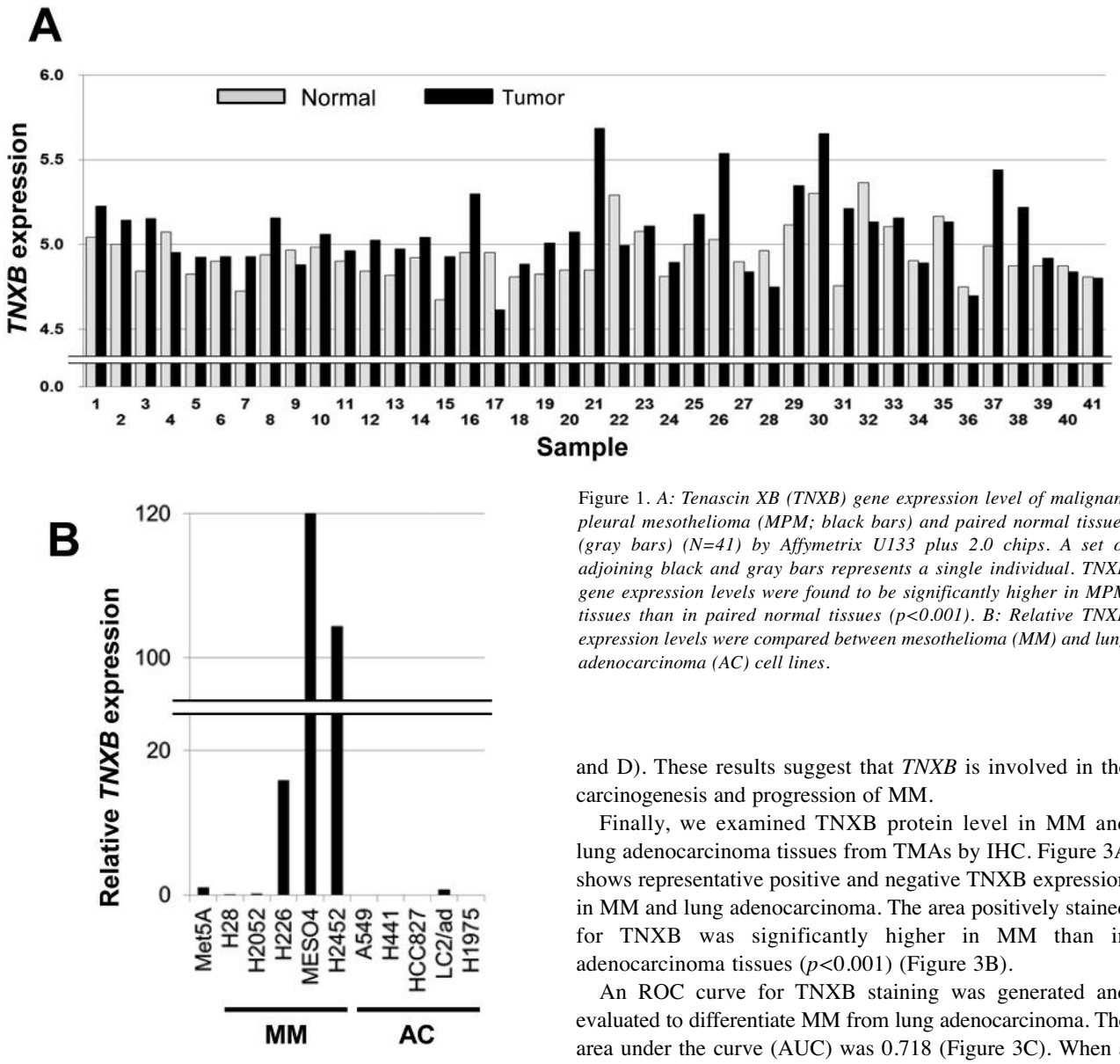

Figure 1. *A: Tenascin XB (TNXB) gene expression level of malignant pleural mesothelioma (MPM; black bars) and paired normal tissues (gray bars) (N=41) by Affymetrix U133 plus 2.0 chips. A set of adjoining black and gray bars represents a single individual. TNXB gene expression levels were found to be significantly higher in MPM tissues than in paired normal tissues ($p<0.001$). B: Relative TNXB expression levels were compared between mesothelioma (MM) and lung adenocarcinoma (AC) cell lines.*

Next, in order to clarify the significance of *TNXB* overexpression in MM, we evaluated whether *TNXB* inhibition affected the proliferation and colony formation abilities of MM. Figure 2A shows the successful suppression of *TNXB* in H2452 and H226 mesothelioma cells using siRNA transfection. After *TNXB* inhibition, proliferation was dramatically reduced on day 3 in H2452 and H226 cells (Figure 2B). A colony formation assay was also performed on H2452 and H226 cells after *TNXB* siRNA treatment. After the suppression of *TNXB*, the colony formation ability was significantly repressed in H2452 cells ($p<0.05$), and showed a tendency to be repressed in H226 cells ($p=0.12$) (Figure 2C and D). These results suggest that *TNXB* is involved in the carcinogenesis and progression of MM.

Finally, we examined TNXB protein level in MM and lung adenocarcinoma tissues from TMAs by IHC. Figure 3A shows representative positive and negative TNXB expression in MM and lung adenocarcinoma. The area positively stained for TNXB was significantly higher in MM than in adenocarcinoma tissues ($p<0.001$) (Figure 3B).

An ROC curve for TNXB staining was generated and evaluated to differentiate MM from lung adenocarcinoma. The area under the curve (AUC) was 0.718 (Figure 3C). When a positively stained area of 1.4% was established as the cut-off, 24 out of 30 MM cases and 21 out of 69 lung adenocarcinoma cases were TNXB-positive (Figure 3D) (Table I). The sensitivity was 80.0% and the specificity was 69.5% (Table II).

Calretinin is widely used in clinical settings as a diagnostic marker for MM. Therefore, we also conducted calretinin IHC staining in serial sections of TMA slides. Figure 3A shows representative positive and negative calretinin staining. When the calretinin-stained area was more than 0.80%, tissues tended to be judged visually as positive. As shown in Table I, 6 out of 30 MM cases had positive calretinin staining using TMA MS801a. In contrast, only four out of 69 adenocarcinoma cases were positive. The sensitivity and specificity for calretinin staining were 20.0% and 94.2%, respectively (Table II). Interestingly, TNXB was positive in 19

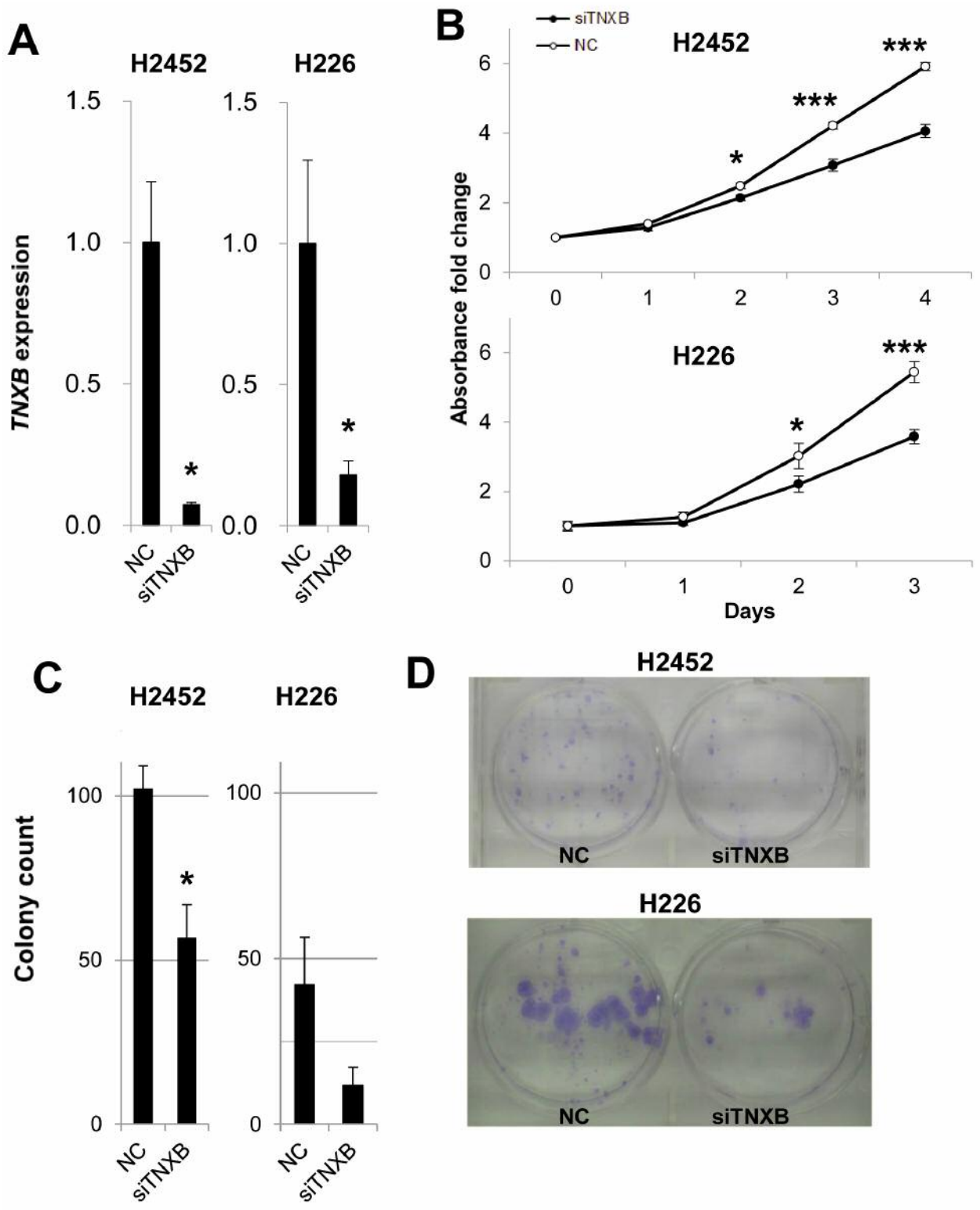

Figure 2. *A: Tenascin XB (TNXB) gene expression in H2452 and H226 cells after treatment with small interfering RNA against TNXB (siTNXB) and in negative control (NC) cells. siTNXB significantly suppressed TNXB expression (*p<0.05). B: Cell growth of H2452 and H226 cells significantly decreased after siTNXB treatment at *p<0.05 and ***p<0.0001. C: Colony formation after siTNXB treatment significantly decreased in H2452 and H226 cells (*p<0.05). D: Representative images of plates after siTNXB treatment of H2452 and H226 cells. Data show the mean±SE. Proliferation and colony formation assay were performed independently three times.*

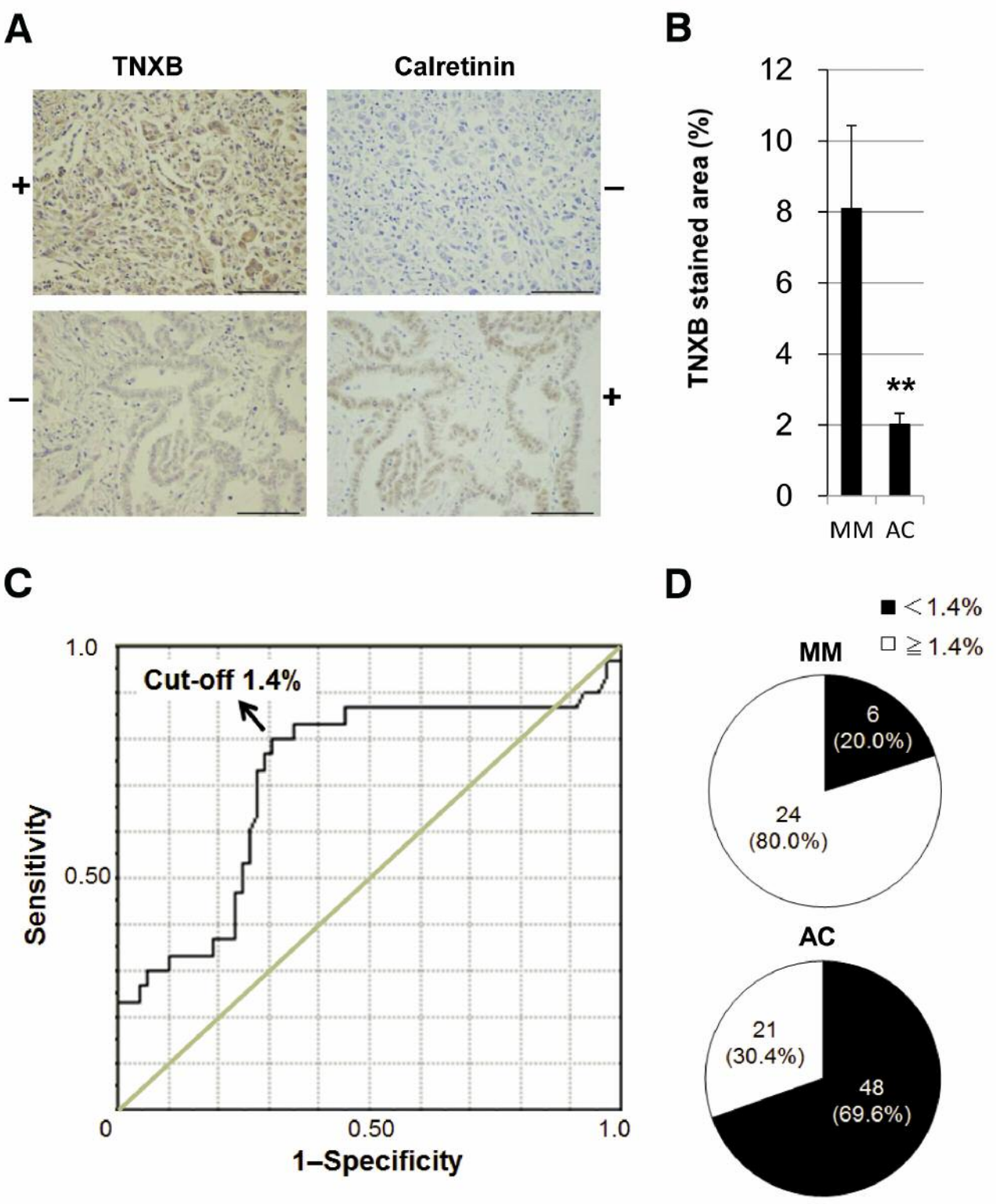

Figure 3. *Tenascin XB (TNXB) and calretinin protein expression in malignant mesothelioma (MM) and lung adenocarcinoma (AC) tissues by immunohistochemistry. A: Representative cases from MM are shown in the upper and lower panels. Scale bar=100 μm. B: TNXB expression by immunohistochemistry in MM and AC. The proportion of positively stained area in MM (n=30) was significantly higher than that for AC (n=69) (**p<0.001). C: Receiver operating characteristic (ROC) curve for TNXB staining for the detection of MM and AC. The area under the ROC curve was 0.718. D: The pie graphs show the result of classification into MM and AC using a cutoff value of 1.4% for TNXB staining.*

out of 24 MM cases without calretinin staining (Table I). In addition, TNXB was negative in only one out of four lung adenocarcinoma with calretinin-positive staining. When the detection of TNXB was combined with that of calretinin, 83.3% (25 out of 30 patients) of MM cases were detected. The sensitivity and specificity of the combination of TNXB and calretinin were 83.3% and 68.1%, respectively (Table II). These findings suggest that TNXB might be used as a diagnostic biomarker for MM. A combination of detecting TNXB with calretinin may be applicable for the differential diagnosis of MM from lung adenocarcinoma.

Discussion

In addition to the poor prognosis of patients with MM, the difficulty of diagnosing MM is a major clinical issue (14). In particular, the differential diagnosis of MM from the pleural involvement of lung adenocarcinoma is still difficult. Several pleural markers including calretinin, WT-1 and D2-40 are available for the diagnosis of MM; however, their sensitivity and specificity are poor (15-19). Therefore, novel diagnostic markers that distinguish MM from lung adenocarcinoma need to be established for the optimal treatment of patients. In this study, we found that TNXB was likely to be involved in the carcinogenesis of MM. The TNXB protein level also served as a diagnostic marker to distinguish MM from lung adenocarcinoma.

Tenascin is an extracellular matrix protein glycoprotein and has four gene family members, TNX, tenascin-C (TNC), tenascin-W, and tenascin-R (20). Tenascin-C is expressed in various cancer types including cholangiocarcinoma, gastric cancer, and breast cancer (21-23). Tenascin-C was also reported to be overexpressed in MM and be associated with metastasis (24-25). In contrast, TNX is involved in collagen organization and matrix integrity (26). TNX has two gene copies *TNXA* and *TNXB*. *TNXB* variants have been associated with Ehlers–Danlos syndrome (27). A previous study reported that *TNXB* mRNA expression was higher in MM compared with ovarian carcinoma, primary peritoneal carcinoma, and breast carcinoma (7). In this study, we found high *TNXB* expression in MM compared with paired normal tissues as well as lung adenocarcinoma. Although the sensitivity of calretinin for detection of MM in this study was lower than in previous studies, we demonstrated that the sensitivity of TNXB was superior to the sensitivity of calretinin as an MM diagnostic marker (15-19). Furthermore, TNXB combined with calretinin improved its diagnostic ability for MM. A definitive diagnosis of MM might allow optimal treatment for patients with MM.

Pemetrexed combined with cisplatin as first-line and nivolumab as second-line treatments are recommended as standard therapies for patients with MPM; however, their effectiveness is limited (4, 5); therefore, new therapeutic agents are required for patients with MPM. TNXB might be both a diagnostic marker and a therapeutic target for MM. In our previous analysis using MM cell lines, lower *TNXB* expression tended to indicate sensitivity to pemetrexed (6). We did not determine the significance of changes in sensitivity to pemetrexed after TNXB inhibition (data not shown). However, the suppression of cell proliferation and decreased colony formation after TNXB inhibition were observed in this study, suggesting TNXB might be a novel therapeutic target.

In conclusion, we found that TNXB is a novel diagnostic marker for MM. The combined use of TNXB and calretinin may be applicable for the differential diagnosis of MM from lung adenocarcinoma. Further studies should be undertaken to evaluate the usefulness and robustness of TNXB combined with calretinin as a diagnostic marker.

Conflicts of Interest

No potential conflicts of interest are disclosed.

Authors' Contributions

Conception and design: K. Nakayama, M. Seike, R. Noro, A. Gemma. Development of methodology: K. Nakayama, S.Takeuchi, R. Noro. Acquisition of data: K. Nakayama, M. Seike. Analysis and interpretation of data (*e.g.* statistical analysis, biostatistics, image analysis): K. Nakayama, M. Seike, R. Noro. Writing, review, and/or revision of the manuscript: K. Nakayama, M. Seike. Administrative, technical, or material support: R. Noro, K. Matsuda, S. Kunugi. Supervisor: K. Kubota, A. Gemma.

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