Wnt1 overexpression associated with tumor proliferation and a poor prognosis in non-small cell lung cancer patients

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Abstract. The Wnt family genes encode multifunctional signaling glycoproteins that are involved in the regulation of a wide variety of normal and pathological processes including tumorigenesis. In order to clarify the clinical significance of the intratumoral Wnt1 expression in non-small cell lung cancer (NSCLC), we performed an immunohistochemical study on the Wnt1 expression in NSCLCs in relation to the tumor proliferation. The intratumoral Wnt1 protein expression appeared in a cytoplasmic staining pattern. Of the 151 NSCLCs studied, 61 carcinomas (40.4%) were Wnt1-positive. Regarding the tumor biology of the intratumoral Wnt1 expression, the Ki-67 proliferation index was significantly higher in Wnt1-positive than in Wnt1-negative tumors (P=0.0062). Furthermore, regarding the expression of c-Myc, one of the proliferation-regulating Wnt targets, the percentage of c-Myc-positive tumor cells was significantly higher in Wnt1-positive than in Wnt1-negative tumors (P=0.0019). The Ki-67 proliferation index was significantly higher in c-Myc-positive than in c-Myc-negative tumors (P=0.0239). The overall survival was significantly lower in patients with Wnt1-positive NSCLCs than in patients with Wnt1-negative NSCLCs (P=0.0003). A Cox regression analysis demonstrated that the Wnt1 status was a significant prognostic factor for NSCLC patients (hazard ratio 1.983; P=0.0061). Our results revealed that the Wnt1 over-expression affects the tumor proliferation in NSCLCs, partly via the upregulation of c-Myc.

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers and the major cause of cancer-related death in North America, Europe and Japan (1). Therefore, it is important to clarify the mechanism of its tumor biology in order to improve the clinical outcome of NSCLC patients (2). Studies on the molecular biology of human cancers have revealed that many molecules affect the progression of malignant tumors. The activation of oncogenes or the inactivation of tumor suppressor genes could initially cause carcinogenesis (3). Then, many molecules associated with angiogenesis or metastasis could produce more aggressive malignant tumors during the subsequent tumor progression (4,5). Considering these facts, the clarification of the mechanism of tumor progression in NSCLCs could potentially lead to the development of a new strategy of treatments for NSCLC patients.

Among the many molecules associated with tumorigenesis, the Wnt family genes are frequently upregulated in many human cancers (6-9). The Wnt family genes encode multi-functional signaling glycoproteins that are involved in the regulation of a wide variety of normal and pathological processes, including embryogenesis, differentiation and tumorigenesis (10-12). In addition, a previous study showed that transfection of the metastatic suppressor gene MRP-1/CD9 can downregulate the expression of various Wnts, including Wnt1 and Wnt5a (13). However, the clinical significance of these Wnt expression levels in human cancers is still unclear. Therefore, an immunohistochemical clinical investigation was conducted on the intratumoral Wnt1 expression in NSCLCs in relation to tumor proliferation. The expression of c-Myc, one of the proliferation-regulating target genes of the canonical Wnt/ß-catenin pathway was evaluated (14).

Materials and methods

Clinical characteristics of the patients. NSCLC patients who underwent surgery at the Faculty of Medicine, Kagawa University from January 1993 to February 2001 were studied. This study was approved by the institutional review board of Kagawa University (14-7, a clinical study of biological markers in NSCLCs) and signed informed consent was obtained from each patient. Tumor-node-metastasis (TNM) staging designations were made according to the postsurgical pathological international staging system. Since advanced stage lung cancer (stage IV) involved several ill-defined factors and had distant metastases, such patients were excluded from this study. In total, 151 patients with lung cancer up to

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stage III, comprising 88 patients with adenocarcinomas, 53 patients with squamous cell carcinomas, and 10 patients with large cell carcinomas, were investigated. The patients' clinical records and histopathological diagnoses were fully documented. This report includes follow-up data as of October 31, 2006.

Immunohistochemistry for Wnt1, c-Myc and Ki-67. We used a rabbit polyclonal antibody for Wnt1 (H-89, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted at 1:200, a mouse monoclonal antibody for c-Myc (9E10, Santa Cruz) diluted at 1:100, and a mouse monoclonal antibody for Ki-67 (MIB-1, Dako, Glostrup, Denmark) diluted at 1:40. Formalin-fixed paraffin-embedded tissue was cut in 4-μm sections and mounted on poly-L-lysine-coated slides. Sections were deparaffinized and rehydrated. The slides were then heated in a microwave for 10 min in a 10-μmol/l citrate buffer solution at pH 6.0 and then cooled to room temperature. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 min, the sections were treated for 2 h at room temperature with 5% bovine serum albumin. Duplicate sections were then incubated overnight with the primary specific antibodies against Wnt1, c-Myc and Ki-67, respectively. The slides were then incubated for 1 h with biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) for Wnt1, and biotinylated anti-mouse IgG (Vector) for c-Myc and Ki-67. The sections were incubated with the avidin-biotin-peroxidase complex (Vector) for 1 h, and antibody binding was visualized with 3,3'-diamino-benzidine tetrahydrochloride. The sections were then lightly counterstained with Mayer's hematoxylin. Sections of resected lung tumors known to express Wnt1 or c-Myc were used as positive controls for immunohistochemical staining. Sections incubated with normal rabbit IgG served as negative reaction controls for staining of Wnt1, and sections incubated with normal mouse IgG served as negative reaction controls for staining of c-Myc.

All the immunostained sections were evaluated by two authors (T.N. and M.U.) who had no knowledge of the patients' clinical status. For protein expression of Wnt1 and c-Myc, the proportion of staining tumor cells in each selected field was determined by counting individual tumor cells at a high magnification. In cases with multiple areas of low intensity that occurred during evaluation of immunostaining, five areas were selected at random and scored. One random field was selected in sections where all staining appeared intense. At least 200 tumor cells were scored per x40 field. The percentage of carcinoma cells with positive staining for Ki-67 in a given specimen was scored as the Ki-67 proliferation index (15).

Statistical analysis. Since the distributions of three variables, including the percentage of Wnt1-positive tumor cells (P=0.8853), the percentage of c-Myc-positive tumor cells (P=0.7008), and the Ki-67 proliferation index (P=0.8583), all showed normal distributions (Kolmogorov-Smirnov analysis), the statistical differences regarding these variables in relation to several clinical and pathological parameters were assessed by the t-test, and analysis of variance with the Bonferroni/Dunn test. As the Wnt1 protein expression cutoff line of 50% demonstrated the greatest significance in relation to the Ki-67 proliferation index, the sample was classified as Wnt1-positive when ≥50% of the carcinoma cells in a given specimen were positively stained for Wnt1. Since the c-Myc protein expression cutoff line of 30% demonstrated the most significance in relation to the Ki-67 proliferation index, the sample was classified as c-Myc-positive when ≥30% of the carcinoma cells in a given specimen were positively stained for c-Myc. Overall survival was defined as the time from the treatment initiation (surgical resection, chemotherapy, or radiation) to the date of death from any cause. 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. All P-values were based on two-tailed statistical analysis, and a P-value <0.05 was considered to indicate a statistical significance.

Results

Intratumoral Wnt1 expression in NSCLCs. The intratumoral Wnt1 protein expression appeared in the form of a cytoplasmic staining pattern (Fig. 1A). The percentage of Wnt1-positive tumor cells varied greatly among NSCLCs (mean, 42.0±28.4%, Table I). Of the 151 NSCLCs studied, 61 carcinomas (40.4%) were Wnt1-positive. Regarding tumor histology, the percentage of Wnt1-positive tumor cells was 40.5±27.8% in adenocarcinomas and 46.9±28.2% in squamous cell carcinomas. There was no difference in the intratumoral Wnt1 expression according to tumor histology. In addition, there was no difference in the intratumoral Wnt1 expression according to tumor status, nodal status or tumor differentiation.

Tumor proliferation of NSCLCs in relation to Wnt1 status. To investigate the tumor proliferation of NSCLCs, we evaluated the Ki-67 proliferation index (Fig. 1B). Of the 151 NSCLCs studied, the Ki-67 proliferation index varied greatly (mean, 44.3±30.2%). Regarding the tumor proliferation in relation to the Wnt1 status, the Ki-67 proliferation index was 52.4±30.6% in Wnt1-positive tumors and 38.8±28.8% in Wnt1-negative tumors. The Ki-67 proliferation index was significantly higher in Wnt1-positive tumors than in Wnt1-negative tumors (P=0.0062, Fig. 2).

c-Myc expression in relation to Wnt1 status. We then studied the intratumoral expression of c-Myc, one of proliferation-regulating Wnt1 targets. The percentage of c-Myc-positive tumor cells varied greatly among the 151 NSCLCs (mean, 36.5±27.3%, Table I and Fig. 1C). Regarding the c-Myc expression in relation to the Wnt1 status, the percentage of c-Myc-positive tumor cells was 44.7±26.2% in Wnt1-positive tumors and 30.8±26.7% in Wnt1-negative tumors. The percentage of c-Myc-positive tumor cells was significantly higher in Wnt1-positive tumors than in Wnt1-negative tumors (P=0.0019, Fig. 3).

Clinical significance of c-Myc expression in NSCLCs. Of the 151 tumors studied, 85 carcinomas (56.3%) were c-Myc-

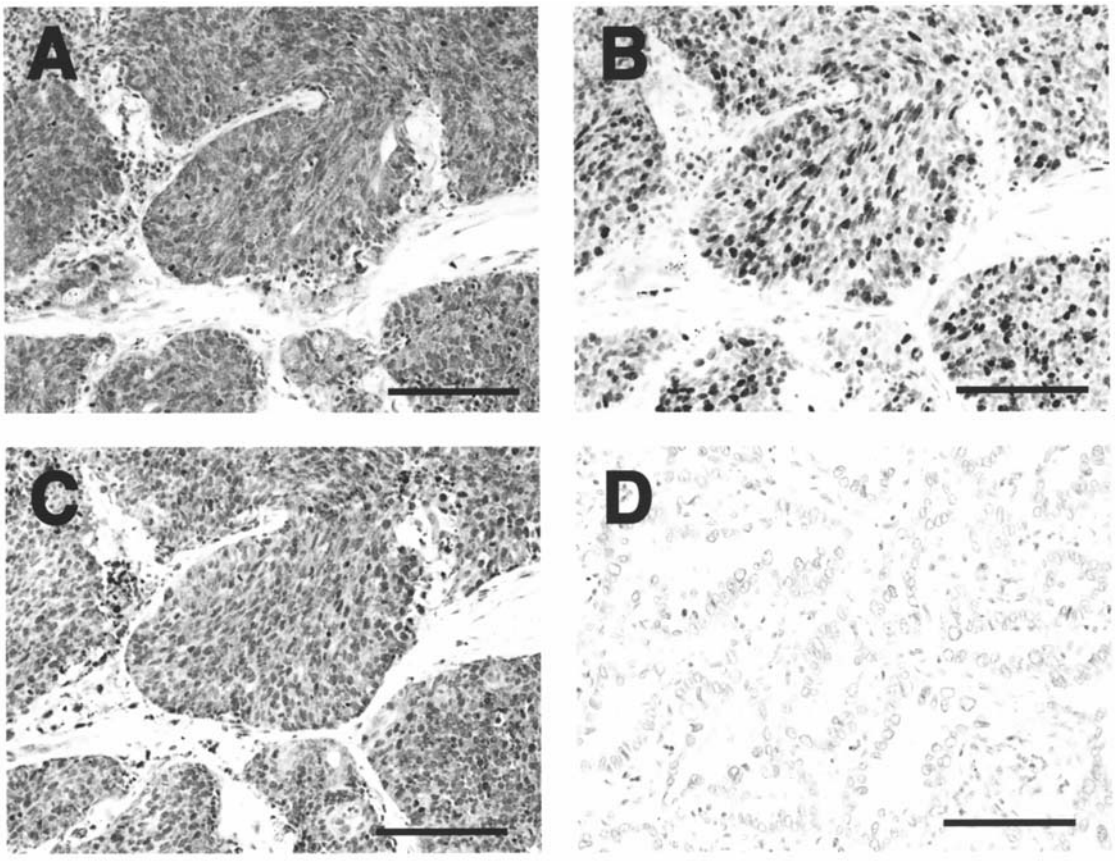

Figure 1. Immunohistochemical staining of human non-small cell lung cancer tissues by the avidin-biotin-peroxidase complex procedure. A carcinoma with (A) positive expression of Wnt1, (B) high Ki-67 index, and (C) positive expression of c-Myc. (D) A carcinoma with negative expression of Wnt1. Bar, 100 μm.

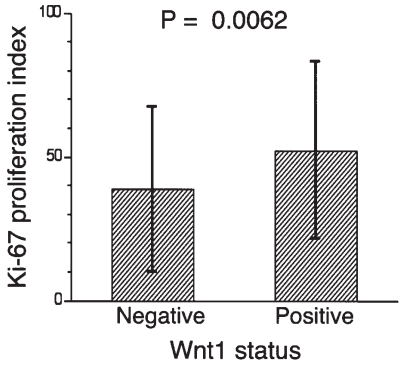

Figure 2. Ki-67 proliferation index in relation to Wnt1 status.

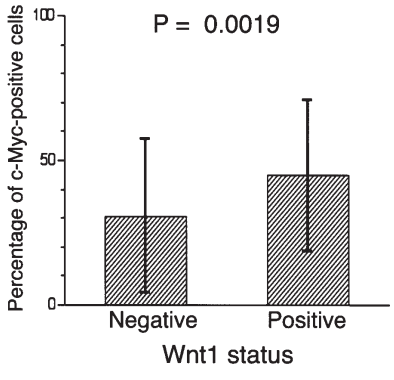

Figure 3. c-Myc expression in relation to Wnt1 status.

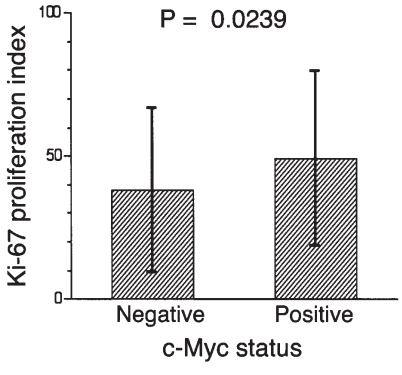

Figure 4. Ki-67 proliferation index in relation to c-Myc status.

positive (Table I). Regarding tumor histology, the percentage of c-Myc-positive tumor cells was 33.2±26.4% in adenocarcinomas and 42.5±28.3% in squamous cell carcinomas. There was no difference in the c-Myc expression according to tumor histology. In addition, there was no difference in the intratumoral c-Myc expression according to tumor status, nodal status or tumor differentiation.

Regarding the tumor proliferation in relation to the c-Myc status, the Ki-67 proliferation index was 49.2±30.6% in c-Myc-positive tumors and 38.0±28.7% in c-Myc-negative tumors. The Ki-67 proliferation index was significantly higher in c-Myc-positive than in c-Myc-negative tumors (P=0.0239, Fig. 4).

Table I. Distribution of Wnt1 and c-Myc status in 151 NSCLC patients.

		Wnt1 status			c-Myc status		
Characteristics	n	%	P-value	Positive	%	P-value	Positive
Smoking							
Non-smoker	57	46.1±27.4	0.1595	26	37.8±27.7	0.6378	34
Smoker	94	39.4±28.8		35	35.6±27.2		51
Tumor status							
T1, T2	111	41.3±27.7	0.6197	43	35.6±27.5	0.5376	59
T3, T4	40	43.9±30.6		18	38.8±27.1		26
Nodal status							
N0	105	39.4±29.0	0.0993	37	35.2±26.1	0.4093	57
N1, N2, N3	46	47.7±26.6		24	39.2±30.0		28
Pathological stage							
Stage I	84	39.2±27.6	0.1662	30	34.4±26.5	0.5771	42
Stage II	16	37.2±31.6		4	37.8±33.2		9
Stage III	51	48.0±28.3		27	39.4±26.9		34
Differentiation							
Well	55	40.5±27.0	0.7738	18	31.3±26.2	0.0790	27
Moderately	58	44.1±28.1		26	43.4±26.9		39
Poorly	38	40.9±31.3		17	33.4±28.0		19
Histology							
Adenocarcinoma	88	40.5±27.8	0.1412	32	33.2±26.4	0.1313	45
Squamous cell carcinoma	53	46.9±28.2		27	42.5±28.3		34
Large cell carcinoma	10	29.0±32.8		2	33.0±27.1		6
Total number of patients	151	42.0±28.4		61	36.5±27.3		85

Table II. Multivariate regression analysis in predicting survival of NSCLC patients.

Variables	Hazard ratio	95% CI	P-value
Wnt1 status	1.983	(1.216-3.236)	0.0061
Smoking	0.945	(0.458-1.947)	0.8775
Gender	0.762	(0.343-1.695)	0.5056
Tumor status	1.407	(1.148-1.725)	0.0010
Nodal status	1.578	(1.222-2.038)	0.0005
Differentiation	1.311	(0.955-1.801)	0.0937

CI, confidence interval.

Overall survival of NSCLC patients in relation to Wnt1 status. The 5-year survival rate of 151 NSCLC patients stratified according to their Wnt1 status is shown in Fig. 5A. Regarding Wnt1 status, the 5-year survival rate was 39.9% in patients with Wnt1-positive NSCLCs and 67.5% in patients with Wnt1-negative NSCLCs. The overall survival was significantly lower in patients with Wnt1-positive NSCLCs than in patients with Wnt1-negative NSCLCs (P=0.0003). Furthermore, regarding the c-Myc status, the overall survival was significantly lower in patients with c-Myc-positive NSCLCs than in patients with c-Myc-negative NSCLCs (P=0.0325, Fig. 5B).

A multivariate analysis using the Cox regression model was performed in order to evaluate prognostic factors for NSCLC patients, as shown in Table II. Three variables, Wnt1 status (hazard ratio 1.983; P=0.0061), tumor status (hazard ratio 1.407; P=0.0010), and nodal status (hazard ratio 1.578; P=0.0005), were significant prognostic factors for NSCLC patients.

Discussion

The Wnt family is a large family of homologous but distinct genes, which have been highly conserved across species during evolution. The Wnt genes encode secreted cysteine-rich proteins with multidirectional biological functions via autocrine or paracrine routes (11). They are involved in the regulation of a wide variety of normal and pathological

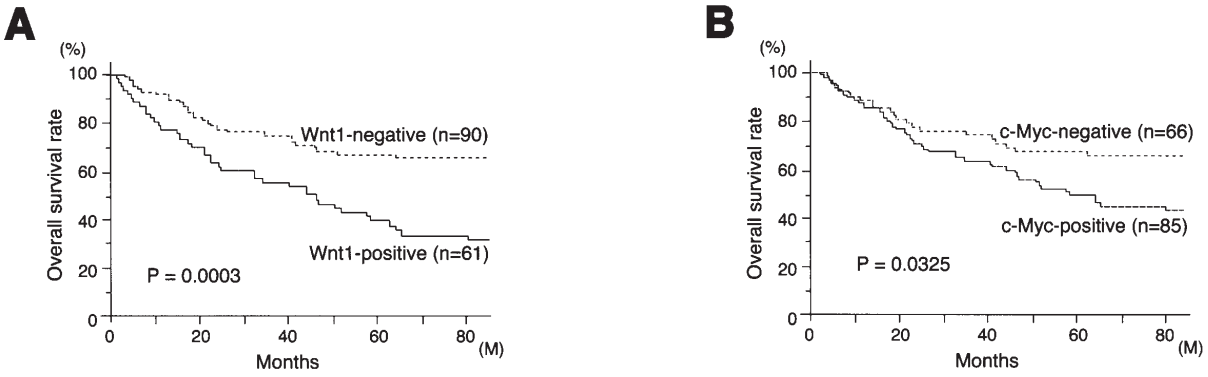

Figure 5. Overall survival in relation to Wnt1 status (A) and c-Myc status (B).

processes including tumorigenesis (10-12). The Wnt genes have been classified into functional groups with separate downstream signaling pathways (16,17). Among them, Wnt1 stimulates the canonical Wnt/ß-catenin signaling pathway, which leads to changes in cell fate and/or cell transformation (18). The canonical Wnt/ß-catenin signaling pathway regulates the transcription of many Wnt-target genes with TCF/LEF1 motifs (19). As a result, the intratumoral Wnt1 expression could affect many biological functions through these Wnt-target genes.

Recent studies have revealed that the upregulation of Wnt1 frequently occurs in many human cancers (6-8,20). However, the clinical significance of the Wnt1 expression in human cancers is still unclear. Therefore, we conducted a clinical study on the intratumoral Wnt1 expression in NSCLCs. Consequently, the present study demonstrated that the intratumoral Wnt1 expression was associated with tumor proliferation in NSCLCs. The tumor proliferation was significantly higher in Wnt1-positive than in Wnt1-negative tumors. In addition, the intratumoral Wnt1 status was a significant prognostic factor for NSCLC patients. A Cox regression analysis demonstrated that the hazard ratio associated with the Wnt1 status was higher than those of tumor status and nodal status in NSCLC patients. The present study is the first clinical report demonstrating the clinical significance of the Wnt1 overexpression in NSCLCs. A recent clinical study in oral squamous cell carcinomas also revealed that the Wnt1 expression was associated with tumor proliferation (8). Experimental studies using cell lines have demonstrated that the canonical Wnt signaling pathway stimulates cell proliferation (21,22). Furthermore, the Wnt inhibitory factor was reported to inhibit the proliferation of lung cancer cells (23).

The canonical Wnt/ß-catenin signaling pathway can affect various biological functions through the induction of many Wnt-target genes containing the TCF/LEF1 motifs (19). This fact makes the clinical significance of the Wnt signaling pathway rather confusing. Therefore, we performed an additional study on the intratumoral expression of c-Myc, one of the target genes of the canonical Wnt/ß-catenin pathway (14). c-Myc is a transcriptional factor that plays a role in cell proliferation, apoptosis and in the development of human cancers (24-26). Although the precise mechanism of c-Myc action is not yet fully understood, c-Myc is considered to be involved in growth control and cell cycle progression by stimulating and repressing the expression of cell cycle regulators. The c-Myc overexpression has been reported to promote the G1 to S transition by activating cyclinE/cdk2 complexes in lung cancer cells (26). Furthermore, experimental studies have also demonstrated that the downregulation of c-Myc can inhibit the proliferation of cell lines, including lung cancer cells (27,28).

The present clinical study demonstrated the intratumoral c-Myc expression to be associated with the intratumoral Wnt1 expression in NSCLCs. The intratumoral c-Myc expression was significantly higher in Wnt1-positive than in Wnt1-negative tumors. The intratumoral c-Myc expression was associated with tumor proliferation. The Ki-67 proliferation index was significantly higher in c-Myc-positive than in c-Myc-negative tumors. In addition, the overall survival was significantly lower in patients with c-Myc-positive NSCLCs than in patients with c-Myc-negative NSCLCs. As a result, the present study demonstrated that the Wnt1 overexpression affects tumor proliferation through the induction of the c-Myc expression, via autocrine or paracrine routes. A recent experimental study has also revealed that the Wnt signaling pathway contributes to c-Myc pro-mutagenic effects in cancer cell lines (22).

The overexpression of c-Myc has been reported in lung cancer cells and tumor samples (29,30). In many clinical studies, including the present study, the c-Myc overexpression has been reported to be a factor of poor prognosis in cancer patients (31-33). Although the gene amplification of c-Myc frequently occurs in NSCLCs (29,34), the present study revealed that the Wnt1 overexpression is another mechanism of the c-Myc overexpression. The present study is the first clinical report demonstrating the association between the Wnt1 expression and the c-Myc expression in NSCLCs. Previous clinical studies have suggested an association between the canonical Wnt/ß-catenin signaling pathway and the c-Myc expression in human cancers (35,36).

Regarding the activation of the canonical Wnt/ß-catenin signaling pathway, it is mainly caused by inactive mutations of the *adenomatous polyposis coli* gene or by activating mutations of the *ß-catenin* gene in the development of colorectal carcinomas (37). However, previous clinical

studies have revealed these mutations to be rare in NSCLCs (38,39). In contrast, experimental studies using cell lines have demonstrated that the Wnt1 expression is regulated by various molecules (6,13,40). Therefore, the intratumoral Wnt1 expression might be secondarily regulated in response to a range of changes of many molecules during tumor progression. As a result, the Wnt1 overexpression can produce more aggressive malignant tumors during progression.

In conclusion, the present study demonstrated that the overexpression of Wnt1 was associated with tumor proliferation in NSCLCs and that its overexpression was a significant prognostic factor for NSCLC patients. Furthermore, the Wnt1 expression was associated with c-Myc expression, a proliferation-regulating Wnt target. These results suggest that the Wnt1 overexpression affects tumor proliferation in NSCLC, partly via the upregulation of c-Myc. These results also indicate that the suppression of this pathway may be a potentially effective treatment for patients with Wnt1-positive NSCLCs (23,27,28). In addition, because the Wnt pathway can affect various biological functions through the induction of many Wnt-target genes, further studies on other Wnt targets are necessary in order to clarify its precise mechanisms in human cancers.

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