SPANDIDOS PUBLICATIONS

Decreased expression of claudin-1 correlates with recurrence status in breast cancer

SATOKO MOROHASHI[1,2], TOMOMI KUSUMI[1], FUYUKI SATO[1], HIROKI ODAGIRI[2], HIROKI CHIBA[1], SHUICHI YOSHIHARA[2], KENICHI HAKAMADA[2], MUTSUO SASAKI[2] and HIROSHI KIJIMA[1]

Departments of [1]Pathology and [2]Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

Received March 8, 2007; Accepted April 12, 2007

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

Correspondence to: Dr Hiroshi Kijima, Department of Pathology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
E-mail: hkijima@cc.hirosaki-u.ac.jp

Abbreviations: CLDNs, claudins; CLDN1, claudin-1; CLDN4, claudin-4

Key words: breast cancer, recurrence, claudin-1, claudin-4, immunohistochemistry

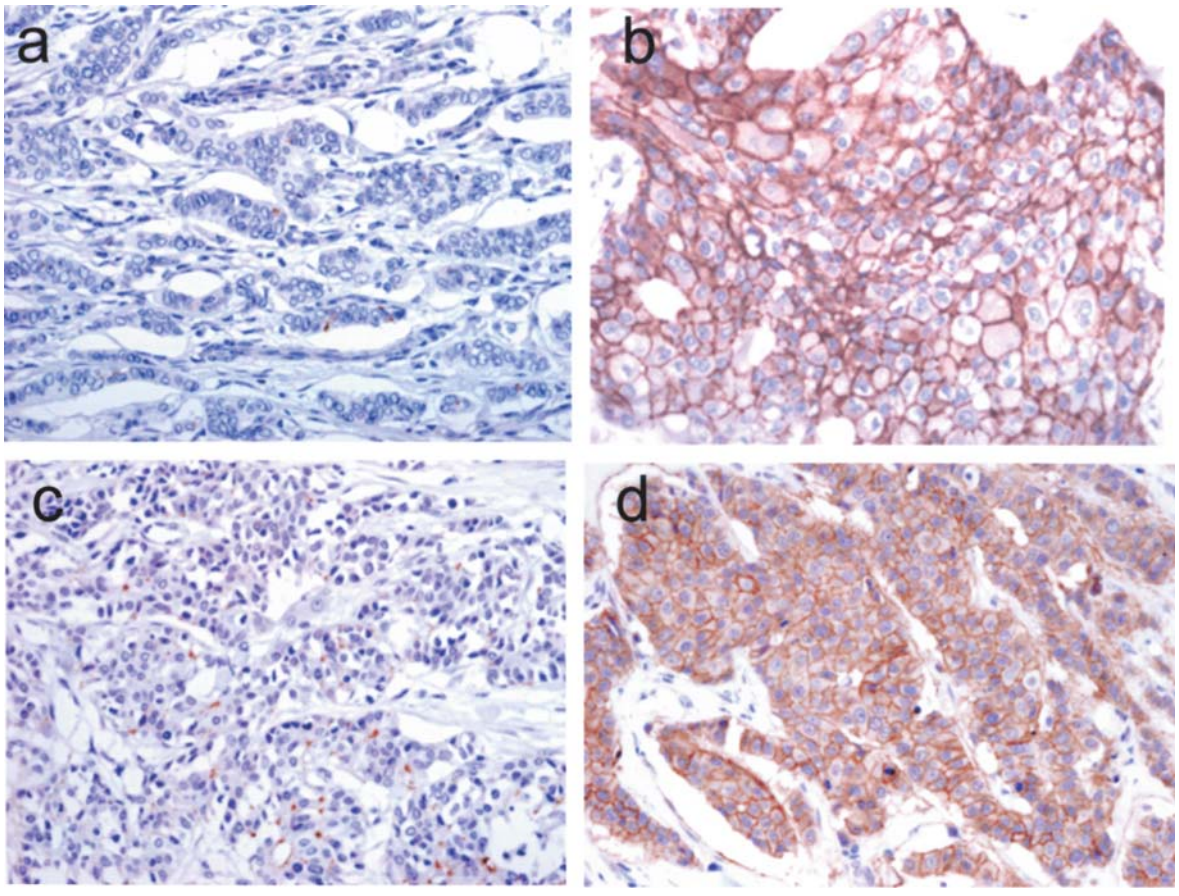

Figure 1. Immunohistochemical expression patterns of claudin-1 (CLDN1) and claudin-4 (CLDN4). (a) CLDN1 negative, ≤10% of cytoplasm and/or membrane staining in tumor cells; (b) CLDN1 positive, >10% of cytoplasm and/or membrane staining in tumor cells; (c) CLDN4 negative, ≤10% of cytoplasm and/or membrane staining in tumor cells; and (d) CLDN4 positive, >10% of cytoplasm and/or membrane staining in tumor cells (streptavidin biotin-peroxidase complex technique, x400).

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 χ^2 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

linicopathological characteristics of breast cancer.

Characteristics	Recurrent group (n=26)	Non-recurrent group (n=57)	p-value
Age, years (mean)	51.3 (37-78)	54.0 (31-80)	
Primary tumor (%)			
T1	2 (8)	12 (21)	
T2	10 (38)	30 (53)	p=0.056
T3	5 (19)	8 (14)	
T4	9 (35)	7 (12)	
Lymph node metastasis-positive	21 (81)	26 (46)	p=0.03[a]
Stage			
I	0 (0)	9 (16)	
II	7 (27)	26 (46)	p=0.045[b]
III	17 (65)	22 (38)	
IV	2 (8)	0 (0)	
Histology			
IDC	23 (88)	53 (92)	
ILC	2 (8)	2 (4)	p=0.285
Mucinous carcinoma	0 (0)	2 (4)	
SCC	1 (4)	0 (0)	
Lymphatic invasion-positive	23 (88)	29 (51)	p=0.01[a]
Venous invasion-positive	10 (38)	7 (12)	p=0.06
Extensive intraductal component-positive	16 (62)	21 (37)	p=0.036[a]
Nuclear grade			
Grade 1	7 (27)	32 (56)	
Grade 2	7 (27)	14 (25)	p=0.041[a]
Grade 3	12 (46)	11 (19)	
Estrogen receptor-positive	11 (42)	39 (68)	p=0.024[a]
Progesterone receptor-positive	12 (46)	34 (60)	p=0.251

[a]Statistical significance. [b]Statistical significance between stage II and III. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; SCC squamous cell carcinoma.

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

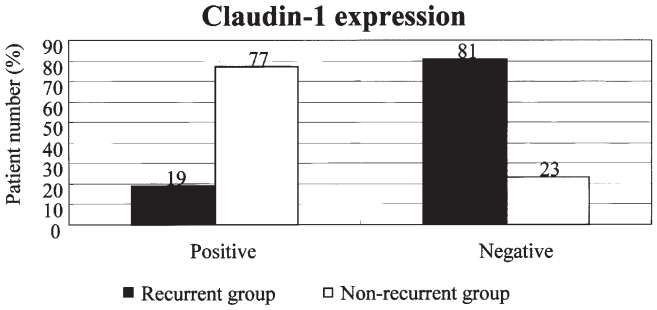

Figure 2. Claudin-1 (CLDN1) expression in recurrent and non-recurrent groups. CLDN1 expression decreased in the recurrent group (p<0.001).

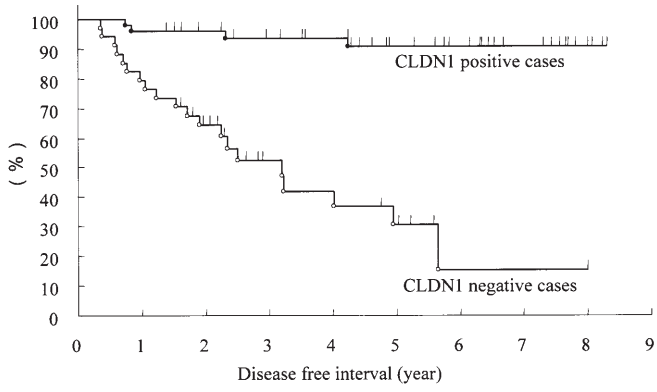

Figure 3. Disease-free intervals in claudin-1 (CLDN1)-positive cases and -negative cases. CLDN1-positive cases showed prolonged disease-free interval (p<0.0001).

(p<0.001); i.e., CLDN1 expression was decreased in the recurrent group (Fig. 2). CLDN1-positive cases had a prolonged disease-free interval (p<0.0001); i.e., decreased expression of CLDN1 correlated with early recurrent status (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

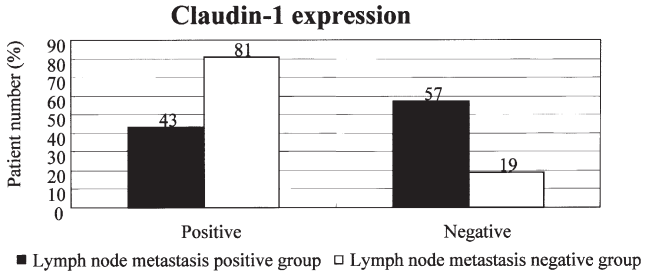

Figure 4. Claudin-1 (CLDN1) expression in lymph node metastasis-positive and -negative groups. CLDN1 expression decreased in the lymph node metastasis-positive group (p=0.001).

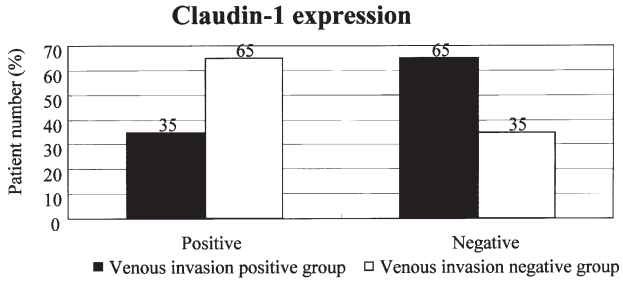

Figure 5. Claudin-1 (CLDN1) expression in venous invasion-positive and -negative groups. CLDN1 expression decreased in the venous invasion-positive group (p=0.031).

studies revealed that CLDNs were important for carcinogenesis and cancer invasion. In this study, we demonstrated that cancer invasion had decreased CLDN1 expression in recurrence status of breast cancer, i.e., decreased expression of CLDN1 correlated with short disease-free intervals. This is the first study to clarify relations between decreased CLDN1 expression and recurrence of breast cancer.

Breast cancer is one of the most common malignancies, and is the second cause of cancer death among women in industrialized countries. The survival time of breast cancer patients with metastasis is approximately 3 years (19). It is well recognized that there is wide variation in the prognosis of patients with primary breast cancer. A number of different prognostic factors for primary breast cancer have been reported to assist in predicting patient outcomes. Among these, axillary nodal status, tumor size, estrogen receptor status, and histological grade are well established and are used routinely in clinical medicine (20-25). However, further studies of prognostic factors are still necessary in breast cancer. Metastasis is the primary cause of fatality in cancer patients. It is widely accepted that the loss of cell-to-cell adhesion is an early 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 (2,29,30). 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 spheroids (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.

References

1. Tsukita S, Furuse M and Itoh M: Structural and signalling molecules come together at tight junctions. Curr Opin Cell Biol 11: 628-633, 1999.
2. Tsukita S, Furuse M and Itoh M: Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2: 285-293, 2001.
3. Furuse M, Fujita K, Hiiragi T, Fujimoto K and Tsukita S: Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 141: 1539-1550, 1998.
4. Tsukita S and Furuse M: Occludin and claudins in tight-junction strands: leading or supporting players? Trends Cell Biol 9: 268-273, 1999.
5. Ivanov AI, Nusrat A and Parkos CA: Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell 15: 176-188, 2004.
6. Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, Rein A, Sauter G, Kallioniemi OP and Sukumar S: Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast. Oncogene 22: 2021-2033, 2003.

r F, White K, Kubbies M, Swisshelm K and Weber BH: ic organization of claudin-1 and its assessment in hereditary and sporadic breast cancer. Hum Genet 107: 249-256, 2000.
8. Miwa N, Furuse M, Tsukita S, Niikawa N, Nakamura Y and Furukawa Y: Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. Oncol Res 12: 469-476, 2000.
9. Hough CD, Sherman-Baust CA, Pizer ES, Montz FJ, Im DD, Rosenshein NB, Cho KR, Riggins GJ and Morin PJ: Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. Cancer Res 60: 6281-6287, 2000.
10. Sawada N, Murata M, Kikuchi K, Osanai M, Tobioka H, Kojima T and Chiba H: Tight junctions and human diseases. Med Electron Microsc 36: 147-156, 2003.
11. Hirohashi S and Kanai Y: Cell adhesion system and human cancer morphogenesis. Cancer Sci 94: 575-581, 2003.
12. Boring CC, Squires TS, Tong T and Montgomery S: Cancer statistics, 1994. CA Cancer J Clin 44: 7-26, 1994.
13. Swisshelm K, Machl A, Planitzer S, Robertson R, Kubbies M and Hosier S: SEMP1, a senescence-associated cDNA isolated from human mammary epithelial cells, is a member of an epithelial membrane protein superfamily. Gene 226: 285-295, 1999.
14. Tokes AM, Kulka J, Paku S, Szik A, Paska C, Novak PK, Szilak L, Kiss A, Bogi K and Schaff Z: Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. Breast Cancer Res 7: 296-305, 2005.
15. Tavassoli FA and Devilee P (eds). Tumours of the Breast and Female Genital Organs. WHO Classification. IARC Press, Lyon, pp18-19, 2003.
16. Yamamoto S, Kijima H, Hara T, Chino O, Shimada H, Tanaka M, Inokuchi S and Makuuchi H: Mucin expression and proliferating cell index of esophageal Barrett's adenocarcinoma. Int J Mol Med 16: 375-380, 2005.
17. Kashiwagi H, Kijima H, Dowaki S, Ohtani Y, Tobita K, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Inokuchi S and Makuuchi H: MUC1 and MUC2 expression in human gallbladder carcinoma: a clinicopathological study and relationship with prognosis. Oncol Rep 8: 485-489, 2001.
18. Kijima H, Kashiwagi H, Dowaki S, Ohtani Y, Tobita K, Matsubayasi H, Ajioka Y, Watanabe H, Tsuchida T, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M and Makuuchi H: Stromal sialyl Le(a) expression is correlated with vascular invasion of human gallbladder adenocarcinoma. Int J Oncol 17: 55-60, 2000.
19. Henderson IC: Chemotherapy for metastatic disease. In: Breast Disease. Harris JR, Hellmann S, Henderson IC and Kinne DW (eds). J.B. Lippincott Company, pp604-665, 1991.
20. Carter CL, Allen C and Henson DE: Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. Cancer 63: 181-187, 1989.
21. Moon TE, Jones SE, Bonadonna G, Valagussa P, Powles T, Buzdar A and Montague E: Development and use of a natural history data base of breast cancer studies. Am J Clin Oncol 10: 396-403, 1987.
22. Lin PP, Allison DC, Wainstock J, Miller KD, Dooley WC, Friedman N and Baker RR: Impact of axillary lymph node dissection on the therapy of breast cancer patients. J Clin Oncol 11: 1536-1544, 1993.
23. McGuire WL, Tandon AK, Allred DC, Chamness GC, Ravdin PM and Clark GM: Treatment decisions in axillary node-negative breast cancer patients. J Natl Cancer Inst Monogr 11: 173-180, 1992.
24. Rosen PP, Groshen S, Kinne DW and Norton L: Factors influencing prognosis in node-negative breast carcinoma: analysis of 767 T1N0M0/T2N0M0 patients with long-term follow-up. J Clin Oncol 11: 2090-2100, 1993.
25. Yamamoto N, Watanabe T, Katsumata N, Omuro Y, Ando M, Fukuda H, Takue Y, Narabayashi M, Adachi I and Takashima S: Construction and validation of a practical prognostic index for patients with metastatic breast cancer. J Clin Oncol 16: 2401-2408, 1998.
26. Peralta Soler A, Knudsen KA, Jaurand MC, Johnson KR, Wheelock MJ, Klein-Szanto AJ and Salazar H: The differential expression of N-cadherin and E-cadherin distinguishes pleural mesotheliomas from lung adenocarcinomas. Hum Pathol 26: 1363-1369, 1995.
27. Wheelock MJ, Soler AP and Knudsen KA: Cadherin junctions in mammary tumors. J Mammary Gland Biol Neoplasia 6: 275-285, 2001.
28. Furuse M, Sasaki H, Fujimoto K and Tsukita S: A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 143: 391-401, 1998.
29. Chiba H, Gotoh T, Kojima T, Satohisa S, Kikuchi K, Osanai M and Sawada N: Hepatocyte nuclear factor (HNF)-4alpha triggers formation of functional tight junctions and establishment of polarized epithelial morphology in F9 embryonal carcinoma cells. Exp Cell Res 286: 288-297, 2003.
30. Katoh M and Katoh M: CLDN23 gene, frequently down-regulated in intestinal-type gastric cancer, is a novel member of CLAUDIN gene family. Int J Mol Med 11: 683-689, 2003.
31. Hoevel T, Macek R, Swisshelm K and Kubbies M: Reexpression of the TJ protein CLDN1 induces apoptosis in breast tumor spheroids. Int J Cancer 108: 374-383, 2004.
32. Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Lohr M, Leder G, Iwamura T, Adler G and Gress TM: Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res 63: 6265-6271, 2003.
33. Sobel G, Paska C, Szabo I, Kiss A, Kadar A and Schaff Z: Increased expression of claudins in cervical squamous intraepithelial neoplasia and invasive carcinoma. Hum Pathol 36: 162-169, 2005.