

## Point Mutation of the E-Cadherin Gene in Invasive Lobular Carcinoma of the Breast

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Reduced or heterogeneous expression of E-cadherin has been demonstrated immunohistochemically in poorly differentiated carcinoma, which frequently shows weak intercellular adhesiveness and marked invasiveness. *In vitro*, not only reduced expression but also structural abnormalities of E-cadherin have been observed in human carcinoma cell lines which grow in a loosely adhering manner. To clarify the participation of structural abnormalities of E-cadherin in cancer invasion *in vivo*, sequence abnormalities were examined in the cadherin domain (exons 5, 6, 7 and 8) including the region essential for E-cadherin specific binding, using the polymerase chain reaction-single-strand conformation polymorphism method and direct sequencing in invasive lobular carcinoma of the breast, in which cancer cells become detached from each other and invade the stroma in a particularly scattered pattern. In 2 (10%) of the 20 cases examined, an identical sequence abnormality was detected in E-cadherin exon 7, i.e. a point mutation of codon 315 (AAT to AGT) which resulted in a single amino acid substitution (asparagine to serine). This mutation may abolish the E-cadherin-mediated cell-cell adhesion and be at least partly responsible for the weak intercellular adhesiveness and scattered histological pattern of the tumor.

Key words: E-cadherin — Genetic abnormality — Invasive lobular carcinoma of breast

The initial step of cancer invasion is the dissociation of cells from cancer nests. In general, when E-cadherin is sufficiently active, epithelial cells cannot disrupt their mutual connection. Therefore, suppression of E-cadherin activity might trigger the release of tumor cells.<sup>1)</sup> In fact, *in vitro* experiments using cell lines have revealed that E-cadherin does have invasion-suppressive properties; non-invasive epithelial cells acquired the ability to invade collagen gels upon addition of antibodies against E-cadherin<sup>2)</sup> or plasmids encoding E-cadherin-specific antisense RNA.<sup>3)</sup> Invasiveness of human carcinoma cell lines has also been reported to be reduced by transfection with E-cadherin cDNA.<sup>3,4)</sup> *In vivo*, immunohistochemical examinations have revealed reduced or heterogeneous expression of E-cadherin in poorly differentiated carcinomas of the stomach,<sup>5-8)</sup> lung,<sup>9)</sup> liver,<sup>10)</sup> head and neck,<sup>11)</sup> esophagus,<sup>12)</sup> prostate,<sup>13)</sup> and breast,<sup>14)</sup> which frequently show weak intercellular adhesiveness and strong invasiveness. Therefore, it is considered that the E-cadherin-mediated invasion suppressor system is inactivated by reduced expression of E-cadherin. On the other hand, we have reported that not only reduced expression but also structural abnormalities of E-cadherin were observed in human cancer cell lines which grew in a loosely adhering manner.<sup>15)</sup> To clarify whether structural abnormalities of E-cadherin *in vivo* do indeed contribute to cancer invasion, sequence mutations in exons 5, 6, 7 and 8<sup>16)</sup> of the cadherin domain, which is essential for E-cadherin-

specific binding,<sup>17)</sup> were examined in invasive lobular carcinoma of the breast, which invades diffusely into the stroma in a particularly scattered manner.<sup>18)</sup> The present study provides the first evidence of E-cadherin structural abnormalities in clinical cases of breast cancer.

### MATERIALS AND METHODS

**Patients and tissue specimens** Fresh tissue samples of primary breast cancer and non-cancerous skin were obtained from patients who underwent mastectomy at the National Cancer Center Hospital. Fresh cancer tissue was embedded in OCT compound (Miles Inc., Elkhart, IN), cut into 5- $\mu$ m-thick sections, and observed microscopically to identify its histological type.

**PCR-SSCP analysis and direct DNA sequencing** High-molecular-weight DNA was isolated from 20 paired specimens of fresh cancer and non-cancerous tissue by phenol-chloroform extraction and dialysis.<sup>19)</sup> The PCR (polymerase chain reaction)-SSCP (single-strand conformation polymorphism) method<sup>20)</sup> was applied for detection of gene mutations of E-cadherin exons 5, 6, 7 and 8. Oligonucleotide primers encompassing exons 6 (GEC 12 and 22) and 7 (GEC 11 and 21) were identical with those used in the previous study.<sup>15)</sup> Primers for amplification of exon 5 (GEC 13: 5'-CCTGACTTGGTTGTGTCGAT-C-3' and GEC 23: 5'-GACCTTTCTTTGGAAACCT-C-3') and exon 8 (GEC 14: 5'-ACCCAGTGTGGGA-TCCTTC-3' and GEC 24: 5'-GTTACCCCGGTGTCA-ACAAG-3') were designed in flanking introns assuming

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that the exon-intron boundaries were conserved between mouse and human E-cadherin.<sup>16)</sup> Each of exons 5–8 of the E-cadherin gene was amplified by 35 cycles of PCR using 5'-end-labeled primers and *Taq* polymerase (Perkin Elmer/Cetus, Norwalk, CT). The PCR products were denatured by heating, and electrophoresed on 6% polyacrylamide gels. Then, abnormally shifted bands detected by SSCP analysis were eluted from the gel, re-amplified by PCR using the same primer pair, subjected to a double-strand cycle sequence system (GIBCO BRL, Gaithersburg, MD) and analyzed on 8% polyacrylamide gel containing 5 M urea.

**Immunohistochemistry** The anti-human E-cadherin mouse monoclonal antibody, HECD-1, and the anti- $\alpha$ -catenin rat monoclonal antibody,  $\alpha$ -18, were produced and characterized previously.<sup>5,8,9)</sup>  $\alpha$ -18 was a gift from Drs. A. Nagafuchi and S. Tsukita. Immunohistochemical staining with HECD-1 and  $\alpha$ -18 was performed for paired cancer and non-cancerous tissue, using AMeX-fixed<sup>21)</sup> and paraffin-embedded specimens, as described elsewhere.<sup>5,8,9)</sup>

**RESULTS**

**Patients and tissue specimens** Cancers from 20 female Japanese patients (cases 1 to 20), all diagnosed histologically as invasive lobular carcinoma, were used in the study.

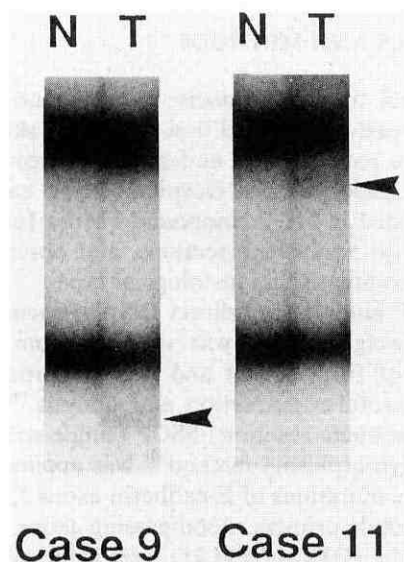


Fig. 1. PCR-SSCP analysis of E-cadherin mutation in invasive lobular carcinoma of the breast. Each of exons 5–8 of the E-cadherin gene was amplified by PCR and electrophoresed on polyacrylamide gels. Cases 9 and 11 showed abnormally shifted bands in exon 7 (arrowheads). N, non-cancerous skin; T, tumorous region. No shifted bands were detected in other exons in any case.

**PCR-SSCP analysis and direct DNA sequencing** Mobility shifts of E-cadherin exon 7 were detected by SSCP in 2 (10%, cases 9 and 11) of the 20 cases (Fig. 1). Since cancerous tissue showing a scattered histological pattern contained a significant amount of stromal cells, the major bands in lane T of cases 9 and 11 in Fig. 1 were considered to have originated from these non-cancerous cells. No mutations were detected in any of the other examined exons in any case. No case showed any mobility shift in DNA from the non-cancerous region. Direct sequencing revealed that the mutations in these two cases were identical, i.e. a point mutation at codon 315, changing AAT to AGT, which resulted in a single amino acid substitution, asparagine to serine (Fig. 2).

**Immunohistochemistry** Cancer cells in cases 9 and 11 showed neither E-cadherin (Fig. 3a) nor  $\alpha$ -catenin (Fig.

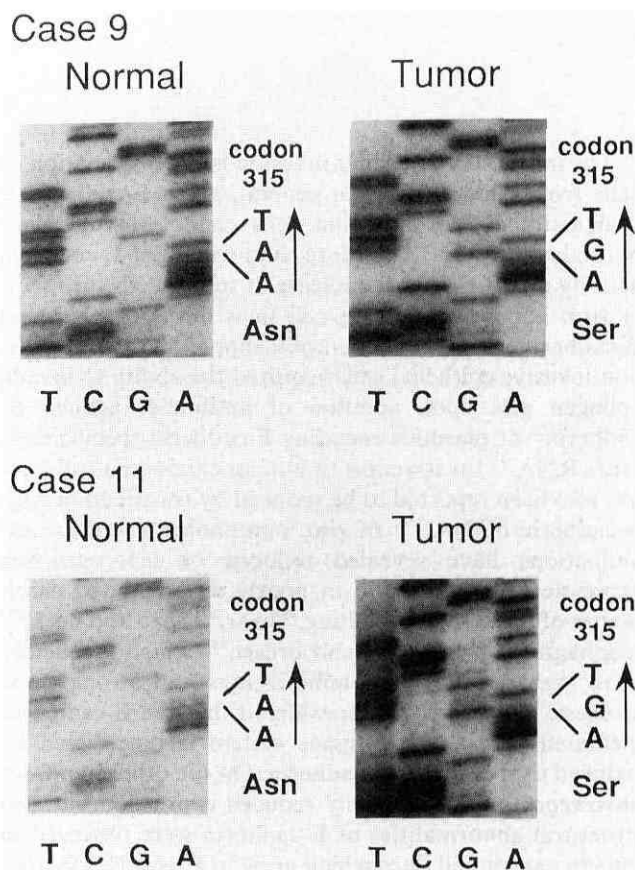


Fig. 2. Direct DNA sequencing of PCR-amplified fragments. Abnormally shifted bands represented in Fig. 1 were eluted from the gels and subjected to direct sequencing analysis. In cases 9 and 11, an identical point mutation in codon 315 (AAT to AGT), which resulted in a single amino acid substitution (asparagine to serine), was observed.

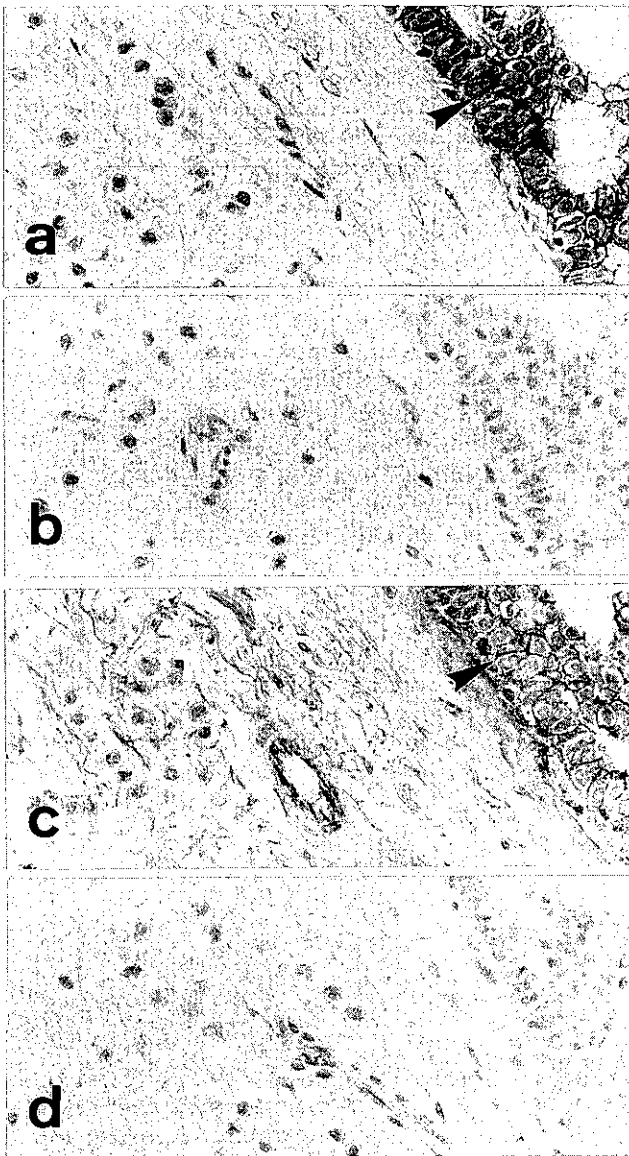


Fig. 3. Immunohistochemical examination of cancerous tissue obtained from case 9, using anti-human E-cadherin mouse monoclonal antibody, HECD-1 (a), and anti- $\alpha$ -catenin rat monoclonal antibody,  $\alpha$ -18 (c). Cancer cells showed neither E-cadherin nor  $\alpha$ -catenin immunoreactivity, whereas both E-cadherin and  $\alpha$ -catenin immunoreactivity were positive at the cell-cell borders in non-cancerous epithelium of the mammary duct (arrowheads). The same procedures were performed using normal mouse serum (b) and normal rat serum (d) for negative controls.

3c) immunoreactivity, whereas non-cancerous epithelial cells in the same specimens showed strong immunoreactivity for both E-cadherin and  $\alpha$ -catenin at the cell-cell borders.

## DISCUSSION

Cancer cells infiltrate into the stroma in a scattered manner in scirrhous-type adenocarcinoma of the stomach and breast.<sup>18, 22)</sup> Especially in invasive lobular carcinoma of the breast, infiltrating cancer cells become detached from each other to form isolated single cells.<sup>18)</sup> Almost all examined cases of invasive lobular carcinoma have been reported to show no E-cadherin or  $\alpha$ -catenin expression.<sup>8, 14)</sup> On the other hand, Ochiai *et al.* reported that 43% of examined scirrhous carcinomas of the stomach and 75% of examined invasive ductal carcinomas of the breast showed loss of  $\alpha$ -catenin, whereas E-cadherin expression was maintained in these cases.<sup>8)</sup> Although dysfunction of the  $\alpha$ -catenin promoter and/or transcriptional elements might have induced the loss of  $\alpha$ -catenin in these cases, invasive lobular carcinoma may provide an *in vivo* example of the post-transcriptional regulation of  $\alpha$ -catenin by E-cadherin reported *in vitro*.<sup>23)</sup> Since mutations in the structural region are known to be responsible for RNA instability,<sup>24, 25)</sup> the possibility that structural abnormalities of E-cadherin in invasive lobular carcinoma reduce the extent of translation of E-cadherin and  $\alpha$ -catenin can be considered.

We have reported mutations of the E-cadherin<sup>15)</sup> and  $\alpha$ -catenin<sup>26)</sup> genes in 3 out of 10 examined human cancer cell lines growing with weak cell-cell adhesion, and suggested that not only reduced expression but also structural abnormalities themselves may result in inactivation of the E-cadherin-mediated cell adhesion system. Whether or not structural abnormalities of E-cadherin exist in invasive lobular carcinoma seems to be an interesting problem in connection with elucidation of the molecular events responsible for the scattered growth pattern of this tumor.

In the present study, we focused on four exons of the cadherin domain, including the region indispensable for  $\text{Ca}^{2+}$ -dependent E-cadherin-specific homophilic binding,<sup>17)</sup> in which we had reported sequence abnormalities of genomic DNA in the human stomach cancer cell lines, MKN 45 and KATO-III.<sup>15)</sup> Since the samples contained significant amounts of stromal cells, we employed the PCR-SSCP method, which can detect DNA abnormalities even in a minor proportion of the sample and enables a nucleotide substitution to be confirmed by direct sequencing.<sup>20)</sup>

Two (10%) of the 20 cases of invasive lobular carcinoma examined showed identical sequence abnormalities in E-cadherin exon 7, which resulted in a single amino acid substitution. Although all cases were examined under the same conditions as those we had employed for successful detection of another point mutation of exon 7 in KATO-III,<sup>15)</sup> the two cases showed different PCR-SSCP migration patterns. We assume that there were two

almost equally stable conformations, and that the different migration patterns shown by the two arose from subtle differences in the temperature and constituents of the gel.

The  $\text{Ca}^{2+}$ -binding motif in exon 7 is regarded as the key element for the function of E-cadherin, since a synthetic molecule with a single amino acid substitution in this motif showed no adhesiveness.<sup>27)</sup> In MKN 45, which lacked tight cell-cell adhesion, we detected a 4-amino-acid deletion at the boundary between exons 6 and 7, which was considered to alter the conformation around the key  $\text{Ca}^{2+}$ -binding motif and to abolish the adhesive property of the E-cadherin molecule.<sup>15)</sup> The single amino acid substitution in the present cases is located near the mutated region of MKN 45 and the key  $\text{Ca}^{2+}$ -binding motif. It is conceivable, therefore, that the mutation in these two cases also abolished the function of E-cadherin, as was the case in MKN 45.

Immunohistochemical examinations revealed neither anti-E-cadherin nor anti- $\alpha$ -catenin immunoreactivity in these two cases (Fig. 3), which may be enough to explain the weak intercellular adhesiveness. In KATO-III, which grew in loosely adhering, floating aggregates, a point mutation of the E-cadherin gene caused a splicing error and affected mRNA stability, resulting in markedly reduced expression of E-cadherin.<sup>15)</sup> Further examination will be necessary in order to reveal the correlation between mutation and reduced expression of E-cadherin and  $\alpha$ -catenin in invasive lobular carcinoma.

Genetic abnormalities of E-cadherin in the examined exons were not detected in all cases, indicating that these were not the only factor determining the tumor invasiveness, which is expected to depend on multifactorial events involving tumor-host interactions. However, it is noteworthy that allele loss on chromosome 16q, which includes the E-cadherin locus,<sup>28)</sup> has frequently been observed in invasive cancer.<sup>29,30)</sup> In MKN 45 and KATO-III, the wild-type allele of the E-cadherin locus was lost, and we have proposed that E-cadherin dysfunction can be mediated by allelic loss coupled with mutation of the remaining allele.<sup>15)</sup> Examination of exons other than the four on which we focused in the present study may reveal that E-cadherin mutation is a more frequent event in breast or other invasive cancers showing loss of heterozygosity on chromosome 16, and should provide further data on the mechanisms by which molecular structural abnormalities influence the invasive character of tumors.

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