Intratumoral Wnt1 expression affects *survivin* gene expression in non-small cell lung cancer

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Abstract. Survivin, a member of the inhibitor of apoptosis protein family, affects tumorigenesis. Recently, survivin is reported to be a target of the canonical Wnt pathway, which activates the transcription of various tumor-associated target genes. One hundred and twenty-two non-small cell lung cancers (NSCLCs) were investigated to evaluate survivin gene expression in relation to the expression of Wnt1 (a novel member of the canonical Wnt pathway) and Wnt5a (a novel member of the non-canonical Wnt pathway). The survivin gene expression was evaluated by semi-quantitative RT-PCR. The protein expression of pan-survivin, Wnt1, and Wnt5a were investigated by immunohistochemistry. The apoptotic index and the Ki-67 proliferation index were also evaluated. Sixty-four tumors (52.5%) were survivin-high tumors, 65 tumors were Wnt1-high tumors, and 67 tumors (54.9%) were Wnt5a-high tumors. The standardized survivin gene expression significantly correlated with the apoptotic index (P<0.0001), the Ki-67 proliferation index (P<0.0001), and patient survival (P=0.0467). Furthermore, the percentage of Wnt1-positive tumor cells significantly correlated with the standardized survivin gene expression (P<0.0001). In contrast, the percentage of Wnt5a-positive tumor cells did not correlate with the standardized survivin gene expression. As a result, intratumoral Wnt1 expression significantly correlated with the apoptotic index (P<0.0001), the Ki-67 proliferation index (P<0.0001), and patient survival (P=0.0355). Intratumoral Wnt1 overexpression could produce more aggressive NSCLCs by induction of survivin.

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common human cancers with a poor prognosis. However, recent molecular biology studies have revealed that many molecules affect the various biological behaviors of malignant tumors. It is therefore important to clarify these tumor biology mechanisms in order to improve the clinical outcome of NSCLC patients (1). Among them, several molecules have proven to be associated with cell cycle regulation, apoptosis, and chemo-radio resistance. These molecules include survivin (2).

On the other hand, the Wnt gene family encodes multifunctional signaling glycoproteins that are involved in the regulation of various normal and pathological processes, including embryogenesis, differentiation, and tumorigenesis (12,13). The Wnt genes have been classified into functional groups with separate downstream signaling pathways (14). Among them, the activation of the canonical Wnt pathway activates the transcription of various tumor-associated Wnt-target genes (15), including c-Myc (16), Cyclin D1 (17), and vascular endothelial growth factor (VEGF) (18). Recently, survivin is also reported to be a target of the canonical Wnt pathway (19,20).

Elucidating the mechanisms of survivin gene regulation could lead to the development of new treatments for NSCLC patients. This study therefore investigated the survivin gene expression in relation to the intratumoral expression of Wnt1 (a novel member of the canonical Wnt pathway) (21) and Wnt5a (a novel member of the non-canonical Wnt pathway) in NSCLC patients (22).

Materials and methods

Clinical characteristics of patients. From January 2001 to June 2004, consecutive NSCLC patients, who underwent surgery at the Department of General Thoracic Surgery,
Breast and Endocrinological Surgery, Kagawa University, were studied. This study was approved by the institutional review board of Kagawa University (14-7, a clinical study of biological markers in non-small cell lung cancers). Signed, written informed consent was obtained from all patients before therapy was initiated. Tumor-node-metastasis (TNM) staging designations were made according to the postsurgical pathological international staging system. In total, 122 patients with NSCLC up to stage IIIB, including 68 patients with adenocarcinomas, 52 patients with squamous cell carcinomas, and 2 patients with large cell carcinomas, were investigated (Table I). The patients’ clinical records and histopathological diagnoses were fully documented.

RT-PCR for survivin gene expression. Quantitative RT-PCR assays with densitometric analyses of gel electrophoresis were used to evaluate the survivin gene expressions because real-time PCR is not an appropriate method to discriminate between survivin (wild-type survivin) and other splice variants such as survivin-2B, and survivin-deltaEx3 (23). The total cellular RNA was extracted from frozen tissue specimens by the acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was performed with 5 μg of total RNA using a cDNA synthesis kit (Pharmacia, Piscataway, NJ). The sense primer for survivin was 5'-CCACCGCATCTCTACAT TCA-3', and the antisense primer of survivin was 5'-TATGT TCTCTTATGGGGTCG-3' (23). All subsequent assays were carried out using the parameters that yielded amplification of survivin band in a given tumor sample was divided by the value ß-actin (the internal control) within a linear range. The reaction mixture of survivin was subjected to 36 PCR amplification cycles of 60 sec at 94˚C, 60 sec at 60˚C, and 90 sec at 72˚C, and that of ß-actin was subjected to 31 PCR amplification cycles of 60 sec at 94˚C, 60 sec at 60˚C, and 90 sec at 72˚C. Preparations of a human adenocarcinoma cell line A549 were used as positive controls for survivin gene expressions. The amplified DNA samples were run on a 1% agarose gel, and the bands were visualized with ethidium bromide. The densitometric value obtained for a survivin gene expression and protein expression of Wnt1 and Wnt5a tumor when the standardized gene expression cut-off (ΔHSCORE) was 1.0, because a standardized survivin gene expression cut-off

Immunohistochemistry. The following antibodies were used, along with isotype antibodies as negative controls: a mouse monoclonal antibody against pan-survivin (sc17779, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted at 1:50, a rabbit polyclonal antibody for Wnt1 (H-89, Santa Cruz) diluted at 1:200, a goat polyclonal antibody for Wnt5a (C-16, Santa Cruz) 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-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. After quenching the endogenous peroxidase activity with 0.3% H2O2 (in absolute methanol) for 30 min, the sections were treated for 2 h with 5% bovine serum albumin to block non-specific staining. Duplicated sections were incubated overnight with primary antibodies, respectively. Slides were then incubated for 1 h with biotinylated secondary antibodies (Vector Laboratories Inc., Burlingame, CA). 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.

All immunostained sections were independently evaluated with two authors (N. Nakashima and C.-L. Huang), without knowledge of the patients’ characteristics. Five areas were selected at random and scored in cases with multiple areas of low intensity. Also, one random field was selected in sections where all staining appeared intense. At least 200 tumor cells were scored per x40 field. Regarding pan-survivin expression, the nuclear staining and the cytoplasmic staining in each section were evaluated, respectively, in a semi-quantitative manner which reflected both the intensity and percentage of cells staining at each intensity, as reported previously (23). The intensity was classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), or +3 (very strong staining). A value designated as the ‘HSCORE’ thus was obtained for each slide by using the following algorithm: HSCORE = Σ (I x PC), where I and PC represent the intensity and the percentage of cells that stain at each intensity, respectively. The percentage of carcinoma cells with positive staining for Ki-67 antigen was scored as the Ki-67 proliferation index.

TUNEL method for apoptosis. The presence of apoptotic cells was detected with the TUNEL method using the In Situ Apoptosis Detection Kit (Takara Biomedicals, Otsu, Japan). After sections were deparaffinized and rehydrated, the slides were treated for 15 min with 20-μg/ml Proteinase K. After quenching the endogenous peroxidase activity with 3% H2O2 for 5 min, the sections were then 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 developed using 3,3'-diaminobenzidine tetrahydrochloride for 15 min. Lastly, the sections were lightly counterstained with Mayer's hematoxylin. Sections incubated with the TUNEL reaction mixture without TdT were used as negative control slides. Apoptotic cells were determined based on observations of TUNEL-staining sections and serial HE-staining sections. TUNEL positive-staining cells, if they represented the histologic features of necrosis in HE-staining sections, were not considered to be apoptotic cells. In each case, a total of 10,000 tumor cells were evaluated at high magnification by two authors (N. Nakashima and D. Liu) independently, without knowledge of the patients’ characteristics. The apoptotic index was defined as the number of apoptotic cells per 1,000 tumor cells.

Statistical analysis. The statistical significances of survivin gene expression and protein expression of Wnt1 and Wnt5a in relation to several parameters were assessed by the t-test or the χ2 test. The sample was classified as a survivin-high tumor when the standardized survivin gene expression was >1.0, because a standardized survivin gene expression cut-off
line of 1.0 demonstrated the greatest significance in relation to the apoptotic index and the Ki-67 proliferation index (23). The sample was classified as a Wnt1-high tumor when the percentage of Wnt1-positive tumor cells was >50%, and the sample was classified as a Wnt5a-high tumor when the percentage of Wnt5a-positive tumor cells was >30%, as reported previously (24, 25).

The overall survival was defined as the time from 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 univariate analysis was performed using the Cox regression model to study the effects on survival. All P-values were based on two-tailed statistical analysis and a P<0.05 was considered to indicate statistical significance.

Results

Survivin gene expression in NSCLCs. The standardized survivin gene expression ratio varied greatly among the 122 NSCLCs (mean ± SD, 1.14±0.65; Fig. 1). Immunostaining using the antibody against pan-survivin showed various patterns of nuclear staining and cytoplasmic staining (Fig. 2A and E). The standardized survivin gene expression ratio significantly

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Figure 1. Agarose gel electrophoresis of RT-PCR-amplified. (A) Survivin cDNA; (B) β-actin cDNA (internal PCR control). Lane 1, size marker; lane 2, A549 (positive control); lanes 3-5, survivin-low tumors; lanes 6-8, survivin-high tumors.
Figure 2. Immunostaining of lung cancers. A carcinoma with (A) positive expression of pan-survivin, (B) low apoptotic index, (C) high Ki-67 proliferation index and (D) positive Wnt1 expression. A carcinoma with (E) negative expression of pan-survivin and (F) negative Wnt1 expression. (G) A carcinoma with high apoptotic index. (H) A carcinoma with positive Wnt5a expression. Bar, 50 μm.
correlated with the HSCORE of nuclear staining of pan-survivin ($r=0.420$, $P<0.0001$). The HSCORE of nuclear staining of pan-survivin was significantly higher in survivin-high tumors than in survivin-low tumors (32.6±26.1 vs. 14.7±12.5, $P=0.0016$). Furthermore, the standardized survivin gene expression ratio also significantly correlated with the HSCORE of cytoplasmic staining of pan-survivin ($r=0.464$, $P<0.0001$). The HSCORE of cytoplasmic staining of pan-survivin was significantly higher in survivin-high tumors than in survivin-low tumors (102.3±66.1 vs. 54.4±47.9, $P<0.0001$). Among 122 NSCLCs, 64 tumors (52.5%) were survivin-high tumors (Table I). Regarding clinical characteristics, no significant difference was observed in the survivin gene expression according to tumor status, nodal status, tumor differentiation, or tumor histology.

The clinical significance of the survivin gene expression in NSCLCs. Regarding tumor apoptosis, the apoptotic index ranged from 8.0 to 50.0 (mean ± SD, 19.8±8.4; Fig. 2B and G). The standardized survivin gene expression ratio significantly correlated with the apoptotic index ($r=0.413$, $P<0.0001$). The apoptotic index was significantly lower in survivin-high tumors in comparison to survivin-low tumors (17.1±7.8 vs. 22.7±8.1, $P=0.0001$; Fig. 3A).

Concerning tumor proliferation, the Ki-67 proliferation index ranged from 2.0 to 95.0 (mean ± SD, 47.5±29.8; Fig. 2C). The standardized survivin gene expression ratio significantly correlated with the Ki-67 proliferation index ($r=0.346$, $P<0.0001$). The Ki-67 proliferation index was significantly higher in survivin-high tumors than in survivin-low tumors (54.8±28.2 vs. 39.6±29.7, $P=0.0042$; Fig. 3B).

Regarding survival, the 5-year survival rate was 58.7% in patients with survivin-high tumors, and 77.9% in patients with survivin-low tumors. The overall survival was significantly lower in patients with survivin-high tumors than in patients with survivin-low tumors ($P=0.0417$; Fig. 3C). A univariate analysis using the Cox regression model revealed the survivin gene expression to be a significant prognostic factor for NSCLC patients (hazard ratio, 2.126; $P=0.0467$).

Wnt1 expression in NSCLCs. The intratumoral Wnt1 expression showed a cytoplasmic staining pattern (Fig. 2D and F). Among 122 NSCLCs, 65 (53.3%) were Wnt1-high tumors (Table I). Regarding tumor histology, 34 of the 68 adenocarcinomas (50.0%) were Wnt1-high tumors. Thirty of the 52 squamous cell carcinomas (57.7%) were Wnt1-high tumors. There was no difference in the Wnt1 expression according to tumor histology. In addition, no difference was found in the Wnt1 expression according to tumor status, nodal status or tumor differentiation.

Wnt5a expression in NSCLCs. The intratumoral Wnt5a expression also appeared in a cytoplasmic staining pattern (Fig. 2H). Among 122 NSCLCs, 67 tumors (54.9%) were Wnt5a-high tumors (Table I). Regarding tumor histology, 29 of the 68 adenocarcinomas (42.6%) were Wnt5a-high tumors. Thirty-eight of the 52 squamous cell carcinomas (73.1%) were Wnt5a-high tumors. The percentage of Wnt5a-high tumors was significantly higher in squamous cell carcinomas than in adenocarcinomas ($P=0.0015$). Furthermore, the intratumoral Wnt5a expression was significantly associated with tumor differentiation ($P=0.0039$). However, there was no difference in the Wnt5a expression according to tumor status or nodal status.

Survivin gene expression and survivin protein expression in relation to Wnt1 and Wnt5a status in NSCLCs. Regarding the intratumoral Wnt1 expression, the percentage of Wnt1-positive tumor cells significantly correlated with the standardized survivin gene expression ratio ($r=0.346$, $P<0.0001$). The standardized survivin gene expression ratio was significantly higher in Wnt1-high tumors than in Wnt1-low tumors (1.31±0.63 vs. 0.96±0.63, $P=0.0030$; Fig. 4A). Furthermore,
the HSCORE of nuclear staining of pan-survivin was significantly higher in Wnt1-high tumors than in Wnt1-low tumors (29.4±22.9 vs. 18.1±17.0, P=0.0483; Fig. 4B). The HSCORE of cytoplasmic staining of pan-survivin was also significantly higher in Wnt1-high tumors than in Wnt1-low tumors (100.4±67.8 vs. 55.8±46.4, P<0.0001; Fig. 4C).

In contrast, concerning the intratumoral Wnt5a expression, no correlation was observed between the percentage of Wnt5a-positive tumor cells and the standardized survivin gene expression ratio (r=0.146, P=0.1081). No difference was observed in the standardized survivin gene expression ratio between Wnt5a-high tumors and Wnt5a-low tumors (1.22±0.74 vs. 1.04±0.51, P=0.1300, Fig. 4D). In addition, no difference was observed in the HSCORE of nuclear staining of pan-survivin between Wnt5a-high tumors and Wnt5a-low tumors (26.6±22.8 vs. 21.1±20.0, P=0.3412, Fig. 4E). There was no difference in the HSCORE of cytoplasmic staining of pan-survivin between Wnt5a-high tumors and Wnt5a-low tumors (77.6±65.9 vs. 82.0±59.2, P=0.7056, Fig. 4F).

The clinical significance of the Wnt1 expression in NSCLCs. Regarding tumor apoptosis, the percentage of Wnt1-positive
tumor cells significantly correlated with the apoptotic index ($r=0.380$, $P<0.0001$). The apoptotic index was significantly lower in Wnt1-high tumors than in Wnt1-low tumors (18.2±8.2 vs. 21.6±8.3, $P=0.0254$; Fig. 5A).

Concerning tumor proliferation, the percentage of Wnt1-positive tumor cells significantly correlated with the Ki-67 proliferation index ($r=0.339$, $P<0.0001$). The Ki-67 proliferation index was significantly higher in Wnt1-high tumors than in Wnt1-low tumors (55.2±27.2 vs. 38.8±30.5, $P=0.0021$; Fig. 5B).

Regarding survival, the 5-year survival rate was 55.8% in patients with Wnt1-high tumors, and 79.7% in patients with Wnt1-low tumors. The overall survival was significantly shorter in patients with Wnt1-high tumors than in patients with Wnt1-low tumors ($P=0.0308$; Fig. 5C). A univariate analysis using the Cox regression model revealed the Wnt1 status to be a significant prognostic factor for NSCLC patients (hazard ratio, 2.217; $P=0.0355$).

**Discussion**

Survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family (3). Survivin has a single Baculovirus IAP repeat domain, a motif that inhibits the caspase family, mediators of apoptosis. Experimental studies have demonstrated that the antisense RNA for survivin can induce apoptosis in human tumor cells (26). Furthermore, the nuclear localization of survivin affects cell mitosis through chromosome condensation and segregation (27,28). Therefore, survivin expression is predicted to promote tumorigenesis by regulating not only apoptosis but also the mitosis of tumor cells (2).

Previous clinical studies have revealed that most human malignancies have a ubiquitous expression of survivin (4-8) while survivin expression is undetectable in most terminally differentiated normal tissues (9). Furthermore, its expression is highly detectable not only in early-stage NSCLCs but also in high-grade atypical adenomatous hyperplasia of the lung (29,30). Therefore, survivin expression is associated with the initiation of NSCLCs.

On the other hand, the Wnt genes encode secreted proteins with multi-directional biological functions via autocrine or paracrine routes (12). They are involved in the regulation of various normal and pathological processes, including tumorigenesis (12,13). The Wnt genes have been classified into functional groups with separate downstream signaling pathways, such as the canonical pathway and several non-canonical pathways (14). Among them, the activation of the canonical Wnt pathway results in ß-catenin accumulation in the cytoplasm (15). The cytoplasmic ß-catenin translocates into the nucleus and activates the transcription of various tumor-associated Wnt-target genes with TCF/LEF1 motifs, including c-Myc (16), Cyclin D1 (17) and VEGF (18). Wnt1 is one of the novel members stimulating the canonical Wnt pathway (21). A recent clinical study in NSCLCs revealed that Wnt1 overexpression is associated with the expression of c-Myc, Cyclin D1 and VEGF, resulting in the acceleration of tumor proliferation and tumor angiogenesis to produce more aggressive tumors (24). Therefore, the Wnt1 overexpression promotes tumor progression in NSCLCs.

Recently, survivin is also reported to be a target of the canonical Wnt pathway (19,20). The clarification of mechanisms of survivin gene regulation could lead to the development of a new treatment for NSCLC patients. The present study therefore investigated the survivin expression in relation to the intratumoral expression of Wnt1 and Wnt5a in NSCLCs.

Consequently, the present study demonstrated the intratumoral Wnt1 expression to correlate with the survivin gene expression in NSCLCs. Furthermore, the intratumoral Wnt1 expression was also associated with the expression of pan-survivin protein, both in the nucleus and in the cytoplasm. In contrast, the intratumoral expression of Wnt5a, a novel member of non-canonical pathways (22), was not associated with...
the survivin gene expression in NSCLCs. Furthermore, the intratumoral Wnt5a expression was associated neither with the expression of nuclear pan-survivin protein nor with the expression of cytoplasmic pan-survivin protein.

Regarding the tumor biology, the present study also demonstrated the survivin gene expression to correlate with the inhibition of tumor apoptosis, the acceleration of tumor proliferation, and a poor prognosis in NSCLC patients, as reported previously (4-8). As a result, the intratumoral Wnt1 expression was associated with the inhibition of tumor apoptosis, the acceleration of tumor proliferation, and a poor prognosis in NSCLC patients. Therefore, the present study revealed survivin to act as a tumor-associated target of the canonical Wnt pathway in NSCLCs. In total, intratumoral Wnt1 overexpression could produce more aggressive NSCLCs by induction of various tumor-associated targets including survivin.

The results of the present study suggest that Wnt1 could be a candidate for molecular-target therapy for NSCLC. New strategies of Wnt1-inhibiting treatment, using a small molecule or the RNA inhibition, may be effective for patients with Wnt1-overexpressing NSCLCs by inhibiting various tumor-associated Wnt targets, including survivin. These Wnt1-inhibiting therapies could have bystander effects on tumor tissues because the canonical Wnt1 pathway affects the biological functions via autocrine or paracrine routes (12).

Further studies should be conducted to develop new treatment modalities for Wnt1-overexpressing tumors.

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References