Jpn. J. Cancer Res. 86, 1054–1059, November 1995

Epithelial-cadherin Gene Is Not Mutated in Ductal Carcinomas of the Breast

Masahiro Kashiwaba,[1,2] Gen Tamura,[1] Yasushi Suzuki,[1] Chihaya Maesawa,[1] Satoshi Ogasawara,[1,2] Ken Sakata[3] and Ryoichi Satodate[1]

Departments of [1]Pathology and [2]Surgery, School of Medicine and [3]Department of Oral and Maxillofacial Surgery, School of Dentistry, Iwate Medical University, 19-1 Uchimaru, Morioka 020

We investigated mutations of the epithelial (E)-cadherin gene and loss of heterozygosity (LOH) at flanking loci using three microsatellite markers on the long arm of chromosome 16 in 25 ductal carcinomas of the breast. Expression of E-cadherin was also investigated immunohistochemically. No mutations were detected in exons 6 through 9 of the E-cadherin gene. LOH was observed more frequently (42%) at D16S402 (16q-ter) than at D16S421 (16q22.3-q23.1) (17%), which is located near the E-cadherin gene. Expression of E-cadherin was observed at the cell borders in 92% (11/12) of the tumors examined. The absence of mutations in the E-cadherin gene and its conserved expression suggest that inactivation of E-cadherin does not contribute significantly to the invasion or metastatic potential of ductal carcinomas of the breast. Furthermore, the high frequency of LOH at 16q-ter suggests the existence of another tumor suppressor gene which may play a crucial role in the genesis of ductal carcinomas of the breast.

Key words: Breast carcinoma — E-cadherin — LOH

Molecular analysis has revealed that the genesis and progression of carcinoma is a multi-step process in which several genetic alterations accumulate.[1] Cell adhesion molecules play an important role in the maintenance of cell-to-cell junctions, and thus prevent the detachment of carcinoma cells from the primary site.[2] Epithelial (E)-cadherin, which is distributed predominantly in epithelial tissues, is a member of a family of transmembrane glycoproteins responsible for the Ca^{2+}-dependent cell-to-cell adhesion mechanism, and has been demonstrated to be involved in organogenesis and morphogenesis.[3] The invasive potential of transformed Madin-Darby canine kidney cells has been reported to be promoted by inactivation of E-cadherin.[4] Frequent loss or reduction of E-cadherin expression has been found immunohistochemically in scattered and diffuse-type gastric carcinomas.[5,6] In breast carcinoma, reduction of E-cadherin expression has also been noted in poorly differentiated tumors.[7] In addition, mutations of the E-cadherin gene have been reported in undifferentiated carcinomas of the stomach[8,9] and in lobular carcinomas of the breast.[10] Furthermore, deletion on 16q, where the E-cadherin gene has been mapped, occurs frequently and has been associated with distant metastasis in breast carcinoma.[11] These findings support the proposal that inactivation of E-cadherin is involved in invasion and metastasis of several types of human carcinomas.

To determine whether E-cadherin is inactivated in ductal carcinomas of the breast, the presence of mutations in exons 6 through 9 of the E-cadherin gene was investigated. The examination included the Ca^{2+}-dependent E-cadherin-specific homophilic binding regions,[12] where mutations have been reported.[7,8] Loss of heterozygosity (LOH) flanking the E-cadherin gene loci on 16q was studied. The expression of E-cadherin was also investigated immunohistochemically.

MATERIALS AND METHODS

Tissue samples and DNA extraction Tumors and corresponding normal tissues were obtained from 25 patients with primary breast carcinoma who were treated at the Department of Surgery, Iwate Medical University, between July, 1991 and October, 1993. The clinicopathological findings of these patients are summarized in Table I. The tumors were histologically classified according to the criteria proposed in the General Rules for Clinical and Pathological Recording of Breast Cancer (1992).[13] The specimens were frozen immediately following surgical resection and stored at −80°C until analysis. DNA was extracted by proteinase-K digestion and phenol/chloroform extraction. Portions of 12 tumors were embedded in compound and stored at −80°C for immunohistochemical analysis.

PCR-SSCP analysis The primers used for polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis[8] were designed to include exon-intron boundaries where mutations have previously been reported[8,9] (Table II). Extracted DNA was amplified in 10 μl of a buffer (50 m*M* KCl, 0.01% gelatine and 10 m*M* Tris buffer at pH 8.3) containing 10 pmol of each primer, 1 m*M* $MgCl_2$, 0.2 m*M* of each deoxynucleotide triphosphate, 0.5 μl of [α-^{32}P]dCTP, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus Co.,

Norwalk, CT) by PCR in a thermal cycler (Perkin-Elmer Cetus Co.) for 35 cycles, each consisting of a denaturation step at 94°C for 0.5 min, an annealing step at 55°C for 0.5 min, and an extension step at 72°C for 1 min. A volume of 3 μl of the PCR product was diluted 10-fold with a sequencing gel-loading buffer (98% deionized formamide, 10 mmol/liter EDTA at pH 8.0, 0.025% xylene cyanol and 0.025% bromophenol blue). Electrophoresis was performed on a 6% neutral polyacrylamide gel with 10% glycerol at 30 W for 4 to 5 h at room temperature. The gel was then dried and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at −80°C for 6 to 24 h.

Table I. Clinicopathological Characteristics of the 25 Breast Carcinomas

Clinicopathological characteristics	No. of patients (%)
TNM stage	
I	1 (4)
II	17 (68)
III	6 (24)
IV	1 (4)
Histological type	
papillotubular	12 (48)
solid-tubular	4 (16)
scirrhous	7 (28)
noninvasive ductal	2 (8)
Lymph node metastasis	
positive	9 (36)
negative	16 (64)
Estrogen receptor	
positive	15 (60)
negative	10 (40)
DNA ploidy pattern	
aneuploidy	17 (68)
diploidy	8 (32)
Tumor size	
<2 cm	5 (20)
2–5 cm	15 (60)
>5 cm	5 (20)

TNM classification according to the General Rules for Clinical and Pathological Recording of Breast Cancer.[13]

PCR-microsatellite analysis Three dinucleotide repeat markers on 16q flanking the E-cadherin gene were used. These markers were located at 16q12.1-q13 (D16S408), 16q22.3-q23.1 (D16S421) and 16q-ter (D16S402)[14] (Table II). Extracted DNA was amplified using the procedure described above. Electrophoresis was performed on a 6% neutral polyacrylamide gel with 7 *M* urea at 60 W for 2 to 3 h at room temperature. LOH was defined as loss of the band corresponding to an allele in tumor DNA.

Immunohistochemistry HECD-1 was used for immunohistochemistry at a dilution of 1/500 with buffer containing 20 m*M* TBS at pH 7.4, 1.0% bovine serum albumin, 0.1% sodium azide and 10 m*M* $CaCl_2$. Immunohistochemistry was performed using frozen sections from 12 tumors. Frozen tissues were cut into 4 μm sections, fixed with cold acetone for 10 min, and washed with 0.01 mol/liter Tris-buffered saline (pH 7.4), containing 10 m*M* Ca^{2+} (TBS^+). To block endogenous peroxidase activity, the specimens were immersed in absolute methanol with 0.3% hydrogen peroxide for 30 min and washed with TBS^+. Following incubation with 2% normal mouse serum for 30 min to block nonspecific binding, the sections were incubated overnight in a moist chamber at 4°C with the HECD-1 monoclonal antibody (diluted in TBS^+). These sections were washed with TBS^+ and incubated for 30 min at room temperature with biotinylated anti-mouse IgG diluted in TBS^+. Peroxidase staining was performed for 15 to 30 min in 20 mg of 3,3′-diaminobenzidine tetrahydrochloride in 100 ml of 0.05 mol/liter Tris-HCl buffer with 10% hydrogen per-

Table II. Sequences of the Primers (5′-3′)

Name	Location	Sequences (5′-3′)
GEC 12	E-cadherin	TCCTCATCAGAGCTCAAGTC
GEC 22	exon 6	GGGTCCAAAGAACCTAAGAG
GEC 11	E-cadherin	TGCCCAGTCCCAAAGTGCAG
GEC 21	exon 7	TCCACACCCTCTGGATCCTC
E-CD8a	E-cadherin	AGGTGGCTAGTGTTCCTGG
E-CD8b	exon 8	CCTTTCTTTGGAAACCCTCTAA
E-CD9a	E-cadherin	GACACATCTCTTTGCTCTGC
E-CD9b	exon 9	GGACAAGGGTATGAACAGCT
D16S408a	16q12.1-q13	TCAAGGAGGGGCAATGAGAG
D16S408b		TCTGTCTCTGTCGCCTCTGT
D16S421a	16q22.3-q23.1	AGACTACACATGAACCGATTG
D16S421b		TCCCTATATTTCCTGGTTGTTA
D16S402a	16q-ter	AGAAACTACAAAGCACACATA
D16S402b		ACTTTTTGGCAGGTTCTCACA

oxide. The sections were finally counterstained for 2 s with 2% methylgreen.

Statistical analysis For all comparisons, Fisher's exact test was applied, and a P value <0.05 was considered statistically significant.

RESULTS

PCR-SSCP analysis No mutations were detected in exons 6 through 9 of the E-cadherin gene (Fig. 1).

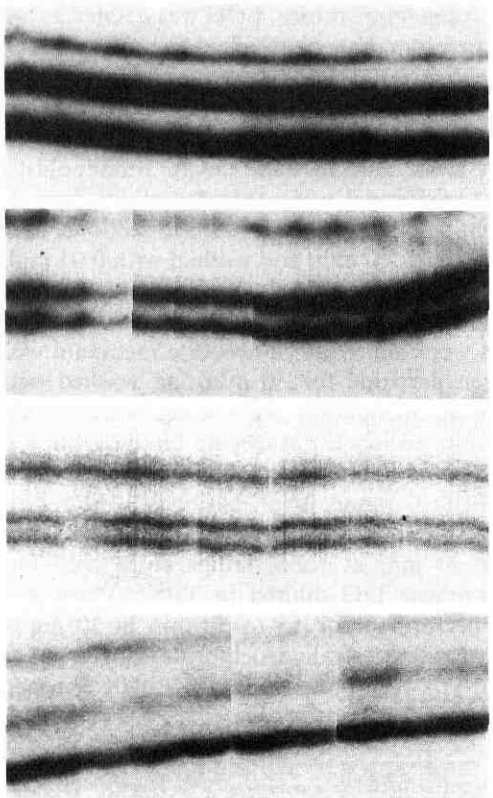

Fig. 1. Representative illustrations of the polymerase chain reaction single-strand conformation polymorphism analyses on exons 6 through 9 of the E-cadherin gene. No mobility shift bands were apparent.

PCR-microsatellite analysis Of the 25 tumors investigated, 46% (13/25) showed LOH in at least one locus (Fig. 2). The most frequent LOH was detected in 42% (5/12 informative cases) of the tumors at D16S402, located on 16q-ter. The incidence of LOH at the examined loci varied from 15% (3/20) (D16S408) and 17% (2/12) (D16S421) to 42% (5/12) (D16S402) (Fig. 3).

Immunohistochemistry Of the 25 tumors, 12 were analyzed immunohistochemically for the expression of E-cadherin. Eleven of the 12 tumors (92%) expressed E-cadherin at the cell-to-cell boundaries irrespective of their histologic type (Fig. 4). The tumor which did not exhibit E-cadherin expression was a papillotubular carcinoma at stage II.

Correlation between LOH and clinicopathological characteristics The correlation between LOH on 16q-ter and

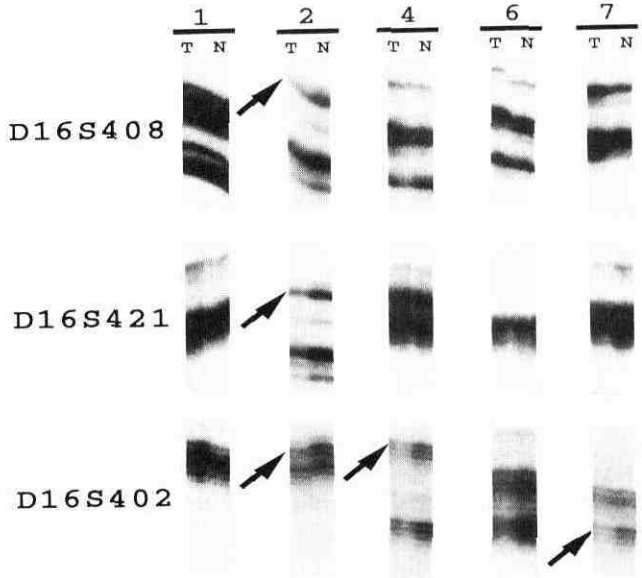

Fig. 2. Several examples of the microsatellite analyses on 16q. D16S408, D16S421 and D16S402 are microsatellite markers localized on 16q12.1-q13, 16q22.3-q23.1, and 16q-ter, respectively. T, tumor DNA; N, normal DNA; LOH, loss of heterozygosity. Arrows indicate LOH.

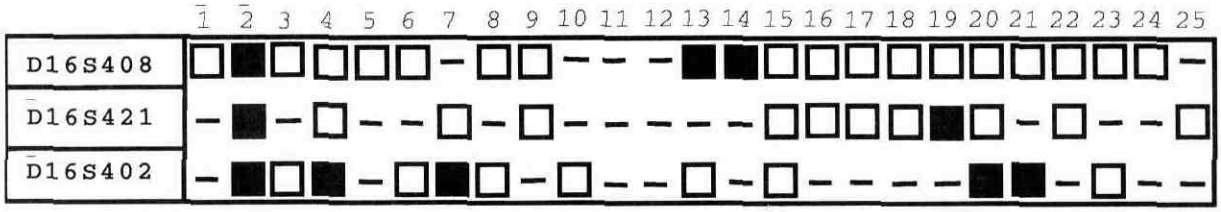

Fig. 3. Results of microsatellite analyses on 16q in 25 ductal carcinomas of the breast. ■, loss of heterozygosity; □, retained heterozygosity; −, not informative.

Fig. 4. Immunohistochemical staining for E-cadherin in a scirrhous carcinoma of the breast. E-Cadherin is uniformly expressed at the surface of the carcinoma cells. ×500, ABC.

Table III. Correlation between LOH on 16q-ter and Clinicopathological Characteristics

Clinicopathological characteristics	Chromosome 16q-ter		
	LOH	Retained	
Stage			
I+II	6	4	*
III+IV	1	1	
Histological type*			
papillotubular	3	3	*
solid-tubular	0	1	
scirrhous	2	2	
noninvasive	0	1	
Lymph node metastasis			
positive	4	3	*
negative	1	4	
Estrogen receptor			
positive	3	1	*
negative	1	4	
DNA ploidy			
aneuploidy	3	6	*
diploidy	2	1	
Tumor size			
<2 cm	1	2	*
2–5 cm	4	5	
>5 cm	0	0	

* Not significant by Fisher's exact test.

clinicopathological characteristics is summarized in Table III. No significant correlations were found by Fisher's exact test.

DISCUSSION

It has been reported that the loss or reduction of E-cadherin expression is associated with invasive and poorly differentiated phenotypes of several human carcinomas, including those of the breast.[15, 16] Furthermore, genetic investigation has revealed that 10% of lobular carcinomas of the breast, which are known to show scattered histologic phenotypes, have somatic mutations of this gene.[10] Scirrhous carcinoma is the most common histologic type of ductal carcinomas of the breast, and also shows scattered growth. However, no report has focused on mutations of the E-cadherin gene in this region. In primary gastric carcinomas, 81% (13/16) of undifferentiated and diffuse regions have been reported to exhibit E-cadherin mutations which were detected by the PCR-SSCP method. Most of these mutations were located in exons 6 through 9, leading to skipping of exon 8 or 9.[9] In the present study, we investigated aberrations of E-cadherin in common ductal carcinomas of the breast by examining mutations, LOH, and immunohistochemical reactivity. No mutations were found in exons 6 through 9 of the E-cadherin gene. We may have missed some mutations because we did not examine all the coding regions of the E-cadherin gene. However, we searched the regions, including the Ca^{2+}-dependent E-cadherin-specific homophilic binding regions,[12] where mutations responsible for adhesive dysfunction[17] have been reported.[7, 8] Alternatively, inactivation of the E-cadherin may be involved in invasive lobular carcinomas, but not in common ductal carcinomas of the breast. The frequency of LOH at 16q-ter was higher than that at 16q22.3-q23.1 which is located closer to the E-cadherin gene. In addition, most (92%) of the tumors exhibited E-cadherin expression immunohistochemically. These findings suggest that disruption of the E-cadherin gene does not play a significant role in the genesis or progression of ductal carcinomas of the breast, and that there may exist some other tumor suppressor gene(s) on 16q-ter. Tsuda *et al.*[18] have also suggested the existence of an unknown tumor suppressor gene involved in the genesis of breast carcinoma on 16q24.2-qter, in the telomeric region from the E-cadherin gene. In agreement with the report by Tsuda *et al.*,[18] we found no significant correlation between LOH on 16q-ter and any clinicopathological characteristic. Therefore, LOH might play a significant role in the genesis of breast carcinoma as an early event.

Invasion and metastasis mediated by the inactivation of cell adhesion systems may not be a simple cascade. Therefore, inactivation of E-cadherin is not the only factor determining tumor invasion and metastasis. E-Cadherin-associated proteins, α-, β- and γ-catenins, which bind directly to the cytoplasmic domain of E-cadherin and the cytoskeleton, have been shown to regulate E-cadherin function.[19–25] Ochiai *et al.*[26] reported that in breast carcinoma E-cadherin expression was con-

served in all scirrhous carcinomas examined, whereas 75% of the tumors showed reduced α-catenin expression by immunohistochemistry. The deletion of a part of the α-catenin gene, which is responsible for cadherin dysfunction, has been reported in human cancer cell lines.[20] Recently, Rimm *et al.*[27] reported that reduction or loss of expression of E-cadherin and α-catenin occurred in 63% and 81% of primary breast carcinomas, respectively. The differences in incidences of immunohistochemical reactivity among reports might result from destabilization of the transcript or protein.[27] However, our genetic analysis indicated that aberrations of the E-cadherin gene are not the major mechanism in disruption of the cell adhesion systems in breast carcinoma. E-Cadherin may lose its function as a cell adhesion molecule without any structural alteration of the gene itself, but with changes in other molecules such as catenins. In addition, cell adhesion molecules have recently been reported to form complexes with the tumor suppressor gene APC[28,29] and oncogene c-*erbB*-2 or epidermal growth factor receptor (EGFR).[30,31] The "deleted in colorectal carcinoma " (DCC) gene, which is a member of the immunoglobulin superfamily of cell adhesion molecules, has also been shown to play a significant role in invasion and metastasis of carcinomas, including those of the breast.[32–34]

In conclusion, inactivation of the E-cadherin gene does not play a significant role in invasion and/or metastasis of ductal carcinomas of the breast. Another tumor suppressor gene which may play a crucial role in the genesis of ductal carcinomas of the breast may exist on 16q-ter.

(Received May 15, 1995/Accepted August 17, 1995)

REFERENCES

1) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1988).
2) Coman, D. R. Adhesiveness and stickiness: two independent properties of the cell surface. *Cancer Res.*, **21**, 1436–1438 (1961).
3) Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*, **251**, 1451–1455 (1991).
4) Behrens, J., Birchmeier, W., Goodman, S. L. and Imhof, B. A. Dissociation of Madin-Darby canine kidney epithelial cells by the monoclonal antibody anti-Arc-1: mechanistic aspects and identification of the antigen as a component related to uvomorulin. *J. Cell Biol.*, **108**, 2435–2447 (1989).
5) Shimoyama, Y. and Hirohashi, S. Expression of E- and P-cadherin in gastric carcinomas. *Cancer Res.*, **51**, 2185–2192 (1991).
6) Mayer, B., Johnson, J. P., Leitl, F., Jauch, K. W., Heiss, M. M., Schildberg, F. W., Birchmeier, W. and Funke, I. E-Cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res.*, **53**, 1690–1695 (1993).
7) Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S., Takeichi, M. and Mori, T. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res.*, **53**, 1696–1701 (1993).
8) Oda, T., Kanai, Y., Oyama, T., Yoshiura, K., Shimoyama, Y., Birchmeier, W., Sugimura, T. and Hirohashi, S. E-Cadherin gene mutations in human gastric carcinoma cell lines. *Proc. Natl. Acad. Sci. USA*, **91**, 1858–1862 (1994).
9) Becker, K.-F., Atkinson, M. J., Reich, U., Becker, I., Nekarda, H., Siewert, J. R. and Höfler, H. E-Cadherin gene mutations provide clues to diffuse type gastric carcinomas. *Cancer Res.*, **54**, 3845–3852 (1994).
10) Kanai, Y., Oda, T., Tsuda, H., Ochiai, A. and Hirohashi, S. Point mutation of the E-cadherin gene in invasive lobular carcinoma of the breast. *Jpn. J. Cancer Res.*, **85**, 1035–1039 (1994).
11) Lindblom, A., Rotstein, S., Skoog, L., Nordenskjöld, M. and Larsson, C. Deletions on chromosome 16 in primary breast carcinomas are associated with development of distant metastases. *Cancer Res.*, **53**, 3707–3711 (1993).
12) Nose, A., Tsuji, K. and Takeichi, M. Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell*, **61**, 147–155 (1990).
13) Japanese Breast Cancer Society. "General Rules for Clinical and Pathological Recording of Breast Cancer," 11th Ed. (1992). Kanehara Co. Ltd., Tokyo.
14) Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissete, J., Millasseau, P., Vaysseix, G. and Lathrop, M. A second-generation linkage map of the human genome. *Nature*, **359**, 794–801 (1992).
15) Shiozaki, H., Tahara, H., Oka, H., Miyata, M., Kobayashi, K., Tamura, S., Iihara, K., Doki, Y., Hirano, S., Takeichi, M. and Mori, T. Expression of immunoreactive E-cadherin adhesion molecules in human cancers. *Am. J. Pathol.*, **139**, 17–23 (1991).
16) Rasbridge, S. A., Gillett, C. E., Sampson, S. A., Walsh, F. S. and Millis, R. R. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J. Pathol.*, **169**, 245–250 (1993).
17) Ozawa, M., Engel, J. and Kemler, R. Single amino acid substitutions in one Ca^{2+} binding site of uvomorulin abolish the adhesion function. *Cell*, **63**, 1033–1038 (1990).
18) Tsuda, H., Callen, D. F., Fukutomi, T., Nakamura, Y. and

Hirohashi, S. Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancers irrespectively of differences in phenotypes and extent of spread. *Cancer Res.*, **54**, 513–517 (1994).
19) Nagafuchi, A. and Tsukita, S. The loss of expression of α-catenin, the 102 kD cadherin associated protein, in central nervous tissues during development. *Dev. Growth Differ.*, **36**, 59–71 (1994).
20) Matsui, S., Shiozaki, H., Inoue, M., Tamura, S., Doki, Y., Kadowaki, T., Iwazawa, T., Shimaya, K., Nagafuchi, A., Tsukita, S. and Mori, T. Immunohistochemical evaluation of alpha-catenin expression in human gastric cancer. *Virchows Arch.*, **424**, 375–381 (1994).
21) Kadowaki, T., Shiozaki, H., Inoue, M., Tamura, S., Oka, H., Doki, Y., Iihara, K., Matsui, S., Iwazawa, T., Nagafuchi, A., Tsukita, S. and Mori, T. E-Cadherin and α-catenin expression in human esophageal cancer. *Cancer Res.*, **54**, 291–296 (1994).
22) Ozawa, M., Baribault, H. and Kemler, R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.*, **8**, 1711–1717 (1989).
23) McCrea, P. D., Turck, C. W. and Gumbiner, B. A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science*, **254**, 1359–1361 (1991).
24) Vestweber, D. and Kemler, R. Some structural and functional aspects of the cell adhesion molecule uvomorulin. *Cell Differ.*, **15**, 269–273 (1984).
25) Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M. and Kemler, R. The uvomorulin-anchorage protein α catenin is a vinculin homologue. *Proc. Natl. Acad. Sci. USA*, **88**, 9156–9160 (1991).
26) Ochiai, A., Akimoto, S., Shimoyama, Y., Nagafuchi, A., Tsukita, S. and Hirohashi, S. Frequent loss of α catenin expression in scirrhous carcinomas with scattered cell growth. *Jpn. J. Cancer Res.*, **85**, 266–273 (1994).
27) Rimm, D. L., Sinard, J. H. and Morrow, J. S. Reduced α-catenin and E-cadherin expression in breast cancer. *Lab. Invest.*, **72**, 506–512 (1995).
28) Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S. and Polakis, P. Association of the APC gene product with β-catenin. *Science*, **262**, 1731–1733 (1993).
29) Su, L.-K., Vogelstein, B., Kinzler, K. W. Association of the APC tumor suppressor protein with catenin. *Science*, **262**, 1734–1737 (1993).
30) Hoschuetzky, H., Aberle, H. and Kemler, R. β-Catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol.*, **127**, 1375–1380 (1994).
31) Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T. and Hirohashi, S. c-*erb*B-2 gene product associates with catenins in human cancer cells. *Biochem. Biophys. Res. Commum.*, **205**, 73–78 (1994).
32) Fearon, E. R., Cho, K. R., Nigro, J. M., Kern, S. E., Simons, J. W., Ruppert, J. M., Hamilton, S. R., Preisinger, A. C., Thomas, G., Kinzler, K. W. and Vogelstein, B. Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science*, **247**, 49–56 (1990).
33) Kikuchi-Yanoshita, R., Konishi, M., Fukunari, H., Tanaka, K. and Miyaki, M. Loss of expression of the DCC gene during progression of colorectal carcinomas in familial adenomatous polyposis patients. *Cancer Res.*, **52**, 3801–3803 (1992).
34) Kashiwaba, M., Tamura, G. and Ishida, M. Frequent loss of heterozygosity at the DCC locus and its association with histologic phenotypes in breast carcinoma. *Virchows Arch.*, **426**, 441–446 (1995).