Aberrant Wnt1/β-Catenin Expression is an Independent Poor Prognostic Marker of Non-small Cell Lung Cancer After Surgery

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Introduction: The Wnt signaling pathway plays a major role in cancer development and progression. As a novel anticancer drug can be developed using inhibitors of this pathway, we investigated the clinical significance of the Wnt signaling pathway molecules in non-small cell lung cancer (NSCLC).

Methods: Immunohistochemical analysis of a tissue microarray with 262 resected NSCLC specimens was performed to study the expression and subcellular localization of Wnt1 in relation to downstream molecules, including GSK-3β, β-catenin, c-Myc, cyclin D1, and p53. These results were correlated with other clinicopathologic features.

Results: Cytoplasmic Wnt1 overexpression was detected in 36.6% (96 of 262) NSCLCs, and aberrant β-catenin staining was identified in 76% (189 of 262) of NSCLCs. There were significant associations between Wnt1 expression and altered expression of β-catenin ($p = 0.034$), overexpression of c-Myc ($p < 0.001$), or overexpression of cyclin D1 ($p = 0.018$). While there was no significant association between Wnt1 or β-catenin and stage, the 5-year survival was significantly lower in patients with Wnt1- and β-catenin-positive NSCLCs than negative NSCLCs ($p < 0.05$, respectively). In multivariate analysis, stage and Wnt1+/β-catenin+ expression were independent prognostic factors of overall survival ($p < 0.05$).

Conclusion: These findings show that Wnt1 expression may be one of the possible mechanisms of the activation of the canonical Wnt/β-catenin signaling pathway in NSCLC, and Wnt1 and altered β-catenin expression are poor prognostic markers, independent of stage.

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Lung cancer is a highly aggressive and challenging cancer that now represents the leading cause of cancer deaths throughout the world.[1] Despite progress in the multimodality treatment of lung cancer in the past several decades, the 5-year survival rate for lung cancer remains poor. Surgery, chemotherapy, and radiation have failed to yield satisfactory results in advanced disease.[2] Further elucidation of the molecular mechanism underlying lung cancer is essential for the development of new effective therapeutic agents. The molecular pathogenesis of lung cancer includes alteration of expression and function of multiple genes including dominant oncogenes and recessive oncogenes/tumor-suppressor genes, alterations in growth-regulatory signaling pathways, and abnormalities in other pathways. Among the many molecules associated with tumorigenesis, the Wingless-type (Wnt) family genes are frequently upregulated in different human cancers, including non-small cell lung cancer (NSCLC).[3,4]

The Wnt gene family encodes the multifunctional signaling glycoproteins that are involved in the regulation of a wide variety of normal and pathological processes, including embryogenesis, differentiation, and tumorigenesis. Wnt signaling is transduced through β-catenin, which is regulated by the adenomatous polyposis coli (APC)/Axin/glycogen synthase kinase (GSK)-3β complex.[5,6] In the absence of Wnt signal (off-state), β-catenin, an integral cell-cell adhesion adaptor protein as well as transcriptional coregulator, is targeted for degradation by the APC/Axin/GSK-3β complex. In the presence of Wnt stimulation (on-state), Drosophila dishevelled (Dvl) is activated, seemingly at least in part by phosphorylation, which in turn recruits GSK-3β away from the degradation complex. This allows stabilization of β-catenin, resulting in the accumulation of free cytosolic β-catenin. The elevated β-catenin can translocate to the nucleus where it forms a complex with T-cell factor/lymphoid enhancer binding factor to stimulate the expression of Wnt target molecules, such as cyclin D1, c-Myc, peroxisome proliferator-activated receptor delta (PPAR-δ), and matrix metalloproteinase

7 (MMP7).[7–10] Increasing evidence indicates that aberrant activation of the Wnt signaling pathway is associated with tumor development and/or progression, suggesting that Wnt signaling functions in oncogenesis, possibly through antiapoptotic mechanisms.[8,11,12] Therefore, inhibition of Wnt signaling has become an attractive strategy for cancer therapeutics.[13,14] Inhibitors of Wnt/β-catenin signaling are under consideration or development, initially for treatment of cancers—a promising avenue given that Wnt/β-catenin signaling seems to be involved in cancer progression.[15–17] However, it has been known that the Wnt signaling pathway activation mechanism in lung cancer is different from that in colorectal cancer.[18] In particular, mutations of APC or β-catenin are rarely found in lung cancer, and the Wnt pathway activation may occur upstream of β-catenin.[18]

It is important to detect specific target molecules of the Wnt pathway as well as to identify and select the patients who would benefit from Wnt pathway inhibitors. It remains to be seen whether or not the inhibitors of specific pathway can be put into clinical practice, and detailed appraisals of biomarker expression in the tumor cells should be preceded. To evaluate the clinical significance of Wnt/β-catenin pathway proteins, we investigated the intratumoral expression of Wnt1 in relation to downstream molecules, including β-catenin, GSK-3β, c-Myc, cyclin D1, and p53 in NSCLC.

PATIENTS AND METHODS

Clinical Data Acquisition

From May 2003 to December 2006, 262 consecutive lung cancer patients who underwent surgical resection in our institution were compiled. The tumors consisted of 165 (63.0%) adenocarcinomas (ADCs) and 97 (37.0%) squamous cell carcinomas (SCCs). The patients given diagnoses other than ADC or SCC were excluded due to their small number. None of the patients had received neoadjuvant chemotherapy or irradiation preoperatively.

Clinical and pathological data were retrieved from the medical records, including follow-up data as of December 2009. This study was approved by Institutional Review Board.

Tissue Microarray

Representative core tissue sections (2 mm in diameter) were taken from paraffin blocks and arranged in new tissue microarray (TMA) blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). In cases with a variety of histologic features, the most representative area was selected for TMA construction. Six cores were sampled and included in the TMA block from each patient.

TABLE 1. Pathological Stage of NSCLCs

p Stage	*n* (%)
Ia	57 (21.8%)
Ib	49 (18.7%)
IIa	49 (18.7%)
IIb	25 (9.5%)
IIIa	64 (24.4%)
IIIb	6 (2.3%)
IV	12 (4.6%)

p stage, pathological stage.

TABLE 2. Clinicopathologic Characteristics According to the Histologic Subtype

Characteristics	Total *n* (%)	ADC *n* (%)	SCC *n* (%)	*p*
Total	262 (100.0)	165 (63.0)	97 (37.0)	
Sex				
Male	184 (70.2)	93 (56.4)	91 (93.8)	<0.001
Female	78 (29.8)	72 (43.6)	6 (6.2)	
Age (yr)				
≤65	147 (56.1)	102 (61.8)	45 (46.4)	0.020
>65	115 (43.9)	63 (38.2)	52 (53.6)	
Smoking				
Nonsmoker	94 (35.9)	89 (53.9)	5 (5.2)	<0.001
Smoker	168 (64.1)	76 (46.1)	92 (94.8)	
Size				
≤3 cm	146 (55.7)	101 (61.2)	45 (46.4)	0.021
>3 cm	116 (44.3)	64 (38.8)	52 (53.6)	
Pleural invasion				
Absent	156 (59.5)	90 (54.5)	66 (68.0)	0.037
Present	106 (40.5)	75 (45.5)	31 (32.0)	
Vascular invasion				
Absent	222 (84.7)	148 (89.7)	74 (76.3)	0.004
Present	40 (15.3)	17 (10.3)	23 (23.7)	
Tumor status				
T1	81 (30.9)	57 (34.5)	24 (24.7)	NS
T2	125 (47.7)	77 (46.7)	48 (49.5)	
T3	41 (15.6)	20 (12.1)	21 (21.6)	
T4	15 (5.7)	11 (6.7)	4 (4.1)	
Nodal status				
N0	143 (54.6)	93 (56.4)	50 (51.5)	NS
N1	60 (22.9)	27 (16.4)	33 (34.0)	
N2	58 (22.1)	45 (27.3)	13 (13.4)	
N3	1 (0.4)	0 (0.0)	1 (1.0)	
p stage				
I	106 (40.5)	78 (47.3)	28 (28.9)	NS
II	74 (28.2)	28 (17.0)	46 (47.4)	
III	70 (26.7)	52 (31.5)	18 (18.6)	
IV	12 (4.6)	7 (4.2)	5 (5.2)	
ECOG PS				
0	170 (64.9)	121 (73.3)	49 (50.5)	<0.001
1, 2	92 (35.1)	44 (26.7)	48 (49.5)	
Recur				
Absent	137 (52.3)	88 (53.3)	49 (50.5)	NS
Present	125 (47.7)	77 (46.7)	46 (47.4)	
Survival				
Alive	194 (74.0)	127 (77.0)	67 (69.1)	0.010
Deceased	68 (26.0)	37 (22.4)	23 (23.7)	

ADC, adenocarcinoma; SCC, squamous cell carcinoma; NS, not significant; p stage, pathological stage; ECOG PS: Eastern Cooperative Oncology Group performance status.

Immunohistochemical Assays

We deparaffinized 4-μm sections from the TMA blocks in xylene and rehydrated them in graded ethanol. The succeeding steps were performed automatically at 37°C using the BenchMark XT Slide Staining System Specifications (Ventana Medical Systems, Tucson, AZ). Endogenous peroxidases were quenched with 1% H_2O_2 for 4 minutes. The sections were incubated with the rabbit polyclonal antibody for Wnt1 (H-89, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:100), the rabbit polyclonal antibody for GSK-3β (H-76, Santa Cruz, dilution 1:100), the mouse monoclonal antibody for β-catenin (5H10, Zymed Laboratories, San Francisco, CA, dilution 1:1000), the mouse monoclonal antibody for c-Myc (9E11, Santa Cruz, dilution 1:5000), the rabbit monoclonal antibody for cyclin D1 (SP4, Thermo Fisher Scientific, Waltham, MA, dilution 1:100), and the mouse monoclonal antibody for p53 (DO-7, Dako, Glostrup, Denmark, dilution 1:1000). The secondary biotinylated antibody was incubated for 8 minutes. The slides were stained using a diaminobenzidine detection kit and counterstained with hematoxylin.

Specimens were evaluated under light microscopy by two independent pathologists (X.X. and J.H.C.) and scored based on a semiquantitative approach of that percentage of positive tumor cells (0–100%), multiplied by staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong). For the expression of Wnt1, GSK-3β, c-Myc, and cyclin D1, a total score range of 0 to 300 was generated for each sample, where 0 to 100 was classified as negative and 101 to 300 was classified as positive. Regarding the β-catenin expression, we used the classification of staining patterns as follows: (1) a membranous pattern, if immunoreactivity was present solely at the cell membranes; (2) a membranous-cytoplasmic pattern, if immunoreactivity was also present in the cytoplasm; (3) a cytoplasmic pattern, if immunoreactivity was chiefly present in the cytoplasm and in less than 20% of the nuclei;

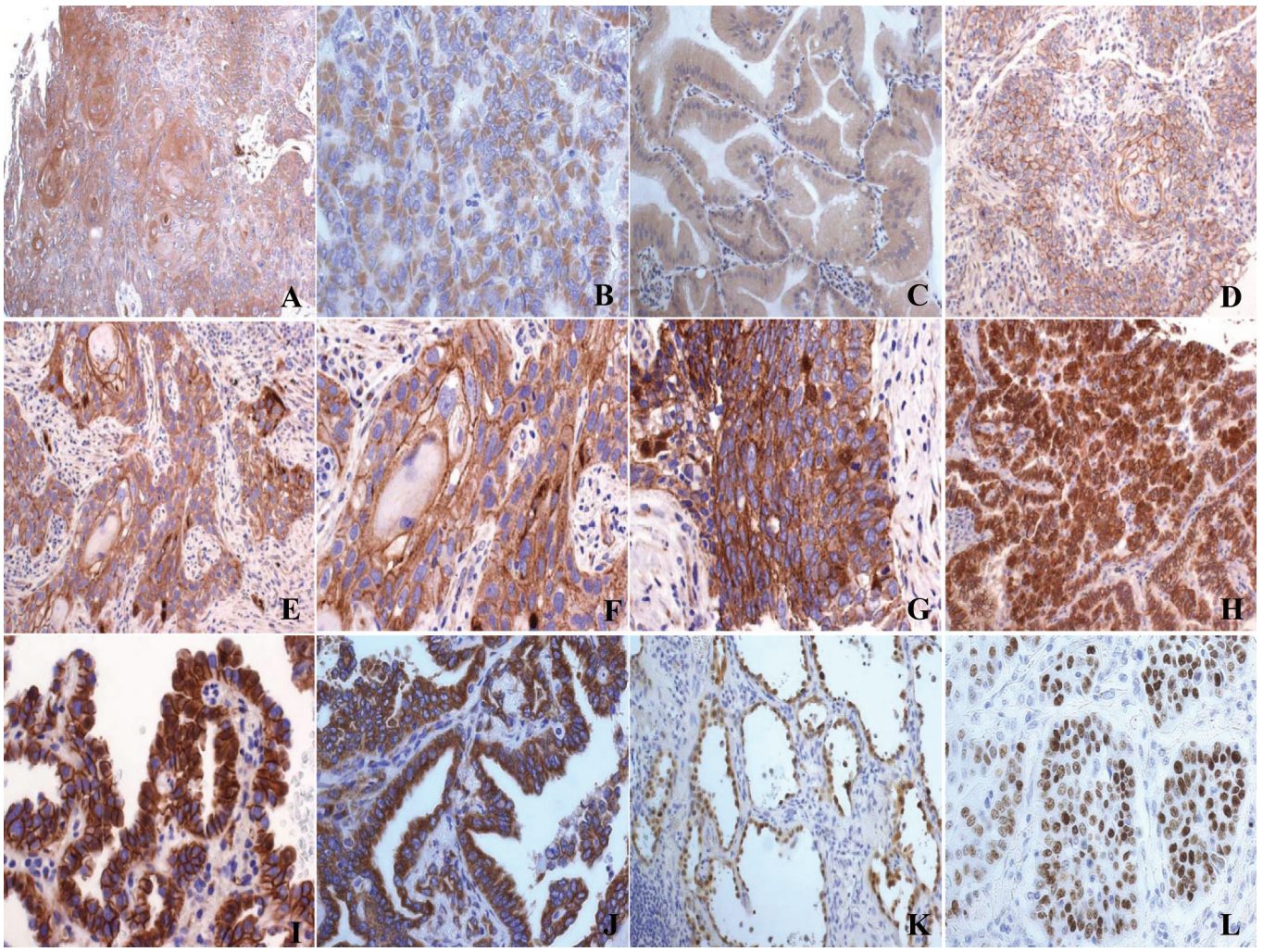

FIGURE 1. Immunohistochemical staining of Wnt pathway proteins in non-small cell lung cancer. Cytoplasmic expression of Wnt1 (*A*, *B*), GSK-3b (*C*), normal membranous pattern of β-catenin (*D*), membranous-cytoplasmic pattern of β-catenin (*E*, *F*), cytoplasmic pattern of β-catenin (*G*), cytoplasmic-nuclear pattern of β-catenin (*H*, *I*), positive expression of c-Myc (*J*), cyclin D1 (*K*), and p53 (*L*).

TABLE 3. Immunohistochemistry Status in NSCLCs According to Clinicopathological Characteristics

		Wnt 1 Status			GSK-3β Status			β-Catenin Status			c-Myc Status			Cyclin D1 Status			p53 Status		
Characteristics	*n*	+	−	*p*	+	−	*p*	+	−	*p*	+	−	*p*	+	−	*p*	+	−	*p*
Total	262	36.6%	63.4%		26.7%	73.3%		76.0%	24.0%		74.8%	25.2%		39.3%	60.7%		45.8%	54.2%	
Sex																			
Female	78	38.5%	61.5%	NS	16.7%	83.3%	0.021	69.2%	30.8%	NS	87.2%	12.8%	0.003	48.7%	51.3%	NS	32.1%	67.9%	0.004
Male	184	35.9%	64.1%		31.0%	69.0%		78.8%	21.2%		69.6%	30.4%		35.3%	64.7%		51.6%	48.4%	
Age (yr)																			
≤65	147	40.1%	59.9%	NS	21.8%	78.2%	0.049	75.5%	24.5%	NS	78.9%	21.1%	NS	41.5%	58.5%	NS	40.8%	59.2%	NS
>65	115	32.2%	67.8%		33.0%	67.0%		76.5%	23.5%		69.6%	30.4%		36.5%	63.5%		52.2%	47.8%	
Smoking																			
Nonsmoker	94	46.8%	53.2%	0.012	18.1%	81.9%	0.020	73.4%	26.6%	NS	86.2%	13.8%	0.002	53.2%	46.8%	0.001	31.9%	68.1%	0.001
Smoker	168	31.0%	69.0%		31.5%	68.5%		77.4%	22.6%		68.5%	31.5%		31.5%	68.5%		53.6%	46.4%	
Histology																			
ADC	165	46.1%	53.9%	<0.001	18.8%	81.2%	<0.001	71.5%	28.5%	0.036	87.3%	12.7%	<0.001	48.5%	51.5%	<0.001	37.0%	63.0%	<0.001
SCC	97	20.6%	79.4%		40.2%	59.8%		83.5%	16.5%		53.6%	46.4%		23.7%	76.3%		60.8%	39.2%	
Vascular invasion																			
Absent	222	35.6%	64.4%	NS	27.0%	73.0%	NS	73.0%	27.0%	0.008	76.6%	23.4%	NS	40.5%	59.5%	NS	44.1%	55.9%	NS
Present	40	42.5%	57.5%		25.0%	75.0%		92.5%	7.5%		65.0%	35.0%		32.5%	67.5%		55.0%	45.0%	
Lymphatic invasion																			
Absent	133	42.9%	57.1%	0.040	24.1%	75.9%	NS	75.2%	24.8%	NS	75.2%	24.8%	NS	43.6%	56.4%	NS	39.1%	60.9%	0.035
Present	129	30.2%	69.8%		29.5%	70.5%		76.7%	23.3%		74.4%	25.6%		34.9%	65.1%		52.7%	47.3%	
Necrosis																			
<10%	171	41.5%	58.5%	0.031	22.2%	77.8%	0.028	74.9%	25.1%	NS	80.7%	19.3%	0.004	43.3%	56.7%	NS	41.5%	58.5%	NS
≥10%	91	25.3%	72.5%		35.2%	64.8%		78.0%	22.0%		63.7%	36.3%		31.9%	68.1%		53.8%	46.2%	

The variables that had no significant differences such as pleural invasion, perineural invasion, tumor status, nodal status, pathological stage, Eastern Cooperative Oncology Group performance status, and recurrence were deleted.
NSCLC, non-small cell lung cancer; NS, not significant; p stage, pathological stage.

TABLE 4. Comparison of Immunohistochemistry in NSCLC

Characteristics	GSK-3β Status			β-Catenin Status			c-Myc Status			Cyclin D1 Status			p53 Status		
	+	−	p	+	−	p	+	−	p	+	−	p	+	−	p
Wnt1 status															
+	24 (25.0)	72 (75.0)	NS	81 (84.4)	15 (15.6)	0.034	85 (88.5)	11 (11.5)	<0.001	47 (49.0)	49 (51.0)	0.018	39 (40.6)	57 (59.4)	NS
−	46 (27.7)	120 (72.3)		121 (72.9)	45 (27.1)		111 (66.9)	55 (33.1)		56 (33.7)	110 (66.3)		81 (48.8)	85 (51.2)	
GSK-3β status															
+				54 (77.1)	16 (22.9)	NS	55 (78.6)	15 (21.4)	NS	25 (35.7)	45 (64.3)	NS	42 (60.0)	28 (40.0)	0.008
−				148 (77.1)	44 (22.9)		141 (73.4)	51 (26.6)		78 (40.6)	114 (59.4)		78 (40.6)	114 (59.4)	
β-Catenin status															
+							148 (73.3)	54 (26.7)	NS	80 (39.6)	122 (60.4)	NS	94 (46.5)	108 (53.5)	NS
−							48 (80.0)	12 (20.0)		23 (38.3)	37 (61.7)		26 (41.3)	37 (58.7)	
c-Myc status															
+										86 (43.9)	110 (56.1)	0.009	80 (40.8)	116 (59.2)	0.007
−										17 (25.8)	49 (74.2)		40 (60.6)	26 (39.4)	
Cyclin D1 status															
+													46 (44.7)	57 (55.3)	NS
−													74 (46.5)	85 (53.5)	

NS, not significant.

and (4) a cytoplasmic-nuclear pattern, if immunoreactivity was present in the cytoplasm and concomitantly in more than 20% of the nuclei.[19] Strongly positive staining specimens of the cytoplasmic-nuclear, membranous-cytoplasmic, and cytoplasmic patterns (≥10%) were taken as positive.[20] Also, the specimens of membranous pattern (<70%) were estimated as the reduced β-catenin expression.[21]

Statistical Analysis

The analyses were performed using the software package Statistical Package for Social Sciences, version 17.0, for Windows (SPSS, Chicago, IL). The χ^2 test was used to evaluate the association between Wnt signaling pathway proteins and each of the clinicopathologic characteristics. p values less than 0.05 were considered to medical statistically significant difference.

Kaplan-Meier analysis was performed for survival curves, and statistical significance was assessed using the log-rank test. Overall survival was defined as the time from the treatment initiation (resection) to the date of death. To evaluate whether a biomarker is an independent prognostic factor of overall survival, multivariate analysis using the Cox proportional hazard regression model was performed. All p values were based on the two-sided statistical analysis, and p value less than 0.05 was considered to indicate statistical significance.

RESULTS

Patient Characteristics

Patients consisted of 184 (70.2%) male and 78 (29.8%) female with a median age of 64 years (range, 20–81), with 94 (35.9%) nonsmokers and 168 (64.1%) smokers. The hematoxylin and eosin-stained slides were reviewed to confirm the original diagnosis by two pathologists (X.X. and J.H.C.) independently, which was based on the World Health Organization criteria.[22] The 262 patients were classified using the 7th Edition of the International Union Against Cancer and American Joint Committee on Cancer TNM classification of Malignant Tumors from the International Association for the Study of Lung Cancer[23] as follows: pathological stage (p stage) I, 106 (40.5%) patients; p stage II, 74 (28.2%) patients; p stage III, 70 (26.7%) patients; p stage IV, 12 (4.6%) patients (Table 1). The male to female ratios were 1.3:1 and 15.2:1 in ADC and SCC patients ($p < 0.001$). The patients with ADC were younger than the patients with SCC ($p = 0.020$), and the tumor size of ADC was smaller than that of SCC ($p = 0.021$). Among the SCC patients, 94.8% had a history of smoking, but only 46.1% of the ADC patients were smokers ($p < 0.01$). There were statistical differences in pleural invasion, vascular invasion, Eastern Cooperative Oncology Group performance status (ECOG PS), and survival between the two histological subtypes ($p < 0.05$, respectively). The detailed clinicopathologic characteristics in this study are summarized in Table 2.

Wnt Pathway Proteins Expression in NSCLCs

The Wnt1 expression appeared in the form of a cytoplasmic staining pattern (Figure 1*A*, *B*). The Wnt1 expression

was negative in the non-neoplastic type I or type II pneumocytes, bronchiolar epithelial cells, mesenchymal cells, and inflammatory cells. Regarding the intratumoral Wnt1 expression, 96 (36.6%) carcinomas were positive. The expression of Wnt1 was significantly higher in the tumors in nonsmokers ($p = 0.012$), with ADC histology ($p < 0.001$), absence of lymphatic invasion ($p = 0.04$), and necrosis less than 10% ($p = 0.031$) (Table 3). No significant differences were observed according to the sex, age, tumor size, pleura invasion, vascular invasion, perineural invasion, tumor, node, metastasis (TNM) stage, ECOG PS, and recurrence.

The GSK-3β expression appeared in the form of a cytoplasmic staining pattern (Figure 1*C*). Regarding the intratumoral GSK-3β expression, 70 (26.7%) carcinomas were positive. The GSK-3β status was significantly higher in male ($p = 0.021$), old age ($p = 0.049$), smokers ($p = 0.020$), SCC histology ($p < 0.001$), and necrosis ≥10% ($p = 0.028$) (Table 3). While the expression pattern of GSK-3β appeared quite different from Wnt1, there was no significant association between Wnt1 and GSK-3β (Table 4).

The β-catenin expression exhibited four staining patterns (Figures 1*D–I*). The β-catenin expression is localized to the membrane in normal epithelium, but reduced expression in membrane, expression in the cytoplasm, and/or nuclear translocation could be considered to be an indication of its aberrant expression. The altered β-catenin expression was associated with SCC histology ($p = 0.036$) and presence of vascular invasion ($p = 0.008$) (Table 3). Among 96 Wnt1-positive tumors, 81 carcinomas were β-catenin positive and 15 were negative. The percentage of β-catenin-positive tumor was significantly higher in Wnt1-positive tumors than in Wnt1-negative tumors ($p = 0.034$, Table 4).

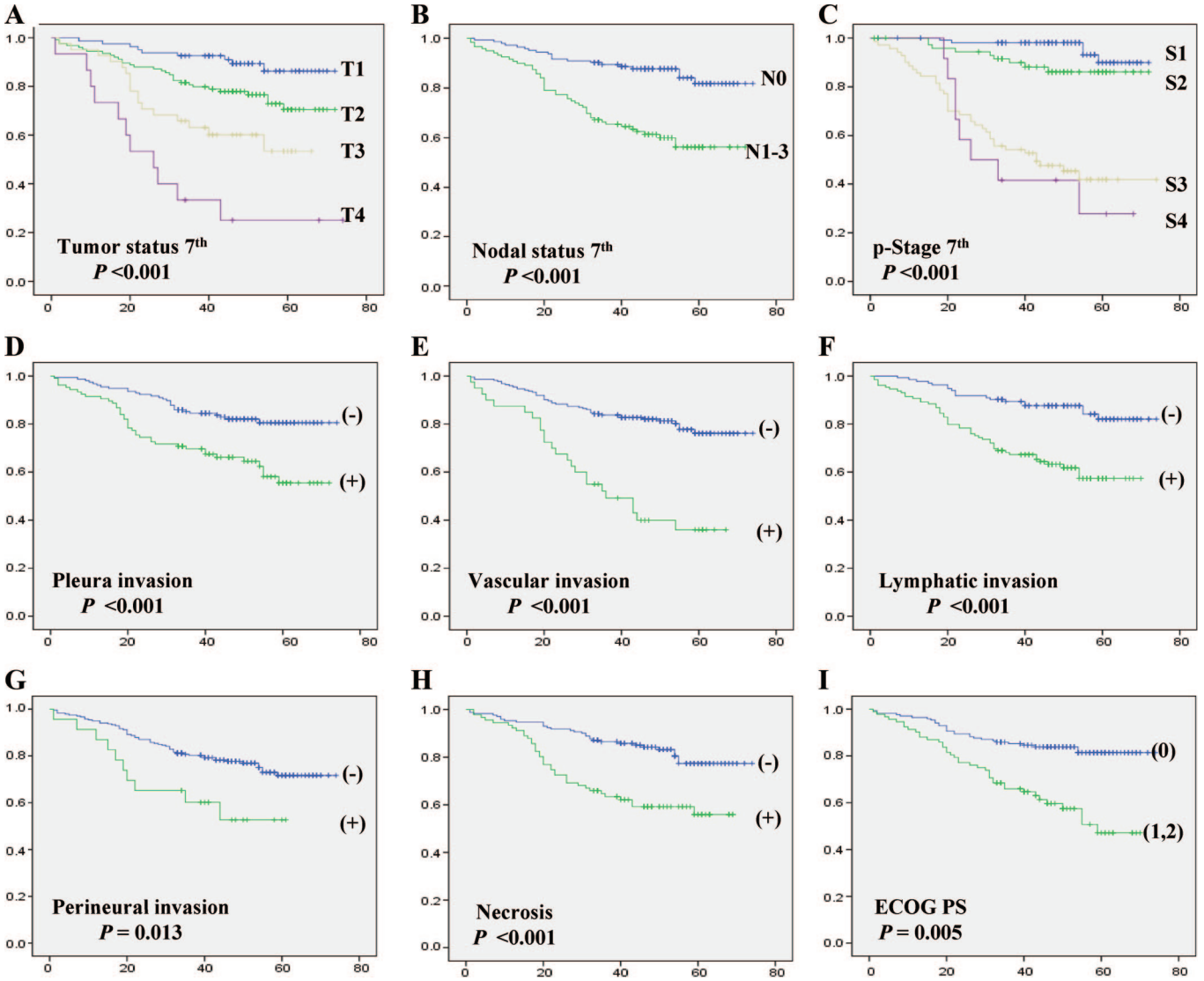

FIGURE 2. Kaplan-Meier univariate analysis of overall survival curves in 262 non-small cell lung cancer patients with the prognostic parameters including tumor status (*A*), nodal status (*B*), p stage (*C*), pleura invasion (*D*), vascular invasion (*E*), lymphatic invasion (*F*), perineural invasion (*G*), necrosis (*H*), and Eastern Cooperative Oncology Group performance status (ECOG PS) (*I*).

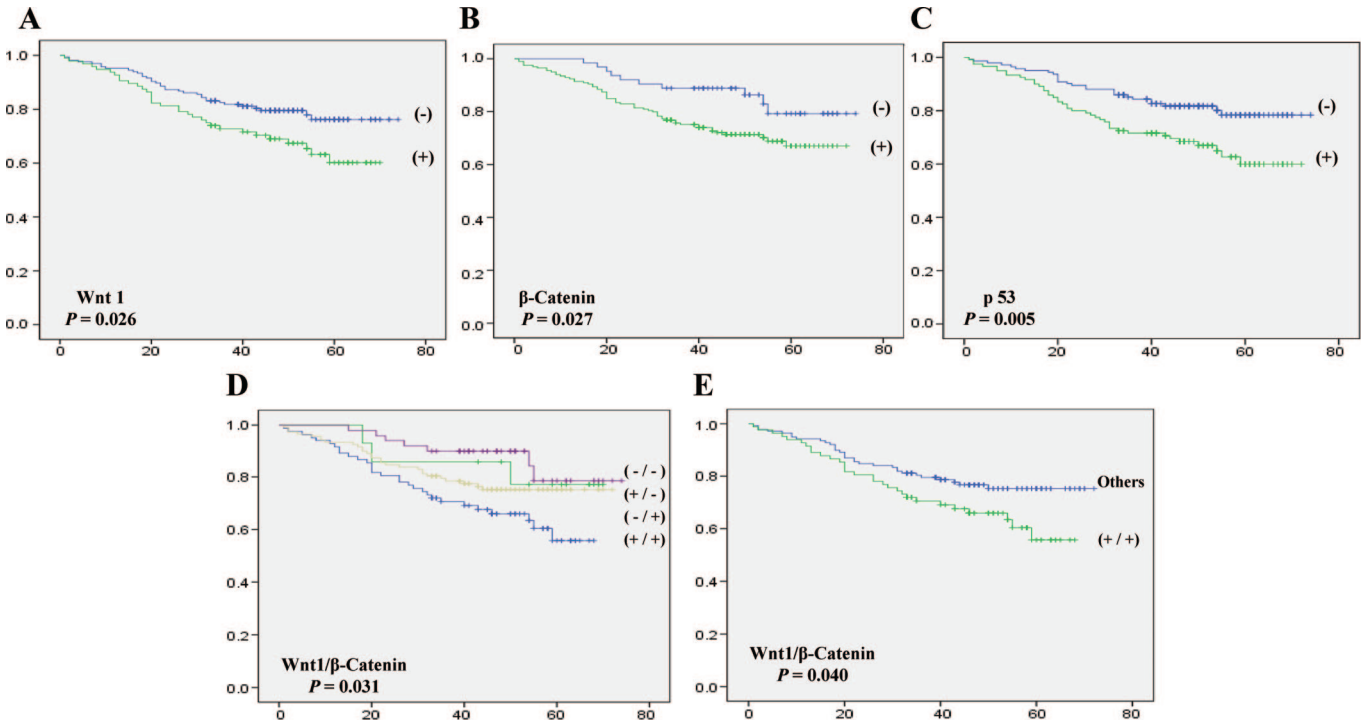

FIGURE 3. Wnt1-positive non-small cell lung cancers (NSCLCs) showed significantly shorter survival than Wnt1-negative NSCLCs (*A*). NSCLCs with β-catenin expression showed significantly shorter survival than β-catenin-negative NSCLCs (*B*). The overall survival was shorter in p53-positive tumors than p53-negative tumors (*C*). Regarding the two variables, Wnt1 and β-catenin, the overall survival is significantly lower in patients with Wnt1+/β-catenin+ than in patients with Wnt1−/β-catenin+, Wnt1+/β-catenin−, and Wnt1−/β-catenin− NSCLCs (*D*, *E*).

Regarding the intratumoral c-Myc expression, 196 (74.8%) carcinomas were positive (Figure 1*J*). The c-Myc overexpression was significantly more frequent in female ($p = 0.003$), nonsmokers ($p = 0.002$), ADC histology ($p < 0.01$), and necrosis less than 10% ($p = 0.004$) (Table 3). The percentage of c-Myc-positive tumor was significantly higher in Wnt1-positive tumors than in Wnt1-negative tumors ($p < 0.001$, Table 4).

The cyclin D1 expression was localized to the nucleus (Figure 1*K*). Regarding the intratumoral cyclin D1 expression, 103 (39.3%) carcinomas were positive. Cyclin D1 status was higher in nonsmokers ($p = 0.001$) and ADC patients ($p < 0.001$) (Table 3). The percentage of cyclin D1-positive tumor cells was higher and significantly correlated with the percentage of Wnt1-positive ($p = 0.018$) and c-Myc-positive tumor cells ($p = 0.009$, Table 4).

p53 Overexpression in Relation to the Wnt Pathway Proteins Expression

Regarding intratumoral p53 expression, 120 (45.8%) carcinomas were positive (Figure 1*L*). The overexpression of p53 protein was significantly related with male gender ($p = 0.004$), smoker ($p = 0.001$), SCC histology ($p < 0.001$), and lymphatic invasion ($p = 0.035$, Table 3). The percentage of p53-positive tumor cells was significantly higher in GSK-3β-positive tumors than negative tumors, while the percentage was significantly lower in c-Myc-positive tumors than in negative tumors ($p < 0.01$, respectively). However, the percentage of p53-positive tumor cells was not statistically related with the percentage of Wnt1-positive tumor cells (Table 4).

Overall Survival Analysis

At the time of analysis, the number of cancer-specific deaths was 78 cases (29.8%). On univariate survival analysis, the conventional prognostic parameters, including tumor status, nodal status, p stage, pleura invasion, vascular invasion, lymphatic invasion, perineural invasion, necrosis, and ECOG PS, reached significance for overall survival (Figure 2). The Wnt1-positive patients had a significantly shorter survival than Wnt1-negative patients, as well as β-catenin ($p < 0.05$, respectively). The patients with Wnt1+/β-catenin+ NSCLC showed inferior survival compared with Wnt1−/β-catenin+, Wnt1+/β-catenin−, and Wnt1−/β-catenin− patients ($p = 0.031$; Figure 3). The overall survival was inferior in p53-positive tumors than negative tumors ($p = 0.005$; Figure 3). Multivariate analysis proved that Wnt1 high expression or β-catenin alteration were independent prognostic factors of overall survival adjusted by stage, pleural invasion, lymphatic invasion, and ECOG PS. The Wnt1+/β-catenin+ expression was also an independent factor adjusted by stage, pleural invasion, lymphatic invasion, ECOG PS or stage, age, gender, histology, and p53 ($p < 0.05$, respectively; Table 5).

TABLE 5. Multivariate Analysis Results of Overall Survival

Variables	HR	95% CI	*p*
p stage I			<0.001
p stage II	1.757	0.735–4.198	0.205
p stage III	4.962	2.242–10.982	<0.001
p stage IV	7.836	2.924–21.000	<0.001
Pleural invasion	1.938	1.172–3.203	<0.001
Lymphatic invasion	2.010	1.118–3.614	<0.001
EOCG PS	3.510	2.125–5.797	<0.001
Wnt1	1.699	1.039–2.778	0.035
β-Catenin	2.165	1.092–4.293	0.027
p stage I			<0.001
p stage II	1.838	0.772–4.378	0.169
p stage III	5.272	2.377–11.690	<0.001
p stage IV	7.611	2.838–20.412	<0.001
Pleural invasion	1.916	1.160–3.167	0.011
Lymphatic invasion	1.938	1.076–3.490	0.028
EOCG PS	3.472	2.109–5.715	<0.001
Wnt1/β-catenin	2.152	1.320–3.507	0.002
p stage I			<0.001
p stage II	2.172	0.885–5.335	0.091
p stage III	9.814	4.670–20.625	<0.001
p stage IV	9.907	3.660–26.815	<0.001
Age	2.164	1.301–3.598	0.003
Gender	0.745	0.403–1.377	0.348
Histology	1.805	1.053–3.096	0.032
Wnt1/β-catenin	2.371	1.421–3.959	0.001
p53	1.559	0.949–2.562	0.080

Variable 1, Wnt1 and β-catenin were independent prognostic factors adjusted by p stage, pleural invasion, lymphatic invasion, and EOCG PS.

Variable 2, $Wnt1^+/\beta\text{-catenin}^+$ expression was an independent factor adjusted by p stage, pleural invasion, lymphatic invasion, and EOCG PS.

Variable 3, $Wnt1^+/\beta\text{-catenin}^+$ expression was an independent factor adjusted by p stage, age, gender, histology, and p53.

HR, hazard ratio; CI, confidence interval; p stage, pathological stage; EOCG PS, Eastern Cooperative Oncology Group performance status.

DISCUSSION

The results of the current study demonstrated that Wnt1 expression correlated with Wnt target proteins, including β-catenin, c-Myc and Cyclin D1, which suggested that Wnt1 is an important member stimulating the Wnt/β-catenin signaling pathway. As a result, high expression of Wnt1 was associated with poor prognosis in NSCLC patients after surgery. Our results corresponded well with a previous study by Huang et al.,[19] which demonstrated that elevated Wnt1 expression was a significant poor prognostic factor in NSCLC. However, there were some different aspects compared with their study. This study showed higher expression of Wnt1 in the ADC histology than SCC and the clinical significance of β-catenin, a pivotal target molecule of Wnt pathway. Although Huang et al. showed significant correlation between Wnt1 and β-catenin expression, they did not mention the prognostic value of β-catenin in relation to Wnt1.

The aberrant expression of β-catenin was higher in Wnt1-positive NSCLCs than in Wnt1-negative NSCLCs, and patients with aberrant β-catenin expression had shorter survival in this study. The correlation of β-catenin expression and cellular localization with the prognosis has been described previously. Strong nuclear or cytoplasmic β-catenin staining in colorectal cancer correlates with more invasive tumor growth, a higher susceptibility to disease recurrence after surgery, and a lower survival rate.[24–26] In esophageal carcinoma, loss of proteins of the E-caderin/β-catenin adhesive complex correlated with poor prognosis.[27,28] On the contrary, analysis of hepatocellular carcinomas revealed that mutation and nuclear staining of β-catenin correlated with less aggressive tumor growth and better survival rates.[29,30] These contradictory results are presumed as follows. First, no standard or universally accepted criteria to evaluate β-catenin expression by immunohistochemistry might generate different results. Second, different activation mechanism of the Wnt signaling pathway in each organ might be present. Therefore, detailed immunohistochemical assessment of β-catenin expression and standardization is recommended.

However, because there is a subgroup of patients who had alteration of β-catenin but negative Wnt1, β-catenin may be activated through other pathways besides Wnt1, as β-catenin is involved not only in the Wnt pathway but also in the cadherin cell adhesion system. β-catenin plays a dual role in cells as a major structural component of cell-cell adherence junctions and as a pivotal signaling molecule in the Wnt pathway, transmitting transcriptional cues into the nucleus. Also, the Wnt gene family encodes at least 19 secreted factors involved in cell growth, differentiation, embryogenesis, and oncogenesis that another Wnt gene besides Wnt1 may cause the alteration of β-catenin.

A novel finding of our study is that the patients with double-positive Wnt1/β-catenin NSCLC showed significantly shorter survival than other groups. We also found that high expression of Wnt1, alteration of β-catenin, and double-positive Wnt1/β-catenin were independent poor prognostic factors in NSCLC regardless of TNM stage.

GSK-3β is an essential component of the Wnt signaling pathway and plays important roles in regulating cell proliferation, differentiation, and apoptosis.[31] However, GSK-3β expression showed no significant association with Wnt1 or β-catenin in NSCLC. This result suggested that GSK-3β might be regulated by another Wnt family member in lung cancer. On the contrary, the overexpression of p53 was significantly associated with GSK-3β expression as well as smoking, SCC histology, and poor prognosis of the patients, whereas it was not associated with the expression of Wnt1/β-catenin in this study.

A possible crosstalk between p53 and β-catenin has been suggested, in which elevated levels of p53 downregulate β-catenin through GSK-3β in variety of cell types, because the ability of p53 to lower β-catenin is blocked by the LiCl, which inhibits the activity of GSK-3β.[10] However, the exact molecular mechanism responsible for the p53 overexpression associated with GSK-3β in this study remains to be elucidated. Furthermore, we have been unable to demonstrate a direct protein-protein interaction between p53 and β-catenin,

suggesting that p53 may operate independently of other aspects of Wnt signaling pathway dysregulation.

In summary, this study has shown that (1) aberrant expression of Wnt1 and its downstream target molecule β-catenin are independent prognostic factors regardless of TNM stage in NSCLC, (2) Wnt1 expression was significantly associated with altered expression of β-catenin and overexpression of c-Myc or cyclin D1, and (3) Wnt1 may be one of the possible mechanisms of the activation of the canonical Wnt/β-catenin signaling pathway in lung cancer.

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