Abstract. Claudins (CLDNs) constitute the major transmembrane proteins of tight junctions. It may be hypothesized that changes in or loss of expression of tight junctional proteins such as CLDNs can lead to cellular disorientation and detachment, which is commonly seen in neoplasia. Recent studies have suggested that claudin-1 (CLDN1) plays an important role in invasion and metastasis and claudin-4 (CLDN4) has a particular role in mammary glandular cell differentiation and carcinogenesis. In this study, we examined 83 breast cancer cases and demonstrated immunohistochemical expression patterns of CLDN1/CLDN4 in recurrent and non-recurrent groups. We found significant results between the recurrent and non-recurrent group for expression of CLDN1/CLDN4. The recurrent group (26 cases) showed decreased expression patterns of CLDN1 (p<0.001), compared to the non-recurrent group (57 cases). Decreased expression of CLDN1 (p<0.0001) correlated with short disease-free interval. The lymph node metastasis-positive group showed decreased expression patterns of CLDN1 (p=0.001). However, there was no significance between the recurrent group and non-recurrent group in CLDN4 expression. There was no significance between histological factors and CLDN4 expression. The results indicated that CLDN1 expression correlated with the recurrence status and malignant potential of breast cancer.

Introduction

Tight junctions of cells serve as a barrier that prevents solutes and water from passing through the paracellular pathway, and as a fence between the apical and basolateral plasma membranes in epithelial cells. Tight junctions consist of transmembrane proteins, such as claudins (CLDNs) and occludin, and many peripheral membrane proteins. As a major transmembrane protein, CLDNs play crucial roles in the formation and maintenance of the tight junctions (1,2). The CLDNs were first discovered in 1998, and the CLDN family consists of at least 24 newly discovered members, the expression of which depends on cell type and tissue (2,3). They are connected with the actin cytoskeleton and participate in intracellular signaling (4,5). In this context, downregulation or upregulation of CLDNs might have a role in cancer development. Alterations of CLDNs have been noted in several tumors, colorectal, ovarian and breast cancer, suggesting their involvement in carcinogenesis (6-11).

Breast cancer is the second leading cause of cancer death among woman in the industrialized world (12). It has been shown that 30% of patients with an initial diagnosis and treatment of breast cancer may have distant metastasis within 10 years. Several reports suggested that claudin-1 (CLDN1) and claudin-4 (CLDN4) correlate with breast cancer carcinogenesis. Downregulation of CLDN1 has been demonstrated in breast cancers compared with normal breast epithelia (6,7), and CLDN1 mRNA expression has been found to be lost or downregulated in most breast cancer cell lines (13). A previous study reported that significant loss of CLDN1 protein in breast cancer cells suggests that CLDN1 may play a role in invasion and metastasis (14). The loss of CLDN4 expression in areas of apocrine metaplasia and in the majority of grade 1 invasive carcinoma also suggests a particular role for this protein in mammary glandular cell differentiation and carcinogenesis (14). However, the relationship between CLDNs and recurrence status of breast cancer has not yet been analyzed.

In this study, we examined the relationship between immunohistochemical expression patterns of CLDN1/CLDN4 and recurrence status in breast cancer.

Materials and methods

Patients' specimens. We analyzed surgically resected breast cancer tissues of 83 patients, after obtaining each patient’s informed consent to examine their clinical records and pathology specimens filed in Hirosaki University Hospital. All patients were female and their mean age was 53.2 years (range, 31-80 years). They underwent simple mastectomy.
and axillary lymph node dissection. They were divided into two groups: non-recurrent group, 57 patients; and recurrent group, 26 patients. The study in this paper was retrospective and followed the principles of the World Medical Association Declaration of Helsinki 1964.

**Histopathological and immunohistochemical examinations.**

For routine histological examination, breast cancer specimens were routinely fixed with formalin, embedded in paraffin, thin-sectioned, and stained with hematoxylin and eosin (H&E). Nuclear grade (nuclear pleomorphism) of the cancer cells was classified into three groups: grade 1 (low grade), grade 2 (moderate grade) and grade 3 (high grade) according to the WHO classification (15).

In each patient, one histological specimen of the main breast cancer lesion was selected for immunohistochemistry (16-18). Sections (4-μm-thick) were mounted on silane-coated glass slides. Primary antibodies of immunohistochemistry were: anti-claudin 1 antibody (rabbit polyclonal, 1:50 dilution; Zymed Laboratories Inc., South San Francisco, CA) and anti-claudin 4 antibody (mouse monoclonal 3E2C1, 1:100, Zymed). All sections were pretreated with autoclave heating (121°C, 10 min) for antigen retrieval. The staining was performed using the streptavidin biotin-peroxidase complex technique with a Histofine kit (Nichirei Co., Tokyo, Japan), according to the manufacturer’s instructions. The sections were reacted with a chromogen, 3, 3'-diaminobenzidine-tetrahydrochloride (Merck KGaA, Darmstadt, Germany), and counter-stained with hematoxylin.

**Evaluation of immunostaining patterns of CLDN1/CLDN4.** In each immunostained specimen, staining patterns of expression were divided into the following two groups: negative, ≤10% of cytoplasm and/or membrane staining in tumor cells; positive, >10% of cytoplasm and/or membrane staining in tumor cells (Fig. 1). Positive staining of cytoplasm and membrane was apparently intense, compared with the surrounding stroma.

**Statistical analysis.** Statistical analyses of immunostainings were performed using a χ² test with SPSS software (version 12.0; SPSS Inc., Chicago, IL). The results were deemed statistically significant for p<0.05. Disease-free intervals were calculated by Kaplan-Meier analysis, and statistically analyzed by the log-rank test using StatView (version 5.0, SAS Institute, Cary, NC).

**Results**

Clinicopathological characteristics of recurrent and non-recurrent groups are summarized in Table I. The following factors showed statistical significances between recurrent and non-recurrent groups: lymph node metastasis (p=0.03), lymph
invasion (p=0.01), estrogen receptor (p=0.024), extensive intraductal component (p=0.036), nuclear grade (p=0.041) and stage between stage II and III (p=0.045).

Immunohistochemical expression patterns of CLDN1 were demonstrated in Fig. 1. We examined expression patterns of CLDN1/CLDN4 between the non-recurrent and recurrent group. CLDN1-positive cases were significantly less frequent in the recurrent group, compared to the non-recurrent group (p<0.001); i.e., CLDN1 expression was decreased in the recurrent group (Fig. 2). CLDN1-positive cases showed prolonged disease-free interval (p<0.0001) (Fig. 3). The cases of decreased expression of CLDN1 were predominantly found in the invasive carcinoma components, compared to the intraductal in situ components. There was no significance between non-recurrent and recurrent groups in CLDN4 expression. CLDN4 expression did not affect disease-free interval.

We also analyzed relationships between histopathological factors and immunohistochemical expression patterns of CLDN1/CLDN4. The factors analyzed were histological classification, lymph node metastasis, lymph invasion, venous invasion, extensive intraductal component, estrogen receptor, progesterone receptor, nuclear grade, tumor size, and stage. CLDN1 expression was significantly decreased in the lymph node metastasis-positive group (p=0.001) (Fig. 4). CLDN1 expression mildly decreased in the venous invasion-positive group (p=0.031) (Fig. 5). There were no statistical significances between the other histological factors and immunohistochemical expression of CLDN1/CLDN4.

**Discussion**

As a major transmembrane protein, CLDNs play crucial roles in formation and maintenance of the tight junction. Recent
that CLDNs were the main proteins responsible for tight proteins in cancer progression (26, 27). Previous studies showed been shown to be a useful prognostic indicator for some function as a tumor suppressor in numerous tissues and has of the adherens junction, E-cadherin. E-cadherin is thought toNumerous studies have focused on the transmembrane protein event in the process of metastasis, allowing the liberation of individual cancer cells from the primary tumor. Cell-to-cell adhesion within the epithelial cell layer is maintained mainly by two types of junction: adherens junction and tight junction. Numerous studies have focused on the transmembrane protein of the adherens junction, E-cadherin. E-cadherin is thought to function as a tumor suppressor in numerous tissues and has been shown to be a useful prognostic indicator for some tumors, illustrating the importance of cell-to-cell adhesion proteins in cancer progression (26, 27). Previous studies showed that CLDNs were the main proteins responsible for tight junction (28), the expression of which depends on cell type and tissue (29, 30). Alteration of CLDNs has been noted in several tumors (8, 9), including those of the breast (6, 7). Downregulation or upregulation of CLDNs might have a role in cancer development (10, 11). Re-expression of the tight junction protein CLDN1 induces apoptosis in breast tumor spheres (31). CLDN4 expression decreases the invasiveness and metastatic potential of pancreatic cancer (32). These studies suggest that CLDNs might be a tumor suppressor in several tissues and be useful prognostic indicators.

Theoretically, recurrent cases have much more metastatic potential than non-recurrent cases. In our study, the recurrent group of breast cancer more frequently showed the clinicopathological factors indicating malignant potential, such as lymph node metastasis (p=0.03), lymph invasion (p=0.01), extensive intraductal component (p=0.036), nuclear grade (p=0.041) and stage II/III (p=0.045); i.e., the recurrent group had higher malignant potential than the non-recurrent group. We hypothesized that CLDN1/CLDN4 expression might be low in the high malignant potential of breast cancer. In this study, we demonstrated decreased expression of CLDN1 in the recurrent group, lymph node metastasis-positive group and venous invasion-positive group. However, CLDN4 expression did not correlate with recurrent status and the clinicopathological factors. We speculated that CLDN1 might affect the individual clinicopathological factors listed above, and would have a greater influence on the recurrent group which had accumulated malignant potentials.

In cervical carcinoma, CLDN1 expression was reported to be stronger in premalignant stages, while a significant decrease was found in invasive cancers. It may serve as a good diagnostic marker for the detection of cervical intraepithelial neoplasia (33). In the present study, expression of CLDN1 was more decreased in the invasive carcinoma components, compared to the intraductal in situ components of breast cancer. This may indicate that CLDN1 is a good diagnostic marker for the detection of breast non-invasive carcinoma components.

In this study, we conclude that decreased CLDN1 expression correlates with the recurrence status, and mean histological malignant potential in breast cancer. Therefore, CLDN1 immunohistochemical expression may be a good indicator of recurrence of breast cancer.

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"INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 20: 139-143, 2007"

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