Disruption of E-Cadherin-mediated Cell Adhesion Systems in Gastric Cancers in Young Patients

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The aim of this study was to elucidate the pathogenetic backgrounds of early-onset gastric cancers. Mutations of the E-cadherin and β-catenin genes were analyzed by subjecting microdissected cancer cells and corresponding non-cancerous epithelial cells obtained from 9 gastric cancer patients under 35 years old to polymerase chain reaction-single strand conformation polymorphism analysis. Somatic, but no germline, E-cadherin gene mutations were detected in 6 (67%) of the patients. The cancer cells of 2 patients were exon 9-deleted E-cadherin molecule-immunoreactive. Neither somatic nor germline mutations in exon 3 of the β-catenin gene were observed in any patient. One patient lacked β-catenin immunoreactivity and the cancer cells of 6 others showed cytoplasmic β-catenin immunoreactivity. The E-cadherin-mediated cell adhesion system in the cancer cells of all the patients examined appeared to be disrupted, indicating that somatically acquired dysfunction of this system plays an important role in early-onset diffuse-type gastric cancers. Helicobacter pylori infection was observed in 6 (67%) of our 9 patients, an incidence higher than the average in young Japanese individuals. Thus, early-onset gastric cancers may be attributable to environmental factors such as Helicobacter pylori infection.

Key words: E-Cadherin — β-Catenin — Gastric cancer — Helicobacter pylori — Laser microdissection

Epithelial (E)-cadherin is a cell surface glycoprotein which is responsible for calcium-dependent cell-cell adhesion.1) Its cytoplasmic domain connects with cytoskeletal actin filaments through β- and α-catenins, which are indispensable for the E-cadherin-mediated cell adhesion system.2, 3) This system plays a critical role in establishing and maintaining the polarity and histological structure of cells. In some human gastric cancer cells lacking tight cell-cell adhesion, the E-cadherin gene is inactivated by a combination of loss of one allele and mutation in the other.4) In surgically resected specimens from gastric cancer patients, E-cadherin gene mutations resulting in skipping of exons 8 and 9 were observed, particularly in diffuse-type gastric cancers, the cells of which lack mutual adhesion.5, 6) Recently, germline mutations of the E-cadherin gene in familial diffuse-type gastric cancer patients from New Zealand and Europe were reported.7, 8) The E-cadherin gene has come to be regarded as one of the tumor suppressor genes.

Early-onset gastric cancers are not particularly rare in countries and areas with high incidences of gastric cancers and most gastric cancers in young patients are classified histologically as the diffuse type.9) It is feasible that genetic backgrounds participate in gastric cancers in young patients. Therefore, in order to elucidate the pathogenetic backgrounds of early-onset gastric cancers, we examined whether the E-cadherin-mediated cell adhesion system was disrupted in 9 Japanese patients under 35 years old with advanced gastric cancers.

MATERIALS AND METHODS

Patients and genomic DNA extraction Nine patients (cases 1 to 9) under 35 years old (mean, 30.1 years) underwent therapeutic surgical resection for advanced gastric cancers at the National Cancer Center Hospital, Tokyo, between 1992 and 1997. They comprised 3 men and 6 women, including one with the Li-Fraumeni syndrome. In two successive generations of all patients, no gastric cancer was diagnosed in individuals under 50 years of age. All the gastric cancers examined were classified histologically as the diffuse type. Cancerous tissues were obtained from the intramucosal regions of surgically resected materials in case 1 and cases 3 to 9, and from the invasive front in case 2. Corresponding non-cancerous mucosae were also obtained from surgically resected materials in all cases. Scattered cancer cells and corresponding non-cancerous epithelial cells were obtained from 10-µm-thick sections of methanol-fixed and paraffin-embedded
tissues using a laser microdissection system (PALM, Wolfrathausen, Germany), taking care to avoid mutual contamination, and contamination with stromal cells. A QIAamp Tissue Kit (QIAGEN, Hilden, Germany) was used to extract genomic DNA from these dissected cells.

**Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing** PCR-SSCP followed by direct sequencing of exons 2 to 16 of the E-cadherin gene and exon 3 of the β-catenin gene were carried out. All the exons, except exons 4 and 5, of the E-cadherin gene were amplified using the intronic primers reported previously. Exons 4 and 5 were amplified using the intronic primer sets, E-cad-4F (5′-CTTGTTCCCTCATCTTTC-3′) and E-cad-4R (5′-ACTTCTGGCACATCCTC-3′), and E-cad-5F (5′-GGGAAGGTTTTCTACAGCATC-3′) and E-cad-5R (5′-GTAAAGCTTCTCATGTGTTTC-3′), respectively. Exon 3 of the β-catenin gene was amplified using the intronic primers reported previously.

The shifted bands were detected by autoradiography and DNA was extracted from them and subjected to PCR using the primers used for the first PCR. The re-amplified PCR products were sequenced using the Bigdye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and analyzed on an Applied Biosystems 310 Genetic Analyzer (Perkin Elmer, Wolfrathausen, Germany), taking care to avoid mutual tissues using a laser microdissection system (PALM, Munich, Germany) and β-catenin (Transduction Laboratories, Lexington, KY; 1:500 dilution) and of non-cancerous gastric mucosae using a monoclonal antibody against H. pylori (Novocastra, Newcastle, UK; 1:100 dilution) was performed. The immunohistochemical method using the avidin-biotin peroxidase complex was carried out as described previously. Briefly, after deparaffinization and rehydration of 4-μm-thick sections of methanol-fixed and paraffin-embedded tissues, the endogenous peroxidase activity was blocked with 0.3% H2O2. Then, the sections in 10 mCitrate buffer (pH 6.0) were heated by two 10-min microwave treatment cycles at 180 W, and allowed to cool to room temperature. Nonspecific binding was blocked with normal swine serum (DAKO Japan, Kyoto). The sections were incubated with the primary antibodies overnight at 4°C, and then for 30 min at room temperature with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), followed by treatment with the Vectastain Elite ABC reagent (Vector Laboratories). 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen and the sections were counterstained with hematoxylin. Cancerous tissues, in which skipping of exon 9 of the E-cadherin gene has been reported previously, served as positive controls for Δ9, and for the negative control preparations, the primary antibodies were omitted from the reaction sequence.

**RESULTS**

**PCR-SSCP analysis and direct sequencing** PCR-SSCP analysis revealed mobility band shifts of the E-cadherin gene in cancer cells from 6 (67%) of the 9 patients, but not in the corresponding non-cancerous epithelial cells (Fig. 1). The results of direct sequencing of the shifted bands are shown in Fig. 1 and Table I. Polymorphism in codon 692 (GCC (Ala) to GCT (Ala)) of the E-cadherin gene was detected in both cancer cells and non-cancerous epithelial cells from case 6 (data not shown). No mobility band shift of exon 3 of the β-catenin gene was detected by PCR-SSCP analysis of either cancer cells or non-cancerous epithelial cells from all the patients (Table I).

**Immunohistochemistry** The cancer cells of cases 6 and 7 showed Δ9-positive signals (Fig. 2A and Table I).
whereas the corresponding non-cancerous epithelial cells did not (Fig. 2B). Neither the cancer cells nor the non-cancerous epithelial cells of the other 7 patients were Δ9-positive. The cancer cells of case 8 lacked β-catenin immunoreactivity (Fig. 3A and Table I), whereas both the cancer cell membranes and cytoplasm of 6 others (Fig. 3C and Table I) and only the cancer cell membranes of the remaining 2 were β-catenin-positive. Only the cell membranes of the non-cancerous epithelial cells of all the patients were β-catenin-positive (Figs. 3, B and D). *H. pylori* immunoreactivity was detected in the gastric crypts of 6 (67%) of the 9 patients (Fig. 4).

**DISCUSSION**

Alterations of the *E-cadherin* gene were first found in sporadic, diffuse-type gastric cancers, and recently, germline mutations of the *E-cadherin* gene in familial diffuse-type gastric cancer patients were reported, indicating that dysfunction of E-cadherin plays an important role in the histogenesis of this type of gastric cancer. In countries with high incidences of gastric cancers, e.g., Japan, early-onset gastric cancers are not particularly rare and most such gastric cancers in young patients are classified histologically as the diffuse type. In order to determine whether germline mutations of the *E-cadherin* gene contribute to early-onset gastric cancers in countries with high incidences of gastric cancers, we examined 9 Japa-

![Fig. 2](image)

**Fig. 2.** Immunohistochemical staining with the monoclonal antibody against the exon 9-deleted E-cadherin molecule, Δ9, of cells from case 7. The cancer cells were Δ9-positive (A), whereas the non-cancerous epithelial cells were not (B, ×400).
Fig. 3. Immunohistochemical staining with the anti-β-catenin monoclonal antibody of cells from cases 8 (A and B) and 4 (C and D). The cancer cells of case 8 lacked immunoreactivity (A), but the cell membranes of the non-cancerous epithelial cells were immunoreactive (B). Both the cell membranes and the cytoplasm of cancer cells of case 4 were immunoreactive (C), whereas only the epithelial cell membranes of the non-cancerous cells were immunoreactive (D, ×400).

Fig. 4. Histological findings (A) and *H. pylori* immunoreactivity (B) of non-cancerous mucosae in the fundic gland region of case 4. (A) Lymphoid and plasma cells have infiltrated among the atrophic fundic glands (×100). (B) *H. pylori* immunoreactivity in the gastric crypt (×1,000).
nese patients who were under 35 years old and had advanced gastric cancers.

PCR-SSCP analysis revealed somatic, but no germline, mutations of the E-cadherin gene in 6 (67%) of the 9 patients. Mutations at the intron-exon boundaries may cause splicing errors\(^4\) and indeed, cancer cells from case 6 were \(\Delta 9\)-positive. Although PCR-SSCP failed to screen gene mutations, cancer cells, but not non-cancerous epithelial cells, of case 7 were also \(\Delta 9\)-positive, indicating that somatic mutation resulted in skipping of exon 9 of the E-cadherin gene. Overall, 7 (78%) of the 9 patients proved to have E-cadherin gene mutations. Although the incidence of E-cadherin mutations in our cohort was higher than that in the previously reported cohort of diffuse-type gastric cancer patients with no age bias,\(^5\) both cohorts showed somatic mutations. There appeared to be no great difference between these two cohorts in the contribution of genetic background to the development of gastric cancers. The locations of the E-cadherin gene mutations in our cohort were similar to those in the previously reported cohorts.\(^5\)–\(^8\)

In human cancers, not only E-cadherin gene alterations, but also structural abnormalities and/or dysfunction of \(\alpha\)- and \(\beta\)-catenins disrupt the E-cadherin-mediated cell adhesion system.\(^15\)–\(^21\) In particular, \(\beta\)-catenin is a homologue of the Armadillo protein of Drosophila, an important element in the Wingless-Wnt signaling, and is regarded as a key molecule in cross talk between the Wingless-Wnt signaling pathway and the pathway transducing signals from the E-cadherin-mediated cell adhesion system.\(^22\),\(^23\) In this study, we performed PCR-SSCP analysis and immunohistochemical staining of \(\beta\)-catenin.

The cancer cells of case 7 lacked \(\beta\)-catenin immunoreactivity, indicating that \(\beta\)-catenin gene mutation resulted in deletion of the epitope and/or reduced gene expression. The cytoplasm of cancer cells of 6 other patients showed \(\beta\)-catenin immunoreactivity. The APC tumor suppressor gene product regulates \(\beta\)-catenin levels by cooperating with glycogen synthase kinase (GSK)-3\(\beta\) through phosphorylation of serine/threonine residues coded on exon 3 of the \(\beta\)-catenin gene.\(^24\),\(^25\) In some colorectal cancers, alterations of either the \(\beta\)-catenin or APC gene inhibit \(\beta\)-catenin degradation and result in \(\beta\)-catenin accumulation in the cytoplasm.\(^12\),\(^26\) In our study, no mutation in exon 3 of the \(\beta\)-catenin gene was detected, not even in cancer cells showing cytoplasmic \(\beta\)-catenin immunoreactivity. Although APC gene alterations in diffuse-type gastric cancers have not been reported to occur frequently,\(^27\) the possibility that the cytoplasmic \(\beta\)-catenin accumulation we observed was attributable to APC mutations cannot be excluded. Moreover, in a previous study, we found that alterations of tyrosine phosphorylation of \(\beta\)-catenin through K-sam gene product activation might cause dysfunction of the E-cadherin-mediated cell adhesion system in diffuse-type gastric cancers showing cytoplasmic \(\beta\)-catenin accumulation.\(^28\) Such mechanisms may have disrupted the system in the gastric cancer cells showing cytoplasmic \(\beta\)-catenin immunoreactivity of 6 of our patients. Furthermore, structural abnormalities of \(\beta\)-catenin itself or some other member(s) of the E-cadherin-catenin complex may partially disrupt the interaction between membrane-spanning-E-cadherin and \(\beta\)-catenin and result in cytoplasmic accumulation of \(\beta\)-catenin.

According to the results of PCR-SSCP and immunohistochemistry, the E-cadherin-mediated cell adhesion system appeared to be disrupted in the gastric cancers from all the patients examined. The disruption occurred even in the intramucosal lesions, indicating that somatically acquired dysfunction of this system plays an important role in early-onset diffuse-type gastric cancers.

All the gastric cancers we examined were surrounded by non-cancerous gastric mucosae showing histological findings compatible with atrophic gastritis. H. pylori infection, which frequently causes gastritis, is considered to be one of the most important factors in human gastric carcinogenesis.\(^29\)–\(^32\) Recently, the incidence of \(H.\) pylori infection in the Japanese younger generation was reported to be getting lower in comparison with that in the older generation, in whom the incidence of gastric cancer was high.\(^33\),\(^34\) The incidence of \(H.\) pylori infection in our cohort (67%) was higher than the average in young Japanese individuals.\(^33\),\(^34\) Some environmental factors such as \(H.\) pylori infection may participate in early-onset gastric cancers, although further studies are needed in order to examine the incidence of \(H.\) pylori infection in some larger cohorts of early-onset gastric cancers and to elucidate the mechanisms by which such environmental factors cause dysfunction of the E-cadherin-mediated cell adhesion system. The possibility of a predisposition to gastric carcinogenesis as a result of germline mutations of genes other than the E-cadherin and \(\beta\)-catenin genes should also be examined.

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REFERENCES

1) Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science, 251, 1451–1455 (1991).
2) 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).
3) Hirano, S., Nose, A., Hatta, K., Kawakami, A. and Takeichi, M. Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. J. Cell Biol., 105, 2501–2510 (1987).
4) Oda, T., Kanai, Y., Oyama, T., Yashiro, 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).
5) 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).
6) Muta, H., Noguchi, M., Kanai, Y., Ochiai, A., Nawata, H. and Hirohashi, S. E-Cadherin gene mutations in signet ring cell carcinoma of the stomach. Jpn. J. Cancer Res., 87, 843–848 (1996).
7) Guilford, P., Hopkins, J., Harraway, J., McLeod, M., McLeod, N., Harawira, P., Taite, H., Scoular, R., Miller, A. and Reeve, A. E. E-Cadherin germline mutations in familial gastric cancer. Nature, 392, 402–405 (1998).
8) Gayther, S. A., Gorringe, K. L., Ramus, S. J., Huntsman, D., Rovio, F., Grehan, N., Machado, J. C., Pinto, E., Seruca, R., Halling, K., MacLeod, P., Powell, S. M., Jackson, C. E., Ponder, B. A. J. and Caldas, C. Identification of germ-line E-cadherin mutations in familial gastric cancer. Nature, 392, 402–405 (1998).
9) Grabiec, J. and Owen, D. A. Carcinoma of the stomach in young persons. Cancer, 56, 388–396 (1985).
10) Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA, 86, 2766–2770 (1989).
11) Berx, G., Clever, A., Nollet, F., de Leeuw, W. J. F., van de Vijver, M. J., Cornillesse, C. and van Roy, F. E-Cadherin is a tumor/invasion suppressor gene mutated in human lobular breast cancers. EMBO J., 14, 6107–6115 (1995).
12) Iwao, K., Nakamori, S., Kameyama, M., Imaoka, S., Kinoshita, M., Fukui, T., Ishiguro, S., Nakamura, Y. and Miyoshi, Y. Activation of the β-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. Cancer Res., 58, 1021–1026 (1998).
13) Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S. and Hirohashi, S. β-Catenin mutation in carcinoma of the uterine endometrium. Cancer Res., 58, 3526–3528 (1998).
14) Risinger, J. I., Berchuck, A., Kohler, M. F. and Boyd, J. Mutations of the E-cadherin gene in human gynecologic cancers. Nat. Genet., 7, 98–102 (1994).
15) Shimoyama, Y., Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S. and Hirohashi, S. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res., 52, 5770–5774 (1992).
16) Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. and Takeichi, M. Identification of a neural α-catenin as a key regulator of cadherin function and multicellular organization. Cell, 70, 293–301 (1992).
17) Oda, T., Kanai, Y., Shimoyama, Y., Nagafuchi, A., Tsukita, S. and Hirohashi, S. Cloning of the human α-catenin cDNA and its aberrant mRNA in a human cancer cell line. Biochem. Biophys. Res. Commun., 193, 897–904 (1993).
18) 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).
19) Oyama, T., Kanai, Y., Ochiai, A., Akimoto, S., Oda, T., Yanagihara, K., Nagafuchi, A., Tsukita, S., Shibamoto, S., Ito, F., Takeichi, M., Matsuda, H. and Hirohashi, S. A truncated β-catenin disrupts the interaction between E-cadherin and α-catenin: a cause of loss of intercellular adhesiveness in human cancer cell lines. Cancer Res., 54, 6282–6287 (1994).
20) Nakanishi, Y., Ochiai, A., Akimoto, S., Kato, H., Watanabe, H., Tachimori, Y., Yamamoto, S. and Hirohashi, S. Expression of E-cadherin, β-catenin and plakoglobin in esophageal carcinomas and its prognostic significance. Oncology, 54, 158–165 (1997).
21) Hirohashi, S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am. J. Pathol., 153, 333–339 (1998).
22) Peifer, M. Regulating cell proliferation: as easy as APC. Science, 272, 974–975 (1996).
23) Nakamura, Y. Cleaning up on β-catenin. Nat. Med., 3, 499–500 (1997).
24) Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. and Polakis, P. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. USA, 92, 3046–3050 (1995).
25) Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. Science, 272, 1023–1026 (1996).
26) Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science, 275, 1787–1790 (1997).
27) Nakatsu, S., Yanagisawa, A., Ichii, S., Tahara, E., Kato, Y., Nakamura, Y. and Horii, A. Somatic mutation of the APC gene in gastric cancer: frequent mutations in very well differentiated adenocarcinoma and signet-ring cell carcinoma. *Hum. Mol. Genet.*, 1, 559–563 (1992).

28) Akimoto, S., Ohtai, A., Inomata, M. and Hirohashi, S. Expression of cadherin-catenin cell adhesion molecules, phosphorylated tyrosine residues and growth factor receptor-tyrosine kinases in gastric cancers. *Jpn. J. Cancer Res.*, 89, 829–836 (1998).

29) Parsonnet, J., Friedman, G. D., Vandersteen, D. P., Chang, Y., Vogelman, J. H., Orentreich, N. and Sibley, R. K. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.*, 325, 1127–1131 (1991).

30) Nomura, A., Stemmermann, G. N., Chyou, P. H., Kato, I., Perez-Perez, G. I. and Blaser, M. J. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N. Engl. J. Med.*, 325, 1132–1136 (1991).

31) Forman, D., Newell, D. G., Fullerton, F., Yarnell, J. W. G., Stacey, A. R., Wald, N. and Sitas, F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *Br. Med. J.*, 302, 1302–1305 (1991).

32) Talley, N. J., Zinsmeister, A. R., Weaver, A., DiMagno, E. P., Carpenter, H. A., Perez-Perez, G. I. and Blaser, M. J. Gastric adenocarcinoma and *Helicobacter pylori* infection. *J. Natl. Cancer Inst.*, 83, 1734–1739 (1991).

33) Kikuchi, S., Wada, O., Nakajima, T., Nishi, T., Kobayashi, O., Konishi, T., Inaba, Y. and the Research Group on Prevention of Gastric Carcinoma among Young Adults. Serum anti-*Helicobacter pylori* antibody and gastric carcinoma among young adults. *Cancer*, 75, 2789–2793 (1995).

34) Haruma, K., Okamoto, S., Kawaguchi, H., Gotoh, T., Kamada, T., Yoshihara, M., Sumii, K. and Kajiyama, G. Reduced incidence of *Helicobacter pylori* infection in young Japanese persons between the 1970s and the 1990s. *J. Clin. Gastroenterol.*, 25, 583–586 (1997).