Abstract. Background/Aim: The expression of Wingless/int-1 (Wnt)-induced secreted protein-1 (WISP-1/CCN4), a member of the Cyr61-CTGF-Nov (CCN) family, has been examined in several types of cancer. However, the correlation between the WISP-1 expression and the clinical features of esophageal squamous cell carcinoma (ESCC) remain to be elucidated. This study aimed to clarify the expression of WISP-1 protein in patients with ESCC and also to examine the function of WISP-1 in esophageal cancer cells in vitro. Patients and Methods: One-hundred and ninety patients with thoracic esophageal carcinoma underwent transthoracic subtotal esophagectomy between 2005 and 2009. All patients that had received previous therapy were excluded and 105 out of the 190 ESCC samples were analyzed immunohistochemically using WISP-1 antibody. The expression of WISP-1 mRNA in esophageal cancer cell lines was analyzed by RT-PCR. Growth assay and invasion assays were performed using WISP-1 transfected cells. Results: The immunohistochemical analysis showed that WISP-1-positive cases were closely associated with tumor size, tumor type, lymph node metastasis and poor prognosis. There were significantly more WISP-1-positive infiltrative type tumors than expanding type tumors. In the esophageal cancer cell lines examined, only TE8 expressed WISP-1 mRNA. The growth of WISP-1- transfected cells was significantly increased in comparison to the control cells, but no differences in the invasion activity were observed between the transfected cells and control cells. Conclusion: The expression of WISP-1 may play an important role in the progression of ESCC. WISP-1 could therefore be a clinical marker for a poor prognosis in patients with ESCC and may also be a therapeutic target to control the progression of ESCC.

Esophageal cancer is the eighth most frequent cancer, and it is the sixth most common cause of cancer death worldwide (1, 2). Despite numerous advances in the diagnosis and treatment of cancer, esophageal cancer is often found as a locally advanced disease and it tends to metastasize to other organs, thus resulting in a poor prognosis. Advanced or metastatic cases often show resistance to systemic therapy including chemotherapy and radiotherapy and are often difficult to cure. Therefore, a new treatment strategy is necessary, based on a better understanding of the molecular events associated with the progression of esophageal cancer.

Wingless/int-1 (Wnt) proteins are extracellular signaling ligands that play a key role in cell growth, motility and differentiation. Wnt signaling is also closely associated with human degenerative diseases and cancer (3). In particular, the Wnt/β-catenin signaling pathway is deeply connected with the pathogenesis of many human carcinomas (4, 5). Several reports have investigated the role of Wnt in esophageal cancer and head and neck squamous cell carcinoma (HNSCC) (6-13). Wnt-1, one of the Wnt ligands, has been shown to function in transcription and cell growth in esophageal squamous cell carcinoma (ESCC) and HNSCC (9, 12, 13).

Wnt-induced secreted protein-1 (WISP-1) is a member of the Cyr61-CTGF-Nov (CCN) family. Six molecules have been identified in this family, including cysteine-rich 61 (Cyr61/CCN1), connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (Nov/CCN3), WISP-1 (CCN4), WISP-2 (CCN5) and WISP-3 (CCN6) (14, 15). These molecules are cysteine-rich extracellular matrix-associated proteins that can act as either matrix components that regulate
adhesion and migration or as growth factors that modulate cell proliferation and differentiation (16). Furthermore, this family of proteins regulates angiogenesis and tumorigenesis. WISP-1 was first identified as a downstream gene of Wnt-1 signaling in colon cancer (17). Previous reports have shown a relationship between WISP-1 protein and myocytes (19), fibroblasts (18, 20, 21), osteoblasts (16, 22), and certain tumor cells (17, 20, 23-28). WISP-1 and its splicing variant WISP-1v have been studied in colon cancer (17, 23, 24), breast cancer (25, 26), scirrhous gastric carcinoma (20), invasive cholangiocarcinoma (27), and lung cancer (28). Mizushima et al. reported that Wnt-1 induces β-catenin/T-cell factor dependent transcription in ESCC (9).

Deng et al. reported that CTGF was overexpressed in ESCC and promoted tumorigenicity through β-catenin/T-cell factor/lymphoid enhancer factor (Lef) signaling (29). No studies have so far investigated WISP-1 or the downstream genes of Wnt-1/β-catenin in ESCC.

Therefore, this study investigated the relationship between WISP-1 protein expression immunohistochemistry and the clinicopathological features of patients with ESCC to determine the potential role of WISP-1 in the development and progression of ESCC. In addition, the expression of WISP-1 mRNA was investigated in esophageal cancer cell lines by RT-PCR and the effect of WISP-1 on cell growth and invasion was evaluated using WISP-1 transfected esophageal cancer cells.

Patients and Methods

Tissue samples and cell lines. One-hundred and ninety patients with thoracic esophageal carcinoma underwent transthoracic subtotal esophagectomy at Kumamoto University Hospital, between 2005 and 2009. Any patients who had undergone previous therapy were excluded and 105 resected specimens of esophageal cancer from the thoracic esophageal carcinoma underwent transthoracic subtotal esophagectomy at Kumamoto University Hospital, between 2005 and 2009. Any patients who had undergone previous therapy were excluded and 105 resected specimens of esophageal cancer from the patients who did not receive any radiotherapy or chemotherapy prior to surgical resection of the esophagus were included in this study. Diagnosis and routine histopathological examinations of hematoxylin and eosin-stained sections were performed at the Department of Surgical Pathology, Kumamoto University Hospital. Alcohol-fixed, paraffin-embedded samples were cut into 4 µm sections for immunohistochemical staining. All of the samples were histopathologically diagnosed as ESCC, and the following information was recorded for each patient: age, gender, clinicopathological staging (TNM system) according to the Unio Internationalis Contra Cancrum (UICC), depth of invasion, tumor size, tumor type, lymphatic invasion, venous invasion and lymph node status. The patients were routinely followed-up clinically after surgery for a median period of 36 months.

The human esophageal cancer cell lines TE1, TE4, TE6, TE8, TE9, TE10, TE11, TE14 and TE15 were obtained from Tohoku University, Sendai, Japan. These cell lines were cultured at 37°C in 5% carbon dioxide, in RPMI medium with 10% fetal bovine serum.

Immunohistochemical staining. Paraffin-embedded tissue sections were stained with rabbit polyclonal antibody to human WISP-1 (Santa Cruz, CA, USA) at 1:50 dilution. Normal rabbit immunoglobulin G was used as a negative control. Immunostaining was performed using an EnVision® System kit (Dako, Copenhagen, Denmark). Sections were deparaffinized in xylene and rehydrated through alcohol. Endogenous peroxidase was quenched with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min, followed by antigen retrieval with 0.01 mol/l citrate buffer (pH 6.0) for 15 min in a microwave oven. Sections were blocked with 3% bovine serum albumin (BSA) in PBS for 40 min, followed by incubation overnight at 4°C with primary antibody and EnVision® horseradish peroxidase for 60 min. The slides were washed in PBS three times for 5 min after each incubation. The color reaction was performed in 3’-diaminobenzidine (DAB) for 5 min, then the slides were counterstained with hematoxylin for 30 s. The slides were dehydrated in alcohol prior to mounting. Immunoreactivity was graded as: 0, negative; 1+, weak homogenous cytoplasmic staining; 2+, strong staining in <30% of tumor cells; 3+, strong staining in >30% of tumor cells. 0 and 1+ were defined as WISP-1-negative; 2+ and 3+ as WISP-1-positive. All of the slides were independently examined by two experienced investigators.

RT-PCR analysis. Total RNA extraction was performed using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using SuperScript III (Invitrogen), according to the manufacturer’s instructions. The initial denaturing was performed at 94°C for 3 min, followed by 27 cycles of amplification (denaturing at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s) and terminal extension at 72°C for 3 min. The primers used for RT-PCR amplification were: human WISP-1; 5’-GAT GCG TCA GCA GCT TG-3’ (forward) and 5’-TAT GGA TGT CCA CAT CGC ATG-3’ (reverse); human GAPDH; 5’- TGA CCA CAG TCC ATG CCA TC- 3’(forward) and 5’-CCA CCC TGT TGCG AGC C-3’ (reverse).

Construction of the WISP-1 expression vector and transfection. The PCR amplified WISP-1 was cloned into a pcDNA3.1/V5-His TOPO vector (Invitrogen). The primer sets for full-length WISP-1 amplification were: human WISP-1; 5’-GAG GTG GTC GCA TCC TCT G-3’ (forward) and 5’-GGT AAC TAA GGC TCA TTG TTG-3’ (reverse). The ligated vector was transformed using a One Shot chemical competent cell kit (Invitrogen). Plasmid DNA was extracted using a QIA filter Plasmid Maxi Kit (QIAGEN, Tokyo, Japan), and sequenced using an ABI 310 auto-sequencer (Applied Biosystems). The expression vectors were transfected using the standard protocol with Lipofectamine 2000 reagent (Invitrogen).
The transfected cells were transferred into 25 cm² flasks after 24 h and used for further analysis. The WISP-1-transfected cell line was designated TE6+WISP-1 and the LacZ (control)-transfected cell line was designated TE6+LacZ.

Western-blot analysis. Proteins were extracted using RIPA buffer (0.5% sodium deoxycholate, 1M Tris, 5M NaCl, 1% NP-40, 0.1% sodium dodecyl sulphate) containing protease inhibitor and phosphatase inhibitor. Each lane of an 11% SDS-PAGE gel was loaded with 20 μg of protein. The proteins were separated by electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in PBS/Tween 20 (0.1%) at room temperature for 1 h and then incubated with anti-rabbit human WISP-1 (SC-25441; Santa Cruz, CA, USA) for 1 h at room temperature. The membrane was rinsed twice for 10 min with PBS/Tween 20 and then incubated with anti-rabbit IgG (Santa Cruz) for 1 h at room temperature. The membrane was rinsed twice for 10 min with PBS/Tween 20 and for 10 min with PBS, incubated with ECL-Plus (GE healthcare) and then exposed to X-ray film and developed.

Growth assay. TE6+LacZ and TE6+WISP-1 cells (2×10³) were plated in a 96-well plate and cultured for 72 h. Cell numbers were measured using a Cell Counting Kit-8 assay kit (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol.

Invasion assay. A Matrigel Invasion Chamber (Becton Dickinson Labware, Bedford, MA, USA) was used to examine the invasion rate. The TE6+LacZ and TE6+WISP-1 cell suspensions were diluted to 1×10⁵ cells/well in a 24-well Matrigel Invasion Chamber using chemoattractant (growth medium supplemented with 10% fetal bovine serum) in the well. The invasive cells were counted 22 h after seeding the cells.

Statistical analysis. The data were analyzed using the Mann–Whitney U-test or Chi-square test. Overall survival (OS) was calculated from the date of esophagectomy to the date of death. Univariate survival analyses were performed using the Kaplan–Meier method and log-rank tests. The Cox proportional hazards regression model was used for the multivariate analysis. The level of significance was defined as being a $p$-value of 0.05 or less. All the statistical analyses were performed using the STATVIEW5.0 software package (SAS Institute Inc., Cary, NC, USA).

Results

Expression of WISP-1 and clinicopathological features in ESCC. The patients’ characteristics are shown in Table I. All the tumors were histopathologically diagnosed as squamous cell carcinoma. Representative immunohistochemical staining for esophageal cancer is shown in Figure 1. The
The cytoplasm of the esophageal cancer cells was positively stained and the stromal components were weakly stained. There was no detectable immunoreactivity on the normal rabbit immunoglobulin G control slide. Fifty-nine out of the 105 patients (56%) with esophageal cancer were WISP-1-positive. The relationship between WISP-1 protein expression and the clinicopathological findings is shown in Table II. WISP-1 protein expression significantly correlated with the depth of invasion, tumor size, tumor type and lymph node metastasis.

Overall survival curves based on WISP-1 protein expression are shown in Figure 2. The WISP-1-positive patients had significantly poorer survival in comparison to the WISP-1-negative patients (p<0.01). The multivariate analysis revealed that lymph node metastasis and WISP-1 expression were independent prognostic factors (Table III).

Expression of WISP-1 in esophageal cancer cell lines. The expression of WISP-1 mRNA examined by RT-PCR in the nine cell lines is shown in Figure 3A. Only one cell line (TE8) showed a clear expression of WISP-1 mRNA. In addition, a transcript smaller than WISP-1 was observed in TE8. Sequence analysis revealed that this transcript corresponded to WISP-1v (data not shown), a splicing variant lacking exon 3 that was previously reported in invasive cholangiocarcinoma and scirrhus gastric carcinoma (20, 27). TE8 is a moderately differentiated squamous cell carcinoma cell line established from a cancer of the esophagus and its morphology is epithelial-like. TE6 had no expression of WISP-1 or WISP-1v mRNA, so this cell line was used in the subsequent experiments.

Effect of WISP-1 on esophageal cancer cell. TE6 cells were transfected with full length WISP-1 (TE6+WISP-1) or LacZ (TE6+LacZ) as a control. TE6+WISP-1 expressed WISP-1 mRNA and protein and TE6+LacZ did not (Figure 3B and 3C). Using the CCK-8 growth assay the rate of growth of TE6+WISP-1 was 3.6-fold greater than TE6+LacZ control cells (Figure 3D). The effect of WISP-1 on cell invasion, evaluated by invasion assay, showed no difference in the invasive cell rate between the TE6+WISP-1 and TE6+LacZ cells (Figure 3E).

Discussion

The WISP-1 protein showed diffuse cytoplasmic staining in the esophageal cancer cells, while WISP-1 immunoreactivity was rarely detected in the adjacent normal squamous epithelium. Fifty-nine out of the 105 esophageal cancer specimens (56%) showed moderate to strong WISP-1 protein expression. WISP-1-positive esophageal cancer was significantly associated with the depth of wall invasion, tumor size, tumor type and lymph node metastasis. These factors resulted in poor prognosis in these cases. It is interesting that the macroscopic tumor type was correlated with WISP-1 protein expression. Most of the advanced tumors that were ulcerative and infiltrative were WISP-1 positive and most of the expanding tumors were WISP-1-negative. Baba et al. have demonstrated that patients with the infiltrative type gastric cancer have lower survival rates than
those with the expanding type (30). Nomiya et al. reported that the infiltrative type esophageal cancer is more radioresistant than the localized type (31). These results suggest that the infiltrative type tumor is more aggressive than an expanding tumor and it seems that WISP-1 may play an important role in such behavior in esophageal cancer. The infiltrative type of esophageal cancer shows a high level of vascular endothelial growth factor (VEGF) expression (31),
and recent studies have reported correlation between CCN1/2 and hypoxia-inducible factor (HIF) 1-α/VEGF (32, 33). It is therefore important to investigate WISP-1-mediated signals in the infiltrative type of esophageal cancer.

WISP-1 protein expression was observed in more than half of the tissue samples; however, the expression of WISP-1 mRNA was observed in only one cell line (TE8). There may be situations where cancer cells express WISP-1 in response to factors released from surrounding cells. This may explain the discrepancy in the protein expression in the tissue samples and the lack of mRNA expression in most of the cell lines.

The WISP-1-transfected esophageal cancer cells showed significantly increased growth in comparison to the control cells. Tanaka et al. showed that WISP-1 stably transfected mouse embryonic fibroblast cell line (NH3T3) cells exhibited piling-up growth (20). Overexpression of WISP-1 in normal rat kidney fibroblasts induced transformation, increased cellular saturation and promoted growth (18). Su et al. demonstrated that WISP-1 activates the anti-apoptotic protein kinase B (Akt) signaling pathway, inhibits the mitochondrial release of cytochrome c, up-regulates antiapoptotic protein Bcl-XL and therefore prevents cells from undergoing p53-mediated apoptosis in response to DNA damage (34). These reports are consistent with the current observation that WISP-1 is related to cancer growth.

On the other hand, the invasion assay showed no significant difference between the WISP-1 stably transfected esophageal cancer cells and control cells. Tanaka et al. revealed that WISP-1v stably transfected NH3T3 cells enhanced the invasive phenotype of co-cultured gastric carcinoma cells, but WISP-1 transfected cells did not (20). They also reported that WISP-1v stimulated the invasive phenotype of cholangiocarcinoma cells by activation of mitogen-activated protein kinase signaling (27). These reports and the present results, suggest that WISP-1 is predominantly related to cancer growth and WISP-1v is related to invasion.

In conclusion, the expression of WISP-1 may play an important role in the progression of esophageal squamous cell carcinoma. Although further study is required, WISP-1 expression could help predict poor prognosis patients and also be a potential candidate molecule for the development of new therapeutic methods for the treatment of esophageal cancer.

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**References**