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Wnt2b and Wnt5a expression is highly associated with M2 TAMs in non-small cell lung cancer

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Abstract. Tumor-associated macrophages (TAMs), particularly M2 macrophages, promote tumor progression, while *Wnt* genes encode a family of multi-functional glycoproteins that serve an important role in tumorigenesis. Immunohistochemical studies were performed to evaluate Wnt2b and Wnt5a expression in tumor and stromal cells in M2 and M1 TAMs and Ki-67 proliferation index in 160 consecutive patients with resected non-small cell lung cancer (NSCLC). Overall, 52 tumors (32.5%) were classified as tumoral Wnt2b-high (Wnt2b-positive tumor cells >30%) and 95 (59.4%) as stromal Wnt2b-high (Wnt2b-positive stromal cells >30%), while 75 (46.9%) were classified as tumoral Wnt5a-high (Wnt5a-positive tumor cells >30%) and 63 (39.4%) as stromal Wnt5a-high (Wnt5a-positive stromal cells >28%). The density of M2 TAMs was significantly higher in the tumoral (P=0.0024) and stromal Wnt2b-high groups (P=0.0054). The density of M2 TAMs was also significantly higher in the tumoral (P=0.0005) and stromal Wnt5a-high groups (P=0.0486). By contrast, no difference in stromal or islet M1 TAM density was observed in relation to tumoral or stromal Wnt2b or Wnt5a status. Furthermore, Ki-67 proliferation index was significantly higher in the tumoral (P=0.0121) and stromal Wnt2b-high (P=0.0019) and tumoral Wnt5a-high (P=0.0088) groups. Overall survival rate was significantly lower in the Wnt2b-high (P=0.0437), Wnt5a-high (P=0.0106) and M2 TAM-high (P=0.0060) groups. Wnt2b and Wnt5a expression in tumor and stromal cells may induce M2 TAMs to produce more aggressive behavior during tumor progression in NSCLC.

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Introduction

Lung cancer remains a challenging health issue and is the leading cause of cancer-associated mortality in numerous developed countries. The age-adjusted mortality rate in the United States was 36.7 per 100,000 persons per year, based on 2015-2019 cases (1). According to treatment strategy, lung cancer is clinically divided into non-small cell lung cancer (NSCLC), which could be treated with surgery, and SCLC, which is chiefly treated with chemotherapy. NSCLC, constituting 85% of all lung cancer cases, has histological subtypes, such as adenocarcinoma and squamous and large cell carcinoma. Advances in molecular biology have led to development of molecular targeted therapies for lung adenocarcinoma, including epidermal growth factor receptor (EGFR)-tyrosine kinase and anaplastic lymphoma kinase inhibitors (2,3). However, these molecular targeted therapies are not available for cancers that do not exhibit mutations of these target genes. On the other hand, immune checkpoint inhibitor (ICI) therapy has become a promising treatment strategy with excellent clinical efficacy against numerous types of cancer, including NSCLC (4,5). However, ICI therapy has been reported to exhibit less efficacy in patients with programmed death-ligand 1 (PD-L1)-negative tumors (6). Therefore, it is essential to elucidate tumor biology for the development of novel therapeutic strategies against NSCLC lacking mutations in receptors such as EGFR or PD-L1.

During tumor progression, infiltrating macrophages in tumors, called tumor-associated macrophages (TAMs), are considered key components of the tumor microenvironment (TME) (7,8). Macrophages can be polarized into separate phenotypes depending on the physiological or pathological context. Generally, M1 macrophages suppress, while M2 macrophages promote, tumor progression (9-11). During tumor progression, M2 TAMs induce angiogenesis by secreting cytokines including vascular endothelial growth factor (VEGF) (12,13), leading to promotion of tumor growth and metastasis. Experimental studies have also demonstrated promotion of tumor cell proliferation by M2 TAMs (14,15). Our previous clinical study also revealed that M2 TAMs may induce increased tumor aggressiveness,

proliferation and metastatic potential, leading to poor prognosis in patients with NSCLC (16). Therefore, it is essential to elucidate the mechanism of M2 polarization of TAMs, which is a multi-factorial, multi-stage and complicated pathological process in the TME (14,15).

Experimental studies have shown that Wnt signaling may be key for M2 polarization of TAMs (17-21). *Wnt* genes encode a family of cysteine-rich glycoproteins that serve roles in the regulation of normal and pathological processes, such as embryogenesis, differentiation and tumorigenesis (22-24). Our previous clinical studies found that various Wnt ligands derived from tumor cells promote tumor progression in NSCLC (25-28). By contrast, recent experimental studies have reported that macrophages express several Wnt ligands, including Wnt2b and Wnt5a (20,21).

Based on the aforementioned findings, to clarify the clinical significance of Wnt2b and Wnt5 expression during tumor progression in NSCLC, the present clinical study assessed Wnt2b and Wnt5a expression in tumor and stromal cells in relation to M2 and M1 TAMs and Ki-67 proliferation index, a prognostic parameter in NSCLC (29).

Materials and methods

Patients. The present study enrolled 160 consecutive patients (mean age 67.8±9.7 years; 88 males and 72 females) with NSCLC who underwent surgery at the Department of Thoracic Surgery, Kitano Hospital, Osaka, Japan, between November 2011 and October 2014, as reported previously (16). The study was approved by the Ethics Committee of the hospital (approval no. P181200300) and written informed consent was provided by each patient. Pathological stage was determined using the 8th tumor node metastasis (TNM) classification system (30). Tumor histology and grade of differentiation were determined based on the classification system developed by the World Health Organization (31). The patient medical records and histopathological diagnoses were documented. The patient records contained follow-up data until August 2021. The median follow-up interval was 61.6 months.

Immunohistochemistry. Immunohistochemical studies were performed to examine Wnt2b and Wnt5a expression in tumor and stromal cells. M2 and M1 TAM density were assessed by CD163 and inducible nitric oxide synthase (iNOS) staining, respectively, and tumor proliferation rate was assessed by Ki-67 proliferation index using the Ventana BenchMark GX system (Ventana Medical Systems; Roche Diagnostics) according to the manufacturer's protocol. The following antibodies were prepared: Mouse monoclonal anti-human Wnt2b (clone C-2; 1:30; Santa Cruz Biotechnology, Inc.), Wnt5a (clone 3A4; 1:100; Sigma-Aldrich; Merck KGaA) and CD163 (clone 760-4437; prediluted; Ventana Medical Systems; Roche Diagnostics) and rabbit polyclonal anti-human iNOS (cat. no. ab3523; 1:50; Abcam) and monoclonal anti-human Ki-67 (clone 30-9; prediluted; Ventana Medical Systems; Roche Diagnostics).

Each tissue of resected tumors was fixed in 10% neutral-buffered formalin for 24 h at room temperature. The tissue samples were embedded in paraffin at 60˚C following dehydration in graded ethanol series followed by xylene at room temperature. Formalin-fixed paraffin-embedded tissue was cut into 4-μm sections and mounted onto poly-L-lysine-coated slides. The sections were deparaffinized and rehydrated at 75˚C with EZ prep (950-100; Ventana Medical Systems; Roche Diagnostics). Antigen retrieval was performed using Cell Conditioner 1 (950-124; Ventana Medical Systems; Roche Diagnostics) for 32 min at 100˚C against Wnt2b, CD163 and iNOS and for 64 min at 100˚C against Ki-67 and Cell Conditioner 2 (950-123; Ventana Medical Systems; Roche Diagnostics) for 40 min at 100˚C against Wnt5a. Sections were incubated with specific primary antibody at 37˚C for 8 (Ki-67), 16 (CD163) or 32 (Wnt2b and iNOS) min or 2 h (Wnt5a). The sections were then incubated with OptiView HQ Linker (760-700; Ventana Medical Systems; Roche Diagnostics) for 8 min at 37˚C and OptiView HRP Multimer (760-700; Ventana Medical Systems; Roche Diagnostics) for 8 min at 37˚C. Lastly, counterstaining was performed using Hematoxylin II (760-2021; Ventana Medical Systems; Roche Diagnostics) for 8 min at 37˚C followed by Bluing Reagent (760-2037; Ventana Medical Systems; Roche Diagnostics) for 4 min at 37˚C.

The stained tissue sections were evaluated by two investigators (RS and CH) who were blinded to patient clinical information, using a light microscope (ECLIPSE Ci-L; Nikon). Discordant cases were collaboratively reviewed until a consensus was obtained. Wnt2b and Wnt5a expression was calculated as the percentage of cytoplasmic staining in tumor or stromal cells throughout the tumor region, irrespective of intensity. A cutoff value of 30% for Wnt2b expression in tumor and stromal cells showed the highest significance with respect to Ki-67 proliferation index, a prognostic parameter in NSCLC (29); samples were classified as tumoral Wnt2b-high when the percentage of Wnt2b-positive tumor cells exceeded 30% and as stromal Wnt2b-high when the percentage of Wnt2b-positive stromal cells exceeded 30%. Similarly, a cutoff value of 30% for Wnt5a showed the highest significance with respect to the Ki-67 proliferation index; thus, samples were classified as tumoral Wnt5a-high when the percentage of Wnt5a-positive tumor cells was >30%, as previously reported (25). On the other hand, samples were classified as stromal Wnt5a-high when the mean percentage of Wnt5a-positive stromal cells exceeded 28% because no difference in Ki-67 proliferation index was observed in relation to the stromal Wnt5a status.

For CD163 and iNOS staining, five of the most representative high-magnification fields (400x; 0.0625 mm^2) of the tumor stroma and islets were selected. Tumor stroma was defined as the region in which tumor stromal cells comprised >70% of all cells (32). Tumor islets were defined as the region in which tumor cells comprised >70% of all cells. The number of CD163-positive cells in each region was manually counted and the mean number of fields/region was calculated. Density of CD163-positive macrophages in the tumor stroma (M2 TAM density) was determined as the number of cells/mm^2. The samples were classified as M2 TAM-high when M2 TAM density was >380 cells/mm^2 due to the highest significance in relation to C-reactive protein, a prognostic biomarker in solid tumors (16,33). The number of iNOS-positive cells in each region was manually counted and mean number of fields per

region was calculated. iNOS-positive macrophage (M1 TAM) density was defined as the number of cells/mm^2 in the tumor stroma (stromal M1 TAM density) and islets (islet M1 TAM density).

Statistical analysis. One section of each resected tumor was evaluated. Data of continuous variables, were distributed normally (by Kolmogorov-Smirnov analysis), and are presented as mean ± standard deviation. Statistical significance was evaluated using unpaired t test, or one-way ANOVA with Bonferroni or Dunn's post hoc test. Correlation was assessed using Pearson's correlation coefficient. Categorical variables were compared by χ^2 test.

Overall survival (OS) was defined as the interval from start of treatment to date of death from any cause. The Kaplan-Meier method was used to evaluate the probability of OS as a function of time, while differences in survival of subgroups of patients were compared using Mantel's log-rank test. A Cox regression model was used to assess the effect of survival. Statistical analysis was performed using SPSS 23.0 for Windows (IBM Corp.). All P-values were based on two-tailed statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Distributions of Wnt2b expression in tumor and stromal cells. The percentage of Wnt2b-positive tumor cells ranged widely between 160 tumor tissue samples (mean, 24.0±30.4%; Fig. 1A, C and E; Table I), with 52 tumors (32.5%) classified as tumoral Wnt2b-high and 108 (67.5%) as tumoral Wnt2b-low. The percentage of Wnt2b-positive tumor cells was significantly highest in large cell carcinoma. There was no significant difference in the percentage of Wnt2b-positive tumor cells in relation to tumor differentiation, tumor or nodal status or pathological stage.

The percentage of Wnt2b-positive stromal cells also showed a high degree of variation between the 160 tumor tissue samples (mean, 42.0±25.8%; Fig. 1A, C and E; Table I); 95 tumors (59.4%) were classified as stromal Wnt2b-high and 65 (40.6%) as stromal Wnt2b-low. The percentage of Wnt2b-positive stromal cells was significantly lowest in adenocarcinoma. No significant difference in the percentage of Wnt2b-positive stromal cells was observed in relation to tumor differentiation, tumor or nodal status or pathological stage.

Distributions of Wnt5a expression in tumor and stromal cells. The percentage of Wnt5a-positive tumor cells was highly variable between the 160 tumor samples (mean, 37.4±27.8%; Fig. 1G; Table II); 75 tumors (46.9%) were classified as tumoral Wnt5a-high and 85 (53.1%) as tumoral Wnt5a-low. The percentage of Wnt5a-positive tumor cells was significantly highest in squamous cell carcinoma. In addition, the percentage of Wnt5a-positive tumor cells was significantly associated with tumor differentiation (well vs. moderate and well vs. poor), nodal status, and pathological stage (stage I vs. III and II vs. III).

Similarly, large variation in the percentage of Wnt5a-positive stromal cells was identified in tumor samples (mean, 27.6±25.0%; Fig. 1G; Table II); 63 tumors (39.4%) were classified as stromal Wnt5a-high and 97 (60.6%) as stromal Wnt5a-low. While no difference in the percentage of Wnt5a-positive stromal cells was observed in relation to tumor histology, the percentage of Wnt5a-positive stromal cells was significantly associated with tumor differentiation. However, there was no significant difference in relation to tumor or nodal status or pathological stage.

M2 TAM density in relation to Wnt2b and Wnt5a expression in tumor and stromal cells. Analysis of M2 TAM density in 160 tumor tissue samples revealed high levels of variation (mean, 407.0±389.2; Fig. 1B, D, F and H), as reported previously (14), with 67 tumors (41.9%) classified as M2 TAM-high and 93 (58.1%) as M2 TAM-low.

The percentage of Wnt2b-positive tumor cells was significantly correlated with M2 TAM density (r=0.167; P=0.034; data not shown). M2 TAM density was significantly higher in the tumoral Wnt2b-high compared with the tumoral Wnt2b-low group (540.2±411.2 vs. 342.9±362.9%; Fig. 2A).

A significant correlation was also observed between the percentage of Wnt2b-positive stromal cells and M2 TAM density (r=0.235; P=0.003; data not shown), with M2 TAM density significantly higher in the stromal Wnt2b-high compared with the stromal Wnt2b-low group (477.3±411.4 vs. 304.2±331.0%; Fig. 2B).

Similarly, the percentage of Wnt5a-positive tumor cells was found to correlate significantly with M2 TAM density (r=0.229; P=0.004; data not shown); M2 TAM density was significantly higher in the tumoral Wnt5a-high compared with the tumoral Wnt5a-low group (518.9±417.8 vs. 308.3±334.4%; Fig. 2C).

Although there was no correlation between the percentage of Wnt5a-positive stromal cells and M2 TAM density (r=0.028; P=0.722; data not shown), M2 TAM density was significantly higher in the stromal Wnt5a-high compared with stromal Wnt5a-low group (482.2±404.2 vs. 358.2±373.1%; Fig. 2D).

Stromal and islet M1 TAM density in relation to Wnt2b and Wnt5a expression in tumor and stromal cells. Analysis of stromal M1 TAM density in 160 tumor tissue samples revealed a high degree of variation (mean, 107.0±114.7; Fig. 1I and J). No difference in stromal M1 TAM density was observed between tumoral Wnt2b-high and -low groups (106.8±121.3 vs. 107.1±112.0%; Fig. 2E) or stromal Wnt2b-high and -low groups (111.8±127.7 vs. 100.1±92.9%; Fig. 2F). Furthermore, no difference in stromal M1 TAM density was observed between tumoral Wnt5a-high and -low groups (110.3±112.3 vs. 104.1±117.3%; Fig. 2G) or stromal Wnt5a-high and -low groups (117.7±121.9 vs. 100.1±109.9%; Fig. 2H).

Analysis of islet M1 TAM density in 160 tumor tissue samples also revealed a high degree of variation (mean, 64.2±90.2; Fig. 1I and J). No difference in islet M1 TAM density was observed between tumoral Wnt2b-high and -low groups (57.3±100.0 vs. 67.6±85.3%; Fig. S1A) or stromal Wnt2b-high and -low groups (66.9±100.6 vs. 60.3±72.7%; Fig. S1B). No difference in islet M1 TAM density was observed between tumoral Wnt5a-high and -low groups (65.3±79.5 vs. 63.3±99.1%; Fig. S1C) or stromal Wnt5a-high or -low groups (69.9±108.5 vs. 60.5±76.3%; Fig. S1D).

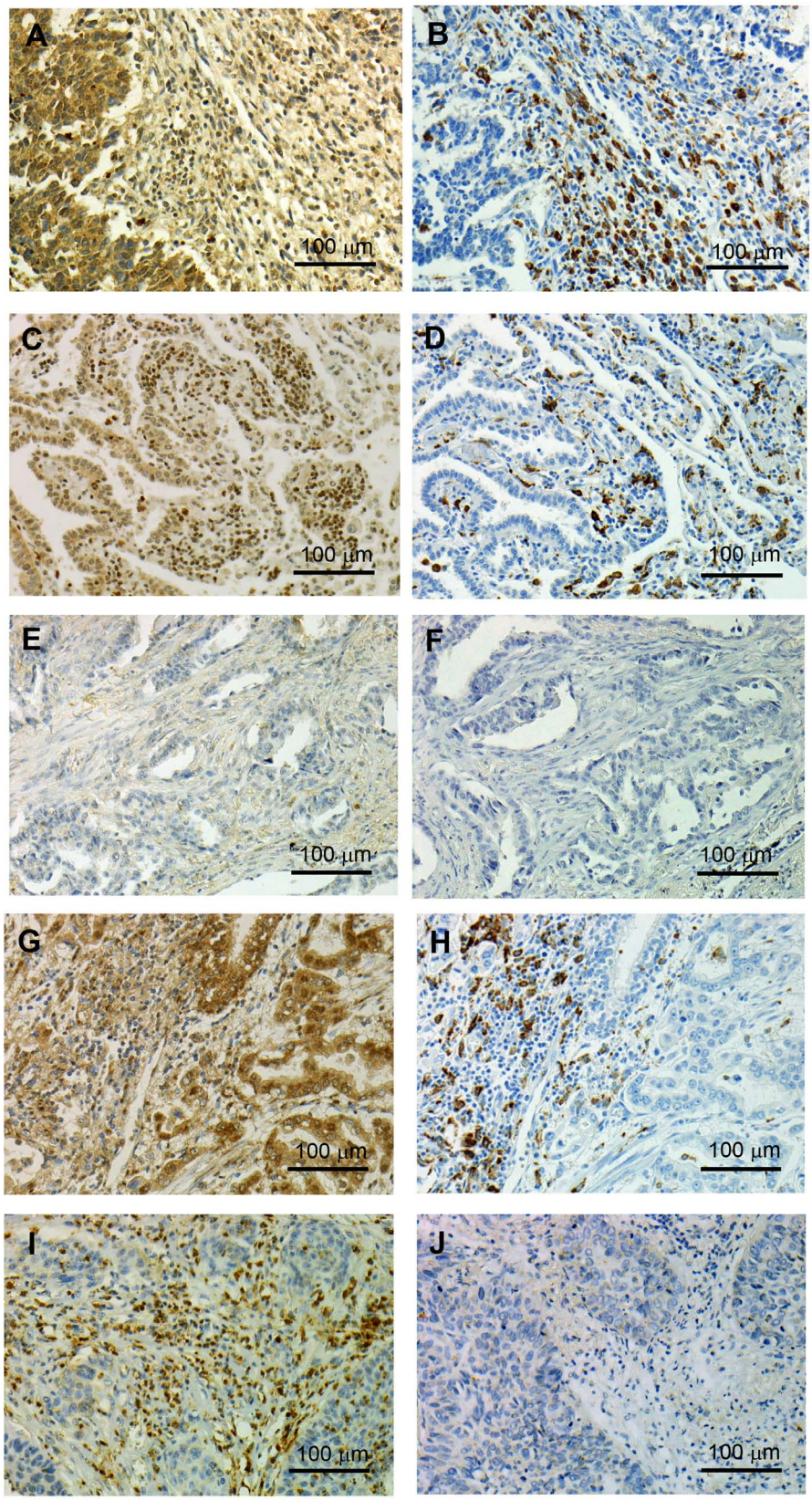

Figure 1. Immunostaining of lung cancer. Carcinoma with (A) positive Wnt2b expression in tumor cells and (B) high density of M2 TAMs. Carcinoma with (C) positive Wnt2b expression in stromal cells and (D) high density of M2 TAMs. Carcinoma with (E) negative Wnt2b expression in tumor and stromal cells and (F) low density of M2 TAMs. Carcinoma with (G) positive Wnt5a expression in tumor and weak Wnt5a expression in stromal cells and (H) high density of M2 TAMs. Carcinoma with (I) high and (J) low density of M1 TAMs. TAM, tumor-associated macrophage.

Ki-67 proliferation index in relation to Wnt2b and Wnt5a expression in tumor and stromal cells. The percentage of Wnt2b-positive tumor cells was significantly correlated with Ki-67 proliferation index (r=0.213; P=0.007). Ki-67

Table I. Wnt2b expression in patients with non-small cell lung cancer.

		Tumor cell, %		Tumoral status			Stromal cell, %		Stromal status		
Characteristic	n	Mean ± SD	P-value	Low	High	P-value	Mean ± SD	P-value	Low	High	P-value
Smoking status											
Smoker	85	22.6±28.5	0.5301[a]	60	25	0.3746[b]	41.8±26.4	0.9255[a]	36	49	0.6357[b]
Non-smoker	75	25.6±32.5		48	27		42.2±25.2		29	46	
Sex											
Male	88	22.8±30.2	0.5812[a]	60	28	0.8387[b]	40.0±25.3	0.2813[a]	37	51	0.6859[b]
Female	72	25.5±30.8		48	24		44.4±26.3		28	44	
Tumor differentiation											
Well	33	23.2±27.4	0.5020[c]	23	10	0.2626[b]	39.3±22.9	0.4494[c]	17	16	0.2706[b]
Moderate	93	22.3±29.9		66	27		41.2±26.7		37	56	
Poor	34	29.4±34.6		19	15		46.7±25.9		11	23	
Tumor status											
T1	80	23.4±30.5	0.8118[a]	55	25	0.7357[b]	44.9±25.4	0.1524[a]	30	50	0.4209[b]
T2-4	80	24.6±30.5		53	27		39.0±26.0		35	45	
Nodal status											
N0	123	25.4±30.2	0.2874[a]	80	43	0.2259[b]	41.3±24.4	0.5492[a]	53	70	0.2472[b]
N1, 2	37	19.3±31.1		28	9		44.2±30.0		12	25	
Pathological stage											
I	107	25.3±30.5	0.1059[c]	71	36	0.6938[b]	42.5±24.3	0.8140[c]	44	63	0.6927[b]
II	24	12.3±20.7		18	6		38.8±29.2		11	13	
III	29	28.8±35.1		19	10		42.7±28.5		10	19	
Tumor histology											
Adenocarcinoma	128	20.4±28.1	0.0009[c,d]	94	34	0.0010[b,d]	39.0±24.9	0.0066[c,d]	59	69	0.0098[b,d]
Squamous cell carcinoma	25	32.6±34.4		1	6		50.6±27.8		0	7	
Large cell carcinoma	7	60.0±31.1		13	12		64.6±16.5		6	19	
Total	160	24.0±30.4		108	52		42.0±25.8		65	95	

[a]P-value determined using t test. [b]P-value determined using χ^2 test. [c]P-value determined by ANOVA followed by Bonferroni or Dunn's test. [d]$P<0.05$.

Table II. Wnt5a expression in patients with non-small cell lung cancer.

		Tumor cell, %		Tumoral status			Stromal cell, %		Stromal status		
Characteristic	n	Mean ± SD	P-value	Low	High	P-value	Mean ± SD	P-value	Low	High	P-value
Smoking status											
Smoker	85	36.7±26.1	0.7287[a]	45	40	0.9604[b]	27.0±24.7	0.7569[a]	51	34	0.8632[b]
Non-smoker	75	38.3±29.8		40	35		28.3±25.5		46	29	
Sex											
Male	88	37.9±27.0	0.8356[a]	44	44	0.3812[b]	26.8±23.2	0.6673[a]	53	35	0.9094[b]
Female	72	36.9±29.0		41	31		28.6±27.1		44	28	
Tumor differentiation											
Well	33	27.0±25.7	0.0278[c,d]	23	10	0.0586[b]	16.4±15.7	0.0087[c,d]	27	6	0.0155[b,d]
Moderate	93	38.5±26.5		48	45		29.3±24.9		53	40	
Poor	34	44.6±31.1		14	20		34.0±29.4		17	17	
Tumor status											
T1	80	36.3±27.3	0.5948[a]	45	35	0.4283[b]	26.5±24.1	0.5793[a]	50	30	0.6274[b]
T2-4	80	38.6±28.5		40	40		28.7±25.9		47	33	
Nodal status											
N0	123	34.8±28.1	0.0258[a,d]	72	51	0.0124[b,d]	27.1±24.6	0.6522[a]	75	48	0.8685[b]
N1, 2	37	46.4±25.4		13	24		29.2±26.6		22	15	
Pathological stage											
I	107	34.8±28.4	0.0219[c,d]	63	44	0.0097[b,d]	26.0±24.8	0.4511[c]	69	38	0.2326[b]
II	24	36.1±25.7		14	10		32.8±23.1		11	13	
III	29	48.2±25.8		8	21		29.3±27.4		17	12	
Tumor histology											
Adenocarcinoma	128	35.8±27.8	0.0465[c,d]	73	55	0.0177[b,d]	27.0±24.6	0.1816[c]	78	50	0.2684[b]
Squamous cell carcinoma	25	47.9±26.9		7	18		34.3±27.0		13	12	
Large cell carcinoma	7	29.7±25.0		5	2		15.9±20.8		6	1	
Total	160	37.4±27.8		85	75		27.6±25.0		97	63	

[a]P-value determined using t test. [b]P-value determined using χ^2 test. [c]P-value determined by ANOVA followed by Bonferroni or Dunn's test. [d]$P<0.05$.

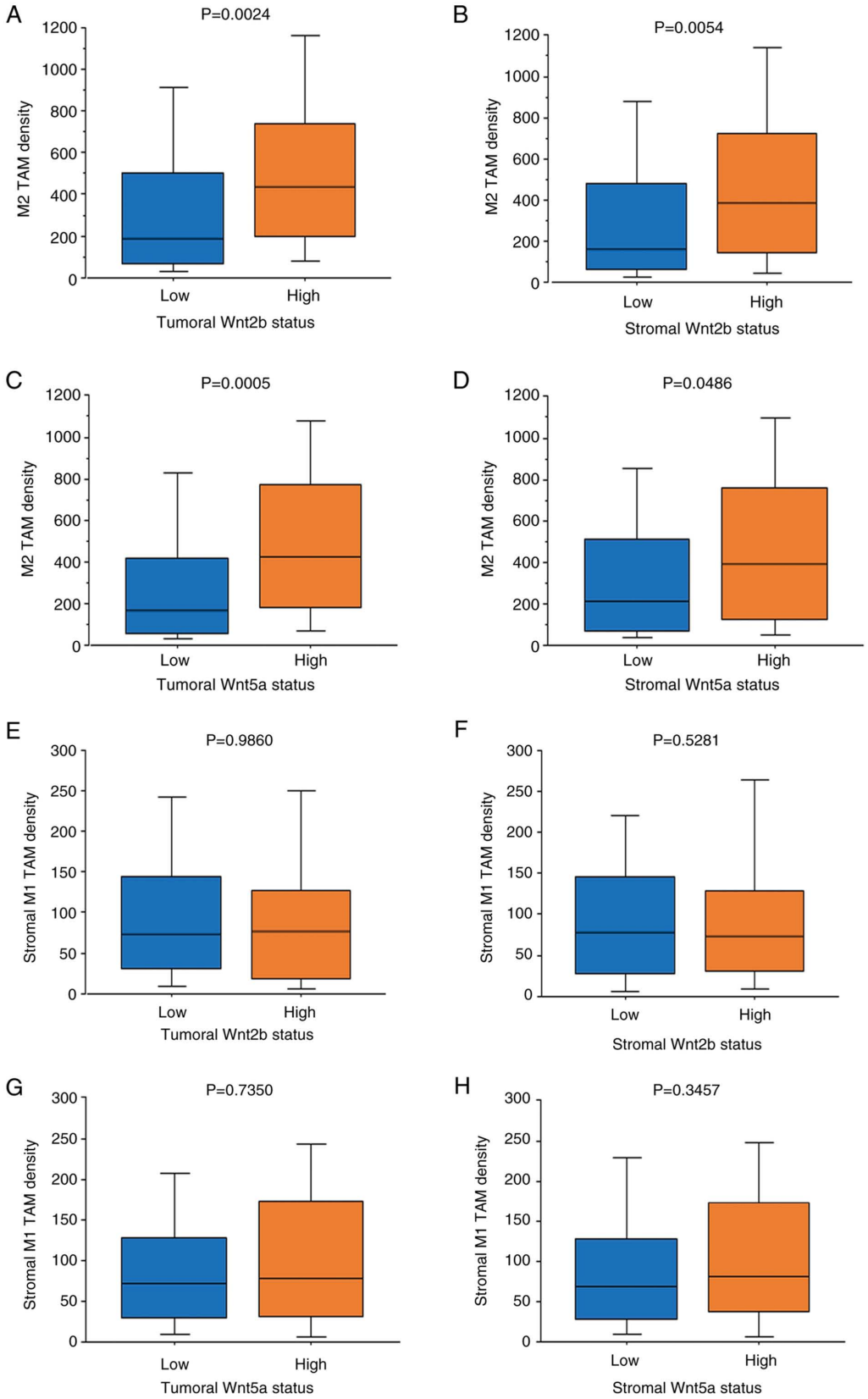

Figure 2. M2 TAM density and stromal M1 TAM density in relation to Wnt status. M2 TAM density in relation to (A) tumoral and (B) stromal Wnt2b and (C) tumoral and (D) stromal Wnt5a status. Stromal M1 TAM density in relation to (E) tumoral and (F) stromal Wnt2b and (G) tumoral and (H) stromal Wnt5a status. TAM, tumor-associated macrophage.

proliferation index was significantly higher in the tumoral Wnt2b-high compared with tumoral Wnt2b-low group (36.0±29.2 vs. 24.3±26.4%; Fig. 3A).

Similarly, the percentage of Wnt2b-positive stromal cells correlated with Ki-67 proliferation index (r=0.238; P=0.002). The Ki-67 proliferation index was significantly higher in the

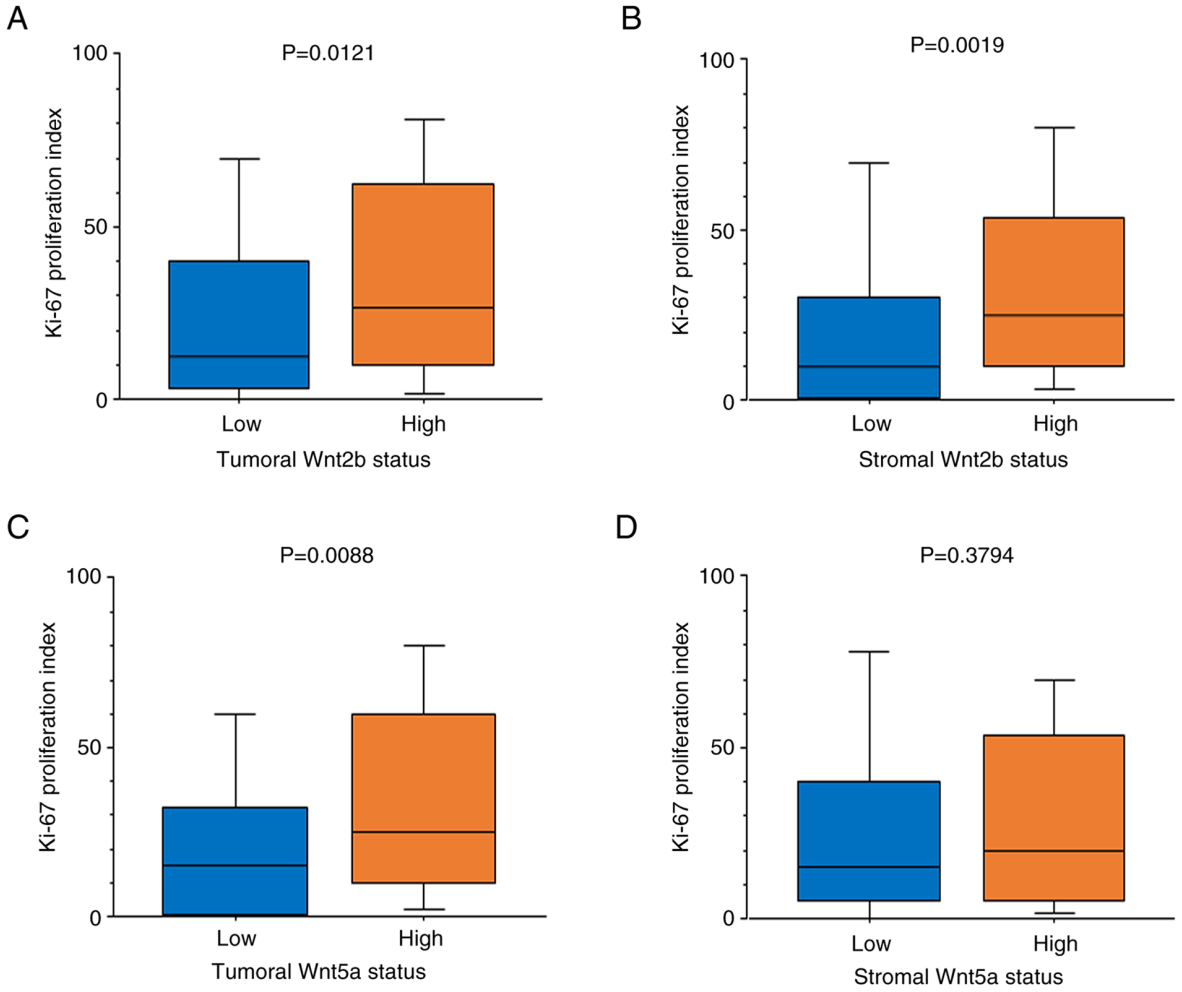

Figure 3. Ki-67 proliferation index in relation to Wnt status. (A) tumoral and (B) stromal Wnt2b and (C) tumoral and (D) stromal Wnt5a status.

stromal Wnt2b-high than in the stromal Wnt2b-low group (33.6±28.0 vs. 19.9±25.6%; Fig. 3B).

The percentage of Wnt5a-positive tumor cells was found to correlate significantly with Ki-67 proliferation index (r=0.226; P=0.004), with a significantly higher proliferation index in the tumoral Wnt5a-high compared with tumoral Wnt5a-low group (34.1±28.9 vs. 22.7±25.8%; Fig. 3C). However, no difference in Ki-67 proliferation index was observed between stromal Wnt5a-high and -low groups (30.5±28.5 vs. 26.5±27.3%; Fig. 3D).

Prognosis of patients with resected NSCLC in relation to Wnt2b, Wnt5a and M2 TAM status. For prognostic analysis, the tumoral and stromal Wnt2b-high groups were combined into a single Wnt2b-high group and remaining cases were classified as Wnt2b-low. Of 160 resected NSCLC samples, 99 tumors (61.9%) were categorized as Wnt2b-high and 61 (38.1%) as Wnt2b-low group. Similarly, tumoral and stromal Wnt5a-high groups were combined to form the Wnt5a-high group, while other cases were classified as Wnt5a-low. Overall, 91 tumors (56.9%) of the resected NSCLC cases were categorized as Wnt5a-high group, while 69 (43.1%) were Wnt5a-low.

OS rate was significantly lower in the Wnt2b-high than in the Wnt2b-low group (Fig. 4A). OS rate was also significantly lower in the Wnt5a-high than in the Wnt5a-low group (Fig. 4B). Furthermore, the OS rate was significantly lower in the M2 TAM-high than in the M2 TAM-low group (Fig. 4C).

Univariate analysis with the Cox regression model showed that both Wnt5a (HR=3.084; 95% CI: 1.242-7.658; P=0.0152) and M2 TAM status (HR=2.865; 95% CI: 1.307-6.283; P=0.0086) were significant predictors for OS of patients with resected NSCLC.

Discussion

During tumor progression, TAMs are considered important components of TME (7,8). TAMs are derived from circulating blood cells, including monocytes. Chemotactic signals from tumor or stromal cells in the TME may mobilize monocytic precursors to the tumor area. Macrophages can be polarized into different phenotypes depending on the physiological or pathological context. Generally, M1 macrophages suppress tumors, while M2 macrophages are known for their tumor-promoting function (9-11). During tumor progression, Th2-derived cytokines secreted by tumor and stromal cells have been shown to induce generation of M2 TAMs in the TME (14). Subsequently, M2 TAMs may induce angiogenesis and promote tumor growth and metastasis (13,14). In addition, M2 TAMs induce epithelial-mesenchymal transition (EMT) of tumor cells, promoting metastasis (34), while EMT-programmed tumor cells remodel

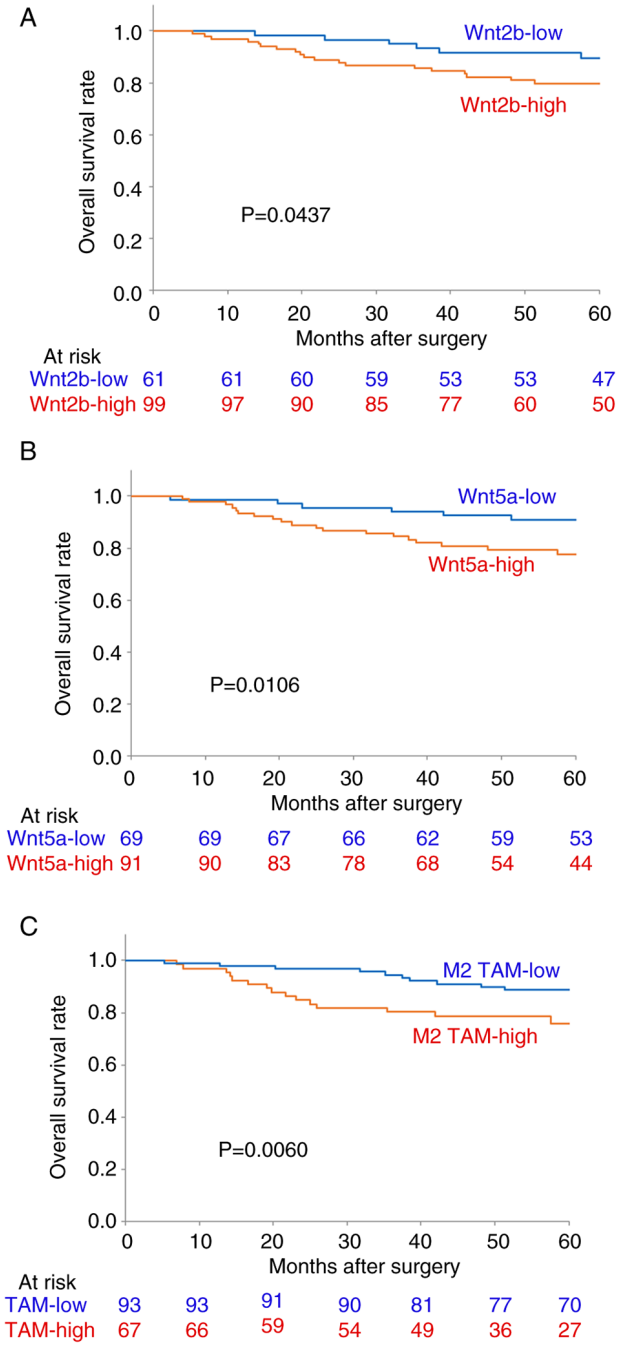

Figure 4. Overall survival of patients with resected NSCLC. (A) in relation to Wnt2b status. (B) in relation to Wnt5a status. (C) in relation to M2 TAM status. TAM, tumor-associated macrophage.

the TME and induce M2 TAM phenotype (35). M2 TAMs may serve a critical role in crosstalk between tumor cells and TME during tumor progression (36,37). Our previous clinical study also revealed that M2 TAMs may induce increased tumor cell aggressiveness, proliferation and metastatic potential, leading to a poor prognosis for patients with NSCLC (16). Therefore, it is essential to clarify the mechanism of M2 polarization of TAMs to control tumor progression.

Both M1 and M2 TAMs are reported to exhibit considerable plasticity in their phenotype and function (38). M2 polarization of macrophages is induced by numerous signaling pathways and cytokines, such as IL-4, IL-10 and TGF-β (11,14,39). Thus, M2 polarization of TAMs is a multi-factorial, multi-stage and complicated pathological process in the TME.

Experimental studies have demonstrated that Wnt signaling serves a key role in M2 polarization of TAMs (17-21). Jiang *et al* (21) reported that Wnt2b expressed by macrophages induces polarization of TAMs to M2-like macrophages by upregulating Wnt2b/β-catenin/c-Myc signaling. Additionally, Liu *et al* (20) reported that Wnt5a-positive TAMs are an M2-like TAM subtype; furthermore, Wnt5a stimulates macrophages to secrete IL-10, which functions in an autocrine and paracrine manner to induce further M2 polarization.

Wnt gene family is involved in numerous physiological, developmental and pathological processes, including tumorigenesis (22,23). The *Wnt* pathway is classified into three branches based on downstream signaling cascades, including canonical Wnt, Wnt/Ca^{2+} (24) and Wnt/planar cell polarity (PCP) signaling pathway (40). In particular, when the canonical Wnt signaling pathway is activated, β-catenin is stabilized and accumulates in the cytoplasm (24); cytoplasmic β-catenin translocates into the nucleus and binds to T-cell factor (TCF)/lymphoid enhancer-binding factor 1 (LEF1) complex to activate transcription of Wnt responsive genes (41). Wnt target genes include molecules associated with tumorigenesis, such as c-Myc, cyclin D1 and VEGF, a key angiogenic factor (42-44).

Previous clinical studies have found that a number of Wnt ligands, including Wnt1, Wnt2b and Wnt5a, are frequently upregulated in numerous types of human cancer, including lung, gastric, pancreatic and breast cancer (25-27,45). Our previous clinical studies also found that numerous Wnt ligands derived from tumor cells promote tumor progression in NSCLC (25-28). Overexpression of Wnt2b in tumor cells is associated with high levels of c-Myc expression in tumor cells, high tumor proliferation rate and low apoptotic index (27). Furthermore, overexpression of Wnt5a in tumor cells is associated with high levels of VEGF expression in stromal cells and high tumor proliferation rate, which suggests tumor-stroma interactions in NSCLCs (25). Using RT-qPCR, the present study evaluated gene expression of Wnts in NSCLC, including *Wnt1*, *Wnt2b* and *Wnt5a*. *Wnt1* gene expression was much lower than *Wnt2b* and *Wnt5a* gene expression (data not shown). Therefore, the present clinical study aimed to examine Wnt2b and Wnt5a expression in tumor and stromal cells in relation to M2 TAMs.

The present study demonstrated that Wnt2b expression both in tumor and in stromal cells was correlated with the M2 TAM density. M2 TAM density was higher in the tumoral and stromal Wnt2b-high group. Furthermore, Ki-67 proliferation rate was also higher in the tumoral and stromal Wnt2b-high group. In addition, similar to the Wnt2b results, Wnt5a expression in tumor cells was correlated with M2 TAM density; M2 TAM density and Ki-67 proliferation rate were higher in the tumoral Wnt5a-high group. M2 TAM density was also higher in the stromal Wnt5a-high group. By contrast, no difference was observed in Ki-67 proliferation rate in relation to stromal Wnt5a status. This result may be partly due to the relatively small number of patients. Therefore, further clinical studies with more patients are required.

Wnt2b is involved in the canonical Wnt signaling pathway, and *VEGF* is a target gene of this pathway (44). In addition, overexpression of Wnt5a in tumor cells is associated with high levels of VEGF expression in stromal cells in NSCLC (25). Therefore, further studies are needed to evaluate the

angiogenesis pathway regarding these biological mechanisms during tumor progression in NSCLC.

The present study evaluated M1 TAMs, which generally suppress tumors (9-11). Because M1 TAMs are widely expressed not only in the tumor stroma but also in tumor islets (10), M1 TAM density was evaluated in both the tumor stroma and islets. Consequently, the present study revealed no difference in stromal or islet M1 TAM density in relation to tumoral and stromal Wnt2b or Wnt5a status in NSCLC.

Previous studies have reported that Wnt ligands are secondarily regulated in response to changes in biological molecules, including nuclear factor-κB, protein kinase C and motility related protein-1 (MRP-1)/CD9 (46-48). Wnt ligands bind to various types of receptor belonging to the Frizzled or the receptor tyrosine kinase-like orphan receptor1 (ROR1)/ROR2 and receptor tyrosine kinase (RYK) family (49). Therefore, Wnt signaling pathways exert effects on tumorigenesis by modulating the TME via fine crosstalk between tumor and infiltrating immune cells. In particular, previous studies have reported that Wnt5a is involved in several downstream signaling cascades, including not only non-canonical Wnt signaling pathways, such as the Wnt/Ca^{2+} and (50,51) and Wnt/PCP signaling pathway (40), but also canonical Wnt signaling pathway (52). Recently, an experimental study reported that the Wnt5a/CaMKII/ERK/CCL2 axis, one of the Wnt/Ca^{2+} signaling pathways, is required for TAMs to promote colorectal cancer progression (50). Further studies are needed to clarify the mechanisms of Wnt5a signaling pathways, including the Wnt5a/CaMKII/ERK/CCL2 axis.

To the best of our knowledge, the present study is the first to demonstrate the clinical significance of Wnt2b and Wnt5a expression in tumor and stromal cells in relation to tumor-promoting M2 TAMs in NSCLC. Overall survival rate was lower in the Wnt2b-high, Wnt5a-high and M2 TAM-high group. Considering both the results in the present study and experimental studies (20,21), Wnt2b and Wnt5a expression in tumor and stromal cells may induce M2 TAMs to produce more aggressive behavior during tumor progression in NSCLC.

Our previous experimental studies found a Wnt2b-inhibiting adenoviral vector to have an effective antitumor effect in lung cancer cells (27,53). Downregulation of Wnt2b and Wnt5a expression may inhibit M2 polarization and control tumor progression. Therefore, combined therapy using Wnt2b- and Wnt5a-inhibiting vector may produce effective antitumor activity. Further research involving non-viral vectors may also be needed to develop novel therapeutic strategies for patients with NSCLC to control tumor progression (54).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RS, CLH and HD designed the study. RS, CLH, HA and TI designed and performed the experiments. RS, CLH and HC collected the data. RS and CLH analyzed and interpreted the data and wrote the manuscript. RS and CLH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of the Kitano Hospital (approval no. P181200300) and written informed consent was provided by each patient. The research was conducted in compliance with the principles outlined in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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