Intratumoral Wnt2B expression affects tumor proliferation and survival in malignant pleural mesothelioma patients

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Abstract. Malignant pleural mesothelioma (MPM) is an aggressive thoracic tumor with a poor prognosis. We performed a comprehensive clinical study on the intratumoral expression of Wnt1, Wnt2B and Wnt5A in MPM. One hundred and seven MPM patients were investigated. Immunohistochemistry was performed to evaluate the intratumoral expression of Wnt1, Wnt2B, Wnt5A, survivin and c-Myc, and the Ki-67 proliferation index. The apoptotic index was evaluated by the TUNEL method. Among the 107 MPMs, 23 MPMs (21.5%) were Wnt1-high tumors, 72 MPMs (67.3%) were Wnt2B-high tumors and 54 MPMs (50.5%) were Wnt5A-high tumors. There was no correlation among the levels of Wnt expression. The percentage of Wnt2B-positive tumors was significantly higher compared to that of the other Wnts ($p<0.0001$). Furthermore, intratumoral Wnt2B expression significantly correlated with the expression of survivin ($p<0.001$) and c-Myc ($p<0.001$). Regarding tumor biology, the Ki-67 proliferation index was significantly higher in the Wnt2B-high tumors than in the Wnt2B-low tumors ($p=0.0438$). In addition, the overall survival was significantly lower in patients with Wnt2B-high tumors than in those with Wnt2B-low tumors ($p=0.0238$). A Cox multivariate analysis also demonstrated the Wnt2B status to be a significant prognostic factor in MPM patients ($p=0.0042$). Intratumoral Wnt2B expression was associated with the expression of survivin and c-Myc, tumor proliferation and patient survival in MPM. Wnt2B is a potential molecular target for the treatment of Wnt2B-overexpressing MPMs.

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Introduction

Malignant pleural mesothelioma (MPM) is a thoracic tumor that arises from surface serosal cells of the pleura. It has been reported to be associated with inhalation exposure to asbestos (1). MPM is characterized by rapidly progressive and diffusely local growth, and a poor prognosis. Unfortunately, the incidence of MPM has been predicted to steadily increase and peak over the next two decades (2). However, there is no known curative modality for MPM (3), and long term survival is rare even with aggressive multimodal therapy including extrapleural pneumonectomy (4). Therefore, a new treatment strategy is required for MPM patients.

Among various molecules, the Wnt gene family encodes multi-functional signaling glycoproteins that are involved in the regulation of a wide variety of normal and pathological processes, including embryogenesis and tumorigenesis (5,6). Recently, we found that three members of the Wnt family, including Wnt1, Wnt2B and Wnt5A, are associated with tumorigenesis (7). In fact, many clinical studies have reported that overexpression of Wnt members is associated with various tumorigenic processes, such as proliferation, angiogenesis and patient survival (8-11). However, there are only a few clinical studies on the molecular biology of human MPM (12).

Therefore, to clarify the tumor biology of MPM, we performed a comprehensive clinical study on the intratumoral expression of Wnt1, Wnt2B and Wnt5A, in relation to the tumor-associated Wnt targets, survivin (13,14) and c-Myc (15).

Materials and methods

Clinical characteristics of patients. One hundred and seven consecutive MPM patients who were diagnosed at Kyoto University Hospital, Hyogo Prefectural Amagasaki Hospital, or Hyogo Prefectural Tsukaguchi Hospital, from January 1998 to December 2010 were studied. This study was approved by the Ethics Committee of Kyoto University, and informed consent was obtained from each patient. All tumors were clinically staged according to the IMIG staging system (16), and histological classification was based on the WHO classification (17). The clinical records and histopathological diagnosis

of all patients were fully documented. This report includes the follow-up data up to December 28, 2010.

Immunohistochemistry. The following antibodies were used: a rabbit polyclonal antibody for Wnt1 (H89; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:200, a rabbit polyclonal antibody for Wnt2B (LS-C31588; LifeSpan Biosciences, Seattle, WA, USA) diluted at 1.5 μg/ml, a goat polyclonal antibody for Wnt5B (C-16) diluted at 1:100, a mouse monoclonal antibody for survivin (sc17779) diluted at 1:50, a mouse monoclonal antibody for c-Myc (9E10) (all from Santa Cruz Biotechnology, Inc.) diluted at 1:100, and a mouse monoclonal antibody for the Ki-67 antigen (MIB-1; Dako, Glostrup, Denmark) diluted at 1:40. Formalin-fixed paraffin-embedded tissue was cut into 4-μm sections and mounted on poly-lysine-coated slides. After deparaffinization and rehydration, the slides were heated in a microwave for 10 min in 10 μmol/l citrate buffer solution at pH 6.0. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 min, the sections were treated with 5% bovine serum albumin. Duplicate sections were incubated overnight with the primary antibodies, respectively. Slides were then incubated for 1 h with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). The sections were incubated with the avidin-biotin-peroxidase complex (Vector) for 1 h, and antibody binding was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Lastly, the sections were lightly counterstained with Mayer's hematoxylin.

The immunostained sections were examined by two authors (M.K. and C.H.) without knowledge of the patient characteristics. Cases with discrepancies were jointly reevaluated until a consensus was reached. At least 200 cells were scored per x40 field about tumour cells. The percentage of carcinoma cells with positive staining for Ki-67 in a given specimen was scored as the Ki-67 proliferation index (9).

Detection of apoptosis. The TUNEL method was performed using the *In Situ* Apoptosis Detection kit (Takara Biomedicals, Otsu, Japan). After deparaffinization and rehydration, the sections were treated with 20 μg/ml proteinase K for 15 min. After quenching the endogenous peroxidase activity with 3% H_2O_2 for 5 min, the sections were incubated for 90 min at 37˚C with the TUNEL reaction mixture, including terminal deoxynucleotidyl transferase (TdT). Next, the sections were incubated for 30 min at 37˚C with anti-FITC horseradish peroxidase conjugate. Staining was detected by 3,3'-diaminobenzidine tetrahydrochloride incubation for 15 min. Lastly, the sections were lightly counterstained with Mayer's hematoxylin. In each case, a total of 10,000 tumor cells were evaluated by two authors (M.K. and C.H.) independently, without knowledge of the patient characteristics. The apoptotic index was defined as the number of apoptotic cells per 1,000 tumor cells (18).

Statistical analysis. The statistical significance of Wnt1, Wnt2B, Wnt5A, survivin, and c-Myc expression was assessed by t-test, ANOVA with Bonferroni/Dunn test or Pearson's correlation coefficient. The Kaplan-Meier method was used to estimate the probability of overall survival as a function of time, and differences in the survival of subgroups of patients were compared by using Mantel's log-rank test. A multivariate analysis was performed using the Cox regression model to study the effects of different variables on survival. The sample was classified as a Wnt1-high tumor when the percentage of Wnt1-positive tumor cells was >50%, and as a Wnt5A-high tumor when the percentage of Wnt5A-positive tumor cells was >30%, as reported previously (8,9). The sample was classified as a Wnt2B-high tumor when the percentage of Wnt2B-positive tumor cells was >50%, a nuclear survivin-high tumor when the percentage of nuclear survivin-positive tumor cells was >20%, and a c-Myc-high tumor when the percentage of c-Myc-positive tumor cells was >30%, as this had the highest significance value in relation to the Ki-67 proliferation index. The sample was classified as a Ki-67-high tumor when the Ki-67 proliferation index was >30% as this had the highest significance value in relation to patient survival. All p-values were based on two-tailed statistical analysis, and a p-value of <0.05 was considered to indicate statistical significance.

Results

Wnt1 expression in MPMs. The Wnt1 expression appeared in a cytoplasmic staining pattern (Fig. 1A). The percentage of Wnt1-positive tumor cells varied greatly among the MPM cases (median, 15.0%; mean ± SD, 25.9±26.4%) (Fig. 2). Regarding clinical and pathological characteristics, the percentage of Wnt1-positive tumors was significantly higher in stage I patients (p=0.0107) (Table I). However, no significant difference was observed in the Wnt1 status according to tumor histology.

Wnt2B expression in MPMs. The Wnt2B expression was also detected in a cytoplasmic staining pattern (Fig. 1B). The percentage of Wnt2B-positive tumor cells also varied greatly among the MPM cases (median, 60.0%; mean ± SD, 54.1±31.7%) (Fig. 2). No significant difference was observed in the Wnt2B status according to pathological stage and tumor histology (Table I).

Wnt5A expression in MPMs. The Wnt5A expression appeared in a cytoplasmic staining pattern (Fig. 1H). The percentage of Wnt5A-positive tumor cells varied greatly among the MPM cases (median, 30.0%; mean ± SD, 32.1±27.9%) (Fig. 2). The percentage of Wnt5A-positive tumors was significantly higher in stage I patients (p=0.0466) (Table I). However, no significant difference was observed in the Wnt5A status according to tumor histology (Table I).

Predominant expression of different Wnt proteins in MPMs. Among the 107 MPMs, 23 MPMs (21.5%) were Wnt1-high tumors, 72 MPMs (67.3%) were Wnt2B-high tumors, and 54 MPMs (50.5%) were Wnt5A-high tumors. There was no correlation between the expression levels of the different Wnt proteins. Furthermore, the percentage of Wnt2B-positive tumors was significantly higher than that of the other Wnt members ($p<0.0001$ vs. Wnt1 and $p<0.0001$ vs. Wnt5A) (Fig. 2).

Survivin expression in MPMs. Immunostaining of the antibody against survivin showed various patterns of nuclear staining

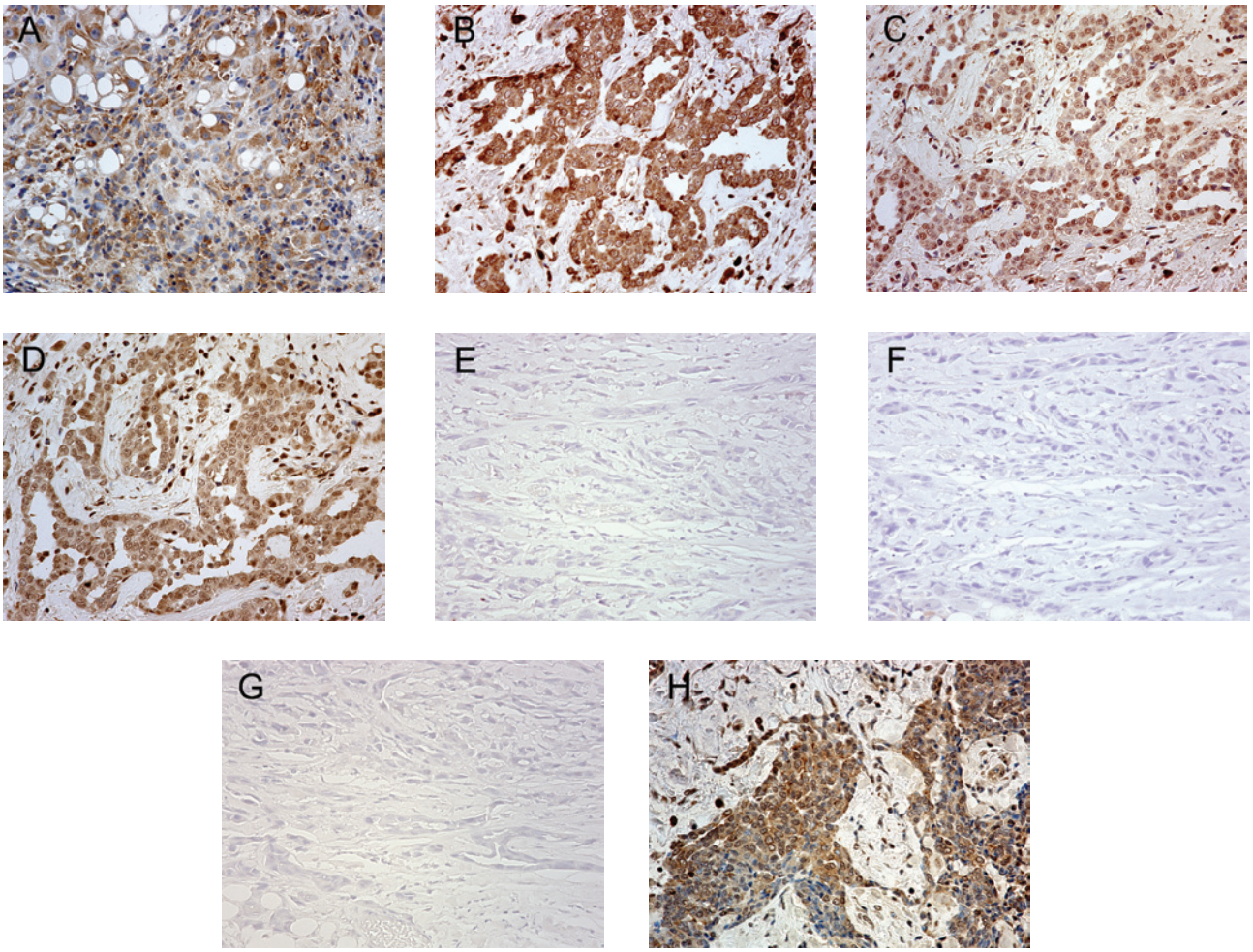

Figure 1. Immunohistochemical staining of human MPM tissue using the avidin-biotin-peroxidase complex procedure. (A) A Wnt1-high tumor. A tumor with positive expression of (B) Wnt2B, (C) nuclear survivin and (D) c-Myc. A tumor with negative expression of (E) Wnt2B, (F) nuclear survivin and (G) c-Myc. (H) A Wnt5A-high tumor. Original magnification, x200.

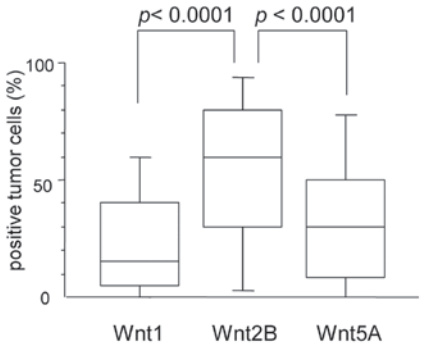

Figure 2. Distribution of Wnt1, Wnt2B and Wnt5A expression in 107 MPMs.

and cytoplasmic staining (Fig. 1C). The percentage of nuclear survivin staining was significantly higher than that of cytoplasmic survivin staining (27.2±30.2 vs. 15.6±25.6%, p=0.0025).

Regarding tumor biology, the Ki-67 proliferation index was significantly higher in nuclear survivin-high tumors than in nuclear survivin-low tumors (60.6±33.8 vs. 37.8±27.8%, p=0.0002) (Fig. 3A). In contrast, there was no difference in the apoptotic index according to the cytoplasmic survivin expression in MPMs (7.0±3.7 in cytoplasmic survivin-high tumors and 5.7±2.3 in cytoplasmic survivin-low tumors).

Regarding the Wnt status, the percentage of survivin-positive tumor cells significantly correlated with the percentage of Wnt2B-positive tumor cells (r=0.335, p<0.001). However, the percentage of survivin-positive tumor cells did not correlated with the percentage of Wnt1-positive tumor cells (p=0.1101) or the percentage of Wnt5A-positive tumor cells (p=0.4631).

c-Myc expression in MPMs. The percentage of c-Myc-positive tumor cells varied greatly among the MPM cases (median, 35.0%; mean ± SD, 37.3±30.3%) (Fig. 1D). Regarding tumor proliferation, the Ki-67 proliferation index was significantly higher in c-Myc-high tumors than in c-Myc-low tumors (53.8±31.4 vs. 39.7±31.7%; p=0.0231) (Fig. 3B). In contrast, there was no difference in the apoptotic index in relation to the c-Myc status in MPMs (5.2±2.5 in c-Myc-high tumors and 7.1±3.0 in c-Myc-low tumors).

In regards to the Wnt status, the percentage of c-Myc-positive tumor cells significantly correlated with the percentage of Wnt2B-positive tumor cells (r=0.364, p<0.001). However, the percentage of c-Myc-positive tumor cells did not correlated with the percentage of Wnt1-positive tumor cells (p=0.3937) or the percentage of Wnt5A-positive tumor cells (p=0.3498).

Table I. Expression of Wnt family members in 107 patients with malignant pleural mesothelioma according to clinicopathological characteristics.

	Wnt1		Wnt2B		Wnt5A	
	Positive tumors (%)	P-value	Positive tumors (%)	P-value	Positive tumors (%)	P-value
Age (years)						
<65	25.5±28.5	0.8504	55.7±33.8	0.6150	34.6±29.8	0.3764
≥65	26.4±24.4		52.6±29.7		29.8±25.8	
Gender						
Male	24.3±25.2	0.2947	54.3±31.4	0.9470	32.4±28.1	0.8772
Female	30.5±29.9		53.8±33.2		31.5±27.6	
Asbestos exposure						
Yes	19.7±22.6	0.0588	52.5±30.2	0.2384	28.3±27.4	0.1411
No	30.3±27.0		60.5±29.6		37.3±26.0	
Smoking						
Non-smoker	25.1±26.3	0.7582	55.7±30.6	0.6461	33.6±26.3	0.6194
Smoker	26.7±26.7		52.8±32.8		30.9±29.3	
Pathological stage						
I	44.6±32.3	0.0107	67.4±29.2	0.2180	46.5±27.1	0.0466
II	29.8±30.0		56.7±27.6		38.3±29.7	
III-IV	21.6±22.9		51.1±32.9		28.0±26.7	
Histology						
Epithelioid	29.2±28.5	0.2649	58.6±31.0	0.1654	34.9±29.0	0.4713
Sarcomatoid	19.1±19.8		47.0±30.1		32.9±25.5	
Biphasic	27.5±26.3		54.9±32.8		23.4±23.4	
Desmoplastic	13.1±21.5		33.9±34.9		32.1±36.3	
Total number of patients	25.9±26.4		54.1±31.7		32.1±27.9	

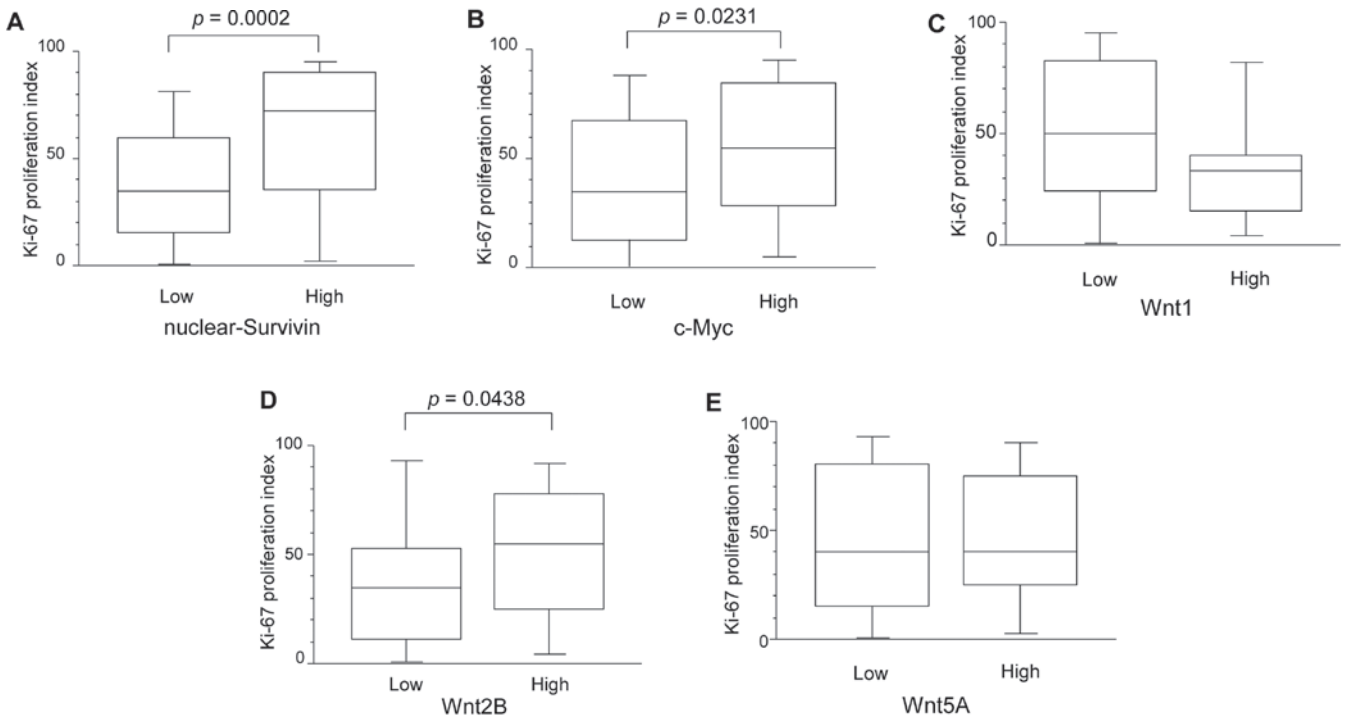

Figure 3. Ki-67 proliferation index of MPMs in relation to (A) nuclear survivin, (B) c-Myc, (C) Wnt1, (D) Wnt2B and (E) Wnt5A status.

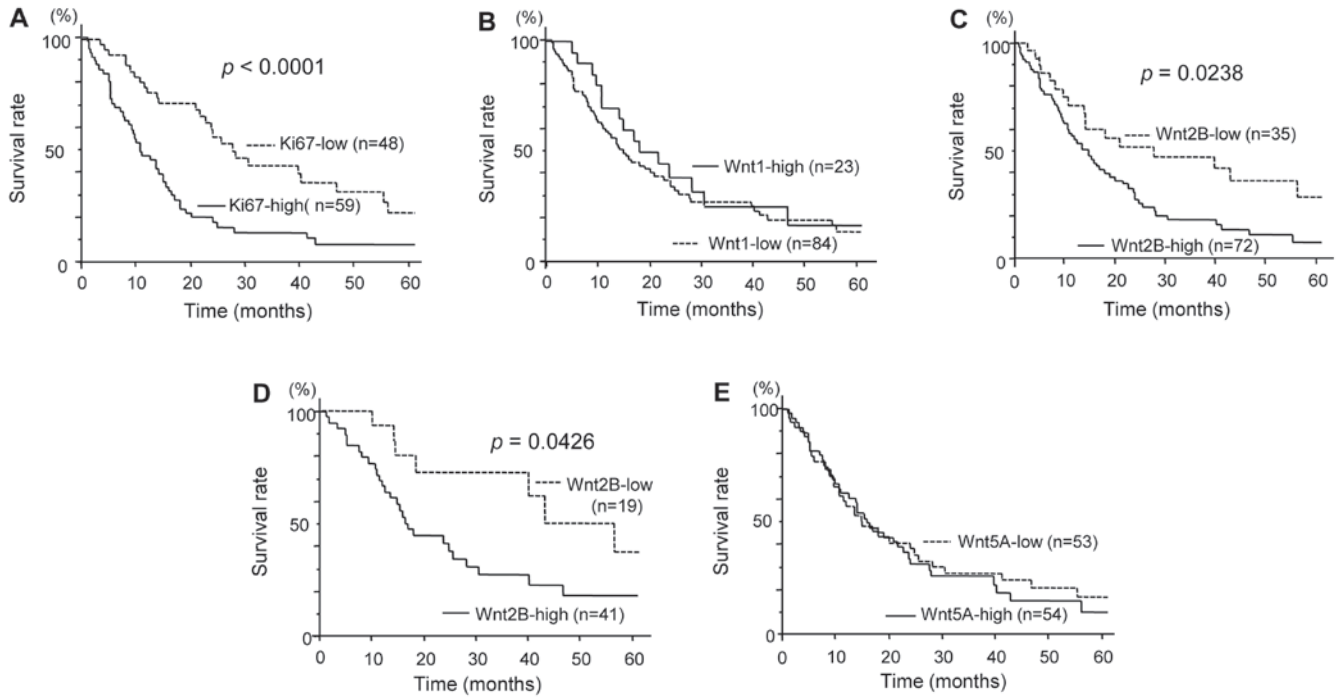

Figure 4. (A) Overall survival of 107 MPM patients in relation to Ki-67 proliferation index, (B) overall survival of 107 MPM patients in relation to Wnt1 status, (C) overall survival of 107 MPM patients in relation to Wnt2B status, (D) overall survival of 60 patients with epithelioid tumors in relation to Wnt2B status, (E) overall survival of 107 MPM patients in relation to Wnt5A status.

Tumor biology and intratumoral Wnt1 status in MPMs. There was no difference in the Ki-67 proliferation index in relation to the Wnt1 status in MPMs (35.4±28.2% in Wnt1-high tumors and 50.1±32.7% in Wnt1-low tumors) (Fig. 3C). Furthermore, there was no difference in the apoptotic index in relation to the Wnt1 status in MPMs (11.2±6.0 in Wnt1-high tumors and 4.7±1.8 in Wnt1-low tumors).

Tumor biology and intratumoral Wnt2B status in MPMs. Regarding tumor proliferation, the Ki-67 proliferation index was 51.3±31.4% in Wnt2B-high tumors, and 37.9±32.4% in Wnt2B-low tumors (Fig. 3D). The Ki-67 proliferation index was significantly higher in Wnt2B-high tumors than in Wnt2B-low tumors (p=0.0438). In contrast, there was no difference in the apoptotic index in relation to the Wnt2B status in MPMs (7.7±2.8 in Wnt2B-high tumors and 2.9±1.3 in Wnt2B-low tumors).

Tumor biology and intratumoral Wnt5A status in MPMs. There was no difference in the Ki-67 proliferation index in relation to the Wnt5A status in MPMs (48.0±31.1% in Wnt5A-high tumors and 45.9±33.6% in Wnt5A-low tumors) (Fig. 3E). In addition, there was no difference in the apoptotic index in relation to the Wnt 5A status in MPMs (5.6±2.6 in Wnt5A-high tumors and 6.6±2.9 in Wnt5A-low tumors).

Survival of MPM patients in relation to the Ki-67 proliferation index and intratumoral Wnt status. Regarding tumor proliferation, the 5-year survival rate was significantly lower in patients with high Ki-67 tumors than in those with low Ki-67 tumors (7.8 vs. 30.4%, p<0.0001) (Fig. 4A). Regarding the intratumoral Wnt status, the 5-year survival rate was 11.6% in patients with Wnt2B-high tumors, and 32.3% in patients with Wnt2B-low tumors (Fig. 4C). The overall survival was significantly lower in patients with Wnt2B-high tumors than in those with Wnt2B-low tumors (p=0.0238). In particular, the overall survival was significantly lower in patients with Wnt2B-high epithelioid tumors than in those with Wnt2B-low epithelioid tumors (20.9 vs. 37.4% at 5-year survival, p=0.0426) (Fig. 4D). In contrast, there was no difference in patient survival according to Wnt1 status or Wnt5A status (Fig. 4B and E). A Cox multivariate analysis demonstrated that Wnt2B status (hazard ratio 2.396; p=0.0042), pathological stage (hazard ratio 1.455; p=0.0439), and tumor histology (hazard ratio 1.973; p=0.0074) were significant prognostic factors for MPM patients (Table II).

Discussion

The clinical outcome of MPM patients is poor even in patients with early-stage MPM (3). In fact, 69.2% (74 of 107) of MPM patients included in the present study had advanced-stage MPM, and the 5-year survival rate was only 22.2% even in stage I patients. The development of new treatment strategies for MPM patients is therefore critical.

The Wnt family is involved in the regulation of a wide variety of normal and pathological processes including tumorigenesis (5,6). We first investigated the expression of Wnt1, Wnt2B, and Wnt5A in tumor tissues from MPM patients. The present study showed that the intratumoral expression of Wnt2B was significantly higher than that of Wnt1 and Wnt5A in MPMs. In addition, Wnt1 and Wnt5A expression levels were significantly lower in MPMs than in non-small cell lung cancers that were

Table II. Multivariate regression analysis for predicting survival of 107 patients with malignant pleural mesothelioma.

Variables	Assigned score	Hazard ratio	95% CI	P-value
Wnt2B status				
Low	0	2.396	1.317-4.359	0.0042
High	1			
Age (years)				
<65	0	1.114	0.664-1.871	0.6821
≥65	1			
Gender				
Male	0	1.434	0.772-2.665	0.2536
Female	1			
Asbestos exposure				
No	0	0.745	0.441-1.259	0.2710
Yes	1			
Smoking				
Non-smoker	0	0.989	0.577-1.696	0.9692
Smoker	1			
Clinical stage				
I	0	1.455	1.010-2.095	0.0439
II	1			
III–IV	2			
Histology				
Epithelioid	0	1.973	1.200-3.245	0.0074
Others	1			

CI, confidence interval.

analyzed concurrently (data not shown). These results suggest that the intratumoral Wnt2B expression has an effect on the tumor biology of MPMs.

Wnt2B is known to stimulate the canonical Wnt/β-catenin pathway (19). The activation of the canonical Wnt/β-catenin pathway leads to the transcription of Wnt-target genes including survivin (13,14), c-Myc (15), and vascular endothelial growth factor-A (20). Therefore, Wnt2B overexpression may affect tumor biology during tumor progression through the induction of these tumor-associated Wnt targets.

Furthermore, the present study clearly demonstrated that the tumor proliferation rate was associated with survival of MPM patients. In fact, MPM is clinically characterized by rapid and diffuse local growth, which results in a poor prognosis. In contrast, the apoptotic index in MPM tissues was significantly lower than that in non-small cell lung cancers that were analyzed concurrently (data not shown). We, therefore, evaluated the expression of survivin (13,14) and c-Myc (15), Wnt-targets associated with proliferation.

Previous studies have shown that survivin not only inhibits the caspase-dependent apoptotic pathway (21,22), but also accelerates cell proliferation (23). In particular, the nuclear localization of survivin affects cell mitosis through chromosome condensation and segregation (24,25). Previous studies revealed that the nuclear expression of survivin is associated with tumor proliferation and a poor prognosis in cancer patients (26-28). The present study also demonstrated that the nuclear expression of survivin was associated with tumor proliferation in MPMs. Furthermore, the survival was significantly lower in patients with nuclear survivin-high tumors than in those with nuclear survivin-low tumors (p=0.0447) (data not shown).

c-Myc is also a target of the canonical Wnt/β-catenin pathway (15). c-Myc is involved in cell cycle progression through the stimulation and repression of the expression of cell cycle regulators (29). Previous studies have revealed that c-Myc overexpression is associated with the malignant phenotype in various human cancers (8,30). The present clinical study also demonstrated that c-Myc expression was associated with the tumor proliferation of MPMs.

Finally, we investigated the clinical significance of Wnt expression in relation to survivin and c-Myc expression. Consequently, the present study has revealed that the intratumoral Wnt2B expression is associated with survivin and c-Myc expression, which results in the acceleration of tumor proliferation. Furthermore, overall survival was lower in patients with Wnt2B-high tumors than in those with Wnt2B-low tumors. To our knowledge, this is the first comprehensive clinical study clearly demonstrating the clinical significance of intratumoral Wnt2B expression in MPMs. In conclusion, the present study demonstrated that intratumoral Wnt2B expression is associated with tumor proliferation and survival of MPM patients through the induction of survivin

and c-Myc. Wnt2B is therefore a potential candidate for molecular-targeted therapy for MPMs. In fact, it was recently demonstrated that an adenoviral vector expressing short hairpin RNA (shRNA) against Wnt2B had a strong antitumor effect against Wnt2B-overexpressing tumors, via the down-regulation of survivin and c-Myc, resulting in the inhibition of tumor proliferation and the induction of apoptosis (31). Therefore, Wnt2B-inhibiting gene therapy, including the intrathoracic administration of viral vectors (32) or non-viral vectors (33), may be effective as a therapeutic strategy for Wnt2B-overexpressing MPMs (34).

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