through the nucleotide excision repair pathway (Reardon *et al.*, 1999; Niedernhofer *et al.*, 2004; Ceppi *et al.*, 2006). *RRM1* is reported to influence cell survival, probably through interaction with the *phosphatase and tensin homolog* (*PTEN*), which is an inhibitor of cell proliferation, and suppresses cell migration and invasion by reducing the phosphorylation of focal adhesion kinase (Gautam *et al.*, 2003; Bepler *et al.*, 2004). In lung cancer, the expression levels of *RRM1* and *ERCC1* are significantly correlated (Bepler *et al.*, 2006; Ceppi *et al.*, 2006).

Gemcitabine is the first line cytotoxic agent for treatment of patients with advanced pancreatic cancer, and it is the only agent with proven benefit in a large adjuvant clinical trial (Oettle *et al.*, 2007). However, it is estimated that only 25% of patients benefit from gemcitabine (Burris *et al.*, 1997). *RRM1* expression appears to be the key determinant of gemcitabine resistance (Dumontet *et al.*, 1999; Goan *et al.*, 1999; Jung *et al.*, 2001). This is partially due to expansion of the dNTP pool, which competitively inhibits the incorporation of gemcitabine triphosphate into DNA (Plunkett *et al.*, 1996). Another mechanism is the direct interaction between *RRM1* and gemcitabine with *RRM1* acting as a 'molecular sink' for gemcitabine (Davidson *et al.*, 2004; Bergman *et al.*, 2005). *ERCC1* is reported to be associated with the repair of cisplatin-induced DNA adducts in ovarian cancer (Li *et al.*, 2000), gastric cancer (Metzger *et al.*, 1998), colorectal cancer (Shirota *et al.*, 2001), lung cancer (Olaussen *et al.*, 2006) and esophageal cancer (Joshi *et al.*, 2005; Kim *et al.*, 2008).

Quantitative analysis of gene expression in pancreatic cancer is challenging because it contains more stromal tissue than other cancers (Sato *et al.*, 2004; Bachem *et al.*, 2005; Infante *et al.*, 2007), which makes laser microdissection a necessity to obtain gene expression of tumor tissue (Giovannetti *et al.*, 2006). Quantitative analysis of the *RRM1* protein had been difficult because of technical limitations. However, an automated, quantitative *in situ* assessment of protein expression was developed recently (Camp *et al.*, 2002), and applied for objective and practical evaluation of *RRM1* and *ERCC1* protein expression levels in tumor specimens (Zheng *et al.*, 2007). In this study, we used the above mentioned technology for gene expression analysis in pancreatic cancer specimens.

We found that the expression levels of *RRM1* and *ERCC1* affected the clinical outcome similar to that described in non-small-cell lung cancer (Zheng *et al.*, 2007). Patients with high levels of expression of both proteins had the best prognosis, including both disease-free survival and overall survival. However, once treatment with gemcitabine was initiated at the time of recurrence, it was only the group of patients with low levels of *RRM1* that benefited significantly from this intervention. In other words, patients with high tumoral *RRM1* levels may as well be treated with other agents, such as S-1 or oxaliplatin plus 5-fluorouracil plus leukovorin (CONKO-003), instead of gemcitabine (Ueno *et al.*, 2005; Okusaka *et al.*, 2008; Saif, 2008). In contrast, patients with low tumoral *RRM1* levels showed improved survival following treatment with gemcitabine (Moore *et al.*, 2007; Boeck and Heinemann, 2008). Many clinical trials of anticancer drugs, including molecular targeting agents, did not result in the improvement of outcome when conducted in unselected groups of patients (Heinemann *et al.*, 2006; Herrmann *et al.*, 2007; Cascinu *et al.*, 2008). However, if patients can be divided into groups with high or low likelihood of benefit from gemcitabine, a more rational design of future trials becomes available (Simon *et al.*, 2007). We believe that future treatment strategies for pancreatic cancer should be more precise and tailored to individual patients, and *RRM1* may be one of the candidate molecules for the stratification. We found that *RRM1* and *ERCC1* were not significantly coexpressed in pancreatic cancer, which is different from several previous reports in non-small-cell lung cancer (Ceppi *et al.*, 2006; Zheng *et al.*, 2007). This discrepancy may be explained by differences in tissue of origin and mechanisms of carcinogenesis between pancreatic cancer and lung cancer.

It is important to carry out prospective tailored therapeutic trials in pancreatic cancer with the goal of improving the clinical outcome, and it is our opinion that *RRM1* and *ERCC1* could play an important role in the design of such trials.

Materials and methods

Patients

Between January 1992 and March 2008, 166 patients underwent surgery for pancreatic cancer at Osaka University Hospital. We excluded 84 patients for the following reasons: (1) tumors were not resectable in 26 patients because of liver metastases or peritoneal carcinomatosis, (2) surgery resulted in R1 (residual microscopic cancer) or R2 (residual macroscopic cancer) resections in 21 patients, (3) chemotherapy or chemoradiotherapy was provided preoperatively to 37 patients and (4) lack of neutral-buffered formalin-fixed and paraffin-embedded tumor blocks or/and clinical follow-up information for study purposes in 14 cases. As the natural history of variant pancreatic neoplasms differs from the usual pancreatic ductal adenocarcinoma, patients with intraductal papillary mucinous neoplasms, mucinous cystic adenocarcinomas and medullary adenocarcinomas were excluded from this study. Supplementary Table 1 summarizes the characteristics of the 68 patients who were enrolled in this study. They included 33 men and 35 women with a mean age of 60.7 ± 7.8 years ($\pm$ s.d.). All patients had R0 (no residual cancer) resections by pancreaticoduodenectomy in 54 patients, distal pancreatectomy in 12 patients and other resections in 2 patients. The histopathological grading showed poorly, moderately, and well-differentiated adenocarcinoma in 10, 32 and 26 patients, respectively. The UICC-TNM classification was 2, 1 and 65 patients with pT1, pT2 and pT3; 29, 33 and 6 patients with pN0, pN1 and pM1lym; and 1, 1, 27, 33 and 6 patients with stage IA, IB, IIA, IIB and IV, respectively. None of the patients had received neoadjuvant therapy preoperatively. All 68 patients were followed until disease recurrence and/or death. The median follow-up period was 16.3 months (range, 4.3–113), the 5-year survival rate was 23.4%, and the recurrence of disease was observed in 50 patients. Treatment with gemcitabine was carried out in 28 patients; 5 patients

received it as adjuvant chemotherapy and 23 patients received it after disease recurrence. Radiation therapy was not carried out during all the follow-up period.

Immunofluorescence and automated quantitative analysis
We carried out immunostaining after constructing a tissue microarray. Immunofluorescence combined with AQUA was used to assess *in situ* expression of the target molecules as described previously (Zheng *et al.*, 2007). Antigens were retrieved by incubating the tissue in a microwave oven. Optimal concentrations of antisera and antibodies were used to detect *RRM1*, *ERCC1* and cytokeratin. The antiserum to *RRM1* was generated from rabbits and affinity-purified (R1AS-6) as described previously (Zheng *et al.*, 2007). Commercially available antibodies were used for the analysis of *ERCC1* (Ab-2 clone 8F1, MS-671-R7, Laboratory Vision Corporation, Fremont, CA, USA) and cytokeratin (antihuman pancytokeratin AE1/AE3, M3515 and Z0622, Dako Cytomation, Glostrup, Denmark) (Zheng *et al.*, 2007). They were visualized with the use of fluorochrome-labeled secondary antibodies. The final slides were scanned with SpotGrabber (HistoRx, New Haven, CT, USA), and images were analysed with AQUA (version 1.6, PM-2000, HistoRx). The AQUA scores ranged from 0 (no expression) to 3000 (maximal observed expression).

Statistical analysis and ethical issues
Data are expressed as mean ± s.d. Differences in continuous values were evaluated by the Student's *t*-test (Table 1). The Fisher's exact probability test was used to compare discrete variables (Table 1). We evaluated correlations between AQUA scores of *RRM1* and *ERCC1* by Pearson's correlation coefficient (Figure 2). Disease-free and overall survival rates were estimated by the Kaplan–Meier method and compared using the log-rank test (Table 1, Figures 3 and 4). Cox's proportional hazard regression model with stepwise comparisons was used to analyse independent prognostic factors (Table 2). The predictive value of *RRM1* was studied by testing the interaction between *RRM1* expression and gemcitabine treatment in the same Cox model. A P-value <0.05 was used to indicate statistical significance.

This study was analysed by the statistical expert in our laboratory and the study protocol was approved by the Human Ethics Review Committee of Osaka University, and a signed consent form was obtained from each subject.

Conflict of interest

The authors declare no conflict of interest.

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Core fucosylation of E-cadherin enhances cell–cell adhesion in human colon carcinoma WiDr cells

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α1,6-Fucosyltransferase (Fut8), an enzyme that catalyzes the introduction of α1,6 core fucose to the innermost *N*-acetylglucosamine residue of the *N*-glycan, has been implicated in the development, immune system, and tumorigenesis. We found that α1,6-fucosyltransferase and E-cadherin expression levels are significantly elevated in primary colorectal cancer samples. Interestingly, low molecular weight population of E-cadherin appeared as well as normal sized E-cadherin in cancer samples. To investigate the correlation between α1,6-fucosyltransferase and E-cadherin expression, we introduced α1,6-fucosyltransferase in WiDr human colon carcinoma cells. It was revealed that the low molecular weight population of E-cadherin was significantly increased in α1,6-fucosyltransferase-transfected WiDr cells in dense culture, which resulted in an enhancement in cell–cell adhesion. The transfection of mutated α1,6-fucosyltransferase with no enzymatic activity had no effect on E-cadherin expression, indicating that core fucosylation is involved in the phenomena. In α1,6-fucosyltransferase knock down mouse pancreatic acinar cell carcinoma TGP49 cells, the expression of E-cadherin and E-cadherin dependent cell–cell adhesion was decreased. The introduction of α1,6-fucosyltransferase into kidney epithelial cells from α1,6-fucosyltransferase $^{-/-}$ mice restored the expression of E-cadherin and E-cadherin-dependent cell–cell adhesion. Based on the results of lectin blotting, peptide *N*-glycosidase F treatment, and pulse-chase studies, it was demonstrated that the low molecular weight population of E-cadherin contains peptide *N*-glycosidase F insensitive sugar chains, and the turnover rate of E-cadherin was reduced in α1,6-Fucosyltransferase transfectants. Thus, it was suggested that core fucosylation regulates the processing of oligosaccharides and turnover of E-cadherin. These results suggest a possible role of core fucosylation in the regulation of cell–cell adhesion in cancer. (*Cancer Sci* 2009; 100: 888–895)

It is generally accepted that glycosylation affects many properties of glycoproteins, including their conformation, flexibility, and hydrophilicity. As a result, it regulates protein sorting, stability, and protein–protein interactions.[1–5] *N*-Glycans have a common core structure, and their branching patterns are determined by glycosyltransferases.[6,7] Fut8 is an enzyme that catalyzes the introduction of α1,6 core fucose on the asparagine-branched *N*-acetylglucosamine residue of the chitobiose unit of complex-type *N*-glycans.[8,9] Fut8 has been investigated especially in terms of oncogenesis, since the α1,6-fucosylation of α-fetoprotein is a well-known marker of hepatocellular carcinoma.[10] In previous studies, our group reported that Fut8 expression is markedly enhanced in several types of cancer cell lines[11] rat hepatoma tissues[12] and in ovarian serous adenocarcinoma cells.[13]

E-cadherin is a 120 kDa type I membrane protein, which belongs to the class of calcium-dependent cell adhesion molecules.[14] It mediates cell–cell adhesion through the assembly of multiprotein complexes linked to the actin cytoskeleton.[15] Several models have been proposed to date for the cadherin homophilic interactions. Examples include the "linear zipper model", which involves Trp-mediated *cis* dimers and *trans* interactions between the outermost domains,[16] a Trp-dimer model, which involves the formation of a Trp-mediated *trans*-homophilic bond,[17] a model which involves *cis*-dimerization at the Ca^{2+}-binding site,[18] and a model that invokes extensive overlap between ectodomains in the adhesive binding interface.[19,20] The extracellular domain of human E-cadherin consists of five repeats of about 110 amino acid residues, referred to as EC1 through 5, and contains four potential *N*-glycosylation sites, two each in EC4 and EC5. It is synthesized in the form of a precursor polypeptide that is glycosylated and the precursor is then processed to the mature polypeptide.[21–23] It has previously been reported that cells expressing unprocessed E-cadherin by mutating recognition site(s) for processing protease showed no E-cadherin-dependent mediated adhesion.[24]

Our previous studies demonstrated that the introduction of GnT-III and the addition of bisecting GlcNAc residues, products of GnT-III, to E-cadherin down-regulated tyrosine phosphorylation of β-catenin, enhanced cell–cell adhesion mediated by E-cadherin, and suppressed lung metastasis in mouse melanoma cells.[25,26] Consistent with these results, Guo *et al.* also reported that the overexpression of GnT-V, which competes with GnT-III for biantennary substrates, decreased cadherin-mediated cell–cell adhesion.[27] On the other hand, the overexpression of Fut8 in hepatoma cells suppressed intrahepatic metastasis after splenic injection into athymic mice.[28] Liwosz *et al.* reported that the status of *N*-glycosylation of E-cadherin is altered in a cell density-dependent manner, and the loss of complex type of *N*-glycan reduces the molecular weight of E-cadherin and enhanced its preferential association with the actin cytoskeleton, leading to the stabilization of E-cadherin scaffolds.[29] These

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Abbreviations: AAL, *Aleuria aurantia* lectin; BSA, bovine serum albumin; CHO, Chinese hamster ovary; ConA, *Concanavalia ensiformis*; DMEM, Dulbecco's modified Eagle's medium; DSA, *Datura stramonium* lectin; EDTA, ethylenediamine tetraacetic acid; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FcγRIIIA, Fcγ receptor IIIA; Fut8, α1,6-fucosyltransferase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GDP-Fucose, guanosine diphosphate-fucose; GlcNAc, *N*-acetyl glucosamine; GnT-III, *N*-acetylglucosaminyltransferase III; LRP-1, lipoprotein receptor-related protein-1; MDCK, Madin-Darby canine kidney; MES-NaOH, 2-morpholinoethane sulfonic acid, memohydrate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PNGase F, peptide *N*-glycosidase F; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TGF-β1, transforming growth factor-β1.

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Table 1. Clinical features of patients with colorectal cancer

Sample number	Age	Sex	Location†	Dukes' stages
1	71	Female	A	C
2	41	Male	R	B
3	77	Male	R	B
4	87	Male	T	B
5	69	Male	S	C
6	71	Male	A	D

†A, ascending colon; R, rectum; S, sigmoid colon; T, transverse colon.

studies suggest that *N*-glycans play a role in modulating E-cadherin status. In the present study, we found that Fut8 and E-cadherin protein levels are significantly increased in colorectal cancer samples. E-cadherin in Fut8 transfected WiDr cells, Fut8 knocked down cells, and Fut8 deficient cells from Fut8$^{-/-}$ mice were examined and our results demonstrate that the activity of Fut8 is involved in the appearance of a low molecular weight population of E-cadherin and regulates the total amount of E-cadherin. We propose the possible involvement of core fucosylation in changing the *N*-glycosylation patterns of E-cadherin, the subsequent stabilization of cell–cell contacts, and the regulation of metastatic potential.

Materials and Methods

Human tissues samples. All experiments were approved by ethical committees both in Osaka University and Osaka National Hospital. Tissues from six cases of primary colorectal cancer were surgically resected (Table 1). Written informed consent was obtained from each patient before surgery. The excised samples were obtained within 1 h after the operation from tumor tissues and corresponding non-tumor tissues 5–10 cm remote from the tumor. All of the excised tissues were placed immediately in liquid nitrogen and stored at –80°C until additional analysis.

Cell lines, culture, and transfection. Human colon carcinoma WiDr cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained in DMEM supplemented with 10% FBS. A Fut8 expression vector was constructed by inserting the open reading frame of human *Fut8* cDNA into a mammalian expression vector pCXN2 which was regulated by the β-actin promoter. Mutant Fut8, which had no enzymatic activity, was produced by mutating arginine 365 to alanine.[30] WiDr cells were transfected with pCXN2/*Fut8* or pCXN2/R365A *Fut8* or pCXN2 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Selection was performed by 2-week incubation in medium containing G418, and G418-resistant colonies were isolated and recloned by serial dilution to ensure clonality. Fut8 knocked down mouse pancreatic aciuar cell catcinoma TGP49 cells were prepared as described previously.[31] Fut8 deficient kidney epithelial cells were prepared from *Fut8*$^{-/-}$ mice as described previously.[32] The cells were serum starved for 8 h before harvest to achieve the cell cycle synchronization.

Activity assay of Fut8. The enzymatic activity of Fut8 was measured by high-performance liquid chromatography using a fluorescence-labeled sugar chain as the substrate, as previously described.[33] A standard mixture included 80 mM MES-NaOH (pH 7.0), 0.5% Triton X-100, 2 μM 4-(2-pyridylamino)butylamine-labeled sugar chain, and 50 μM GDP-Fucose. After incubation at 37°C for 2 h, the reaction was terminated by incubating at 100°C for 1 min. The samples were then centrifuged at 15 000 *g* for 10 min and applied to high-performance liquid chromatography on a TSK-gel, ODS-80TM column (4.6 × 150 mm) (Tosho, Tokyo, Japan). Elution was performed at 55°C with 20 mM sodium acetate buffer, pH 4.0, 0.1% butanol, in an isocratic manner. Fluorescence of the column elutes was detected with a fluorescence spectrometer (model RF 535; Shimadzu Corp., Kyoto, Japan), the excitation and emission wavelengths being 320 and 400 nm, respectively.

Protein extraction, immunoprecipitation, and western blotting. Frozen tissue samples were homogenized in 5 vol. of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% [w/v] Nonidet P-40, 10% [w/v] glycerol, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 5 μg/mL leupeptin, and 1 mM dithiothreitol) using Polytron homogenizer (Kinematica, Littau-Luzern, Switzerland). After centrifugation (15 000 *g*) for 20 min at 4°C the supernatant were collected. Cell cultures were rinsed twice with ice-cold PBS and harvested in lysis buffer. Protein concentrations were determined using a Protein Assay CBB kit (Nacalai Tesque, Kyoto, Japan). For the immunoprecipitation of E-cadherin, whole cell lysates (500 μg) were incubated with 4 μg of mouse anti-E-cadherin antibody (610182; BD Bioscience, San Jose, CA, USA) for 2 h at 4°C, and then with 20 μL of Protein G Sepharose 4 Fast Flow (GE Healthcare Biosciences, Buckinghamshire, UK) for 4 h at 4°C. For western blot analysis, protein samples or immunoprecipitates were subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Mouse anti-E-cadherin antibody (610182; BD Bioscience) or mouse anti-Fut8 antibody[34] was used as primary antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (GE Healthcare Biosciences).

Cell surface biotinylation and immunoprecipitation of E-cadherin. Cell surface biotinylation was performed as described previously.[35] Briefly, cells were incubated with sulfosuccinimidobiotin (s-NHS-biotin; Pierce, Rockford, IL, USA) (1 mg/mL) for 10 min on ice, and the reaction was quenched with 50 mM NH_4Cl. The cell lysate was immunoprecipitated with anti-E-cadherin antibody as described above. The biotinylated proteins were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and an enhanced chemiluminescence kit.

Lectin blot analysis. Lectin blot analysis was performed as described previously.[36] The immunoprecipitated E-cadherin was electrophoresed on 8% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked with 5% BSA (w/v) and then incubated with 2 μg/mL of biotinylated AAL, DSA, or ConA lectin (Seikagaku Corp., Tokyo, Japan) for 30 min at room temperature. After washing, lectin reactive proteins were detected using a Vectastain ABC kit and an enhanced chemiluminescence kit.

Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative real-time PCR. Total RNA was prepared from WiDr cells. cDNAs were synthesized using an SYBY RT-PCR kit (Perfect Real Time; Takara-Bio Inc., Otsu, Japan) and Reverse Transcription Reagent (Takara-Bio Inc.) according to the manufacturer's instructions. A random hexamer was used for cDNA synthesis. Real-time PCR was performed using the SYBR RT-PCR kit and was analyzed on Smart Cycler II system (Cepheid, Sunnyvale, CA, USA). Human E-cadherin was amplified using the primers, sense (5′-GGATTGCAAATTCCTGCCATTC-3′) and antisense (5′-AACGTTGTCCCGGGTGTCA-3′). GAPDH was amplified as a control using the primers, sense (5′-ATTG-CCCTCAACGACCACTT-3′) and antisense (5′-AGGTCCACCA-CCCTGTTGCT-3′). The levels of gene expression were determined using a Delta-Delta Ct method.[37]

Immunofluorescence microscopy. Cells were plated on poly L-lysine-coated glass bottom dish, fixed by incubation with PBS containing 4% paraformaldehyde for 10 min at room temperature and permeabilized in PBS containing 0.1% Triton X-100 for 1 min. After washing with PBS three times for 15 min each, the cells were incubated in ECCD-2 (monoclonal antibody to mouse

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E-cadherin M108; Takara-Bio Inc.) (1:100 dilution) for 1 h at room temperature. Primary antibody binding was detected with a fluorescein isothiocyanate-labeled goat antibody to mouse IgG. Glass bottom dishes were mounted under Permafluor aqueous mounting medium and the stained cells were viewed with a laser scanning confocal microscope (Carl Zeiss, Jena, Germany).

Cell aggregation assay. Cells were washed twice with PBS and dissociated by incubation with PBS containing 2 mM EDTA for 30 min at 37°C. Single cell suspensions were prepared, washed, and resuspended in DMEM containing 1% (w/v) BSA. 1×10^6 cells were incubated on a rotation apparatus for 3 h at 37°C. In some experiments, 2 mM EDTA or 100 μg/mL HECD-1 (M106 monoclonal antibody to human E-cadherin; Takara-Bio Inc.) was added. At the end of the incubation, cells were diluted into single wells of a 24-well plate to prevent further aggregation. After allowing cells to settle for 40 min at 37°C, an equal volume of 7.4% formaldehyde in PBS was added to each well and the plate was incubated for 10 min at room temperature. Photos were taken at random under a phase contrast microscope to count single cells or cell aggregates (four or more cells).

Peptide N-glycosidase F (PNGase F) treatment. Whole cell lysates were boiled in 0.1 M 2-mercaptoethanol and 0.1% SDS for 10 min. After boiling, the 50 μg of proteins were incubated for 16 h at 37°C with 100 mM Tris-HCl (pH 8.6), 1% NP-40, and 40 mU/mL PNGase F (Takara Bio Inc.). Then the samples were subjected to 6% SDS-PAGE as described above.

Metabolic labeling and pulse chase study. Eighty-percent confluent monolayers of Fut8 and mock transfectants in 6-well dishes were preincubated for 2 h at 37°C with methionine, cysteine-free DMEM (Sigma) containing dialyzed 10% FBS. For pulse chase studies, L-[^{35}S]methionine and L-[^{35}S]cysteine (Promix; GE Healthcare Biosciences) were added at a concentration of 200 μCi/mL each to the culture media and incubated for 20 min at 37°C for protein labeling. After rinsing three times with PBS, the cells were incubated at 0, 8, 24, and 32 h in DMEM with 10% FBS. Another experiment was performed under conditions of a long-pulse and a long-chase. For the long-pulse, L-[^{35}S]methionine and L-[^{35}S]cysteine were added at a concentration of 50 μCi/mL each to the culture media, followed by incubation for 24 h at 37°C for protein labeling, and after rinsing, the cells were incubated at 0, 24, and 48 h in DMEM with 10% FBS. E-cadherin was immunoprecipitated and subjected to 8% SDS-PAGE. After electrophoresis, the gel was autoradiographed using imaging plates and a BAS-2500 system (FujiFilm, Tokyo, Japan).

Results

E-cadherin and Fut8 expression were increased in primary colorectal cancers. Abnormal cell–cell adhesion that is observed in many types of cancer could result from the changes in E-cadherin expression. We examined Fut8 and E-cadherin expression levels in primary colorectal cancer, and found that they are significantly increased in five out of six examined samples (Fig. 1). Low molecular weight population of E-cadherin appeared as well as normal sized E-cadherin only in the cancer samples. The relative expression levels of Fut8 and E-cadherin in five samples are 3.5–29 and 3.3–11, respectively.

Establishment of WiDr clones stably expressing Fut8. To investigate the correlation between Fut8 and E-cadherin expression, we introduced Fut8 in WiDr human colon carcinoma cells in which Fut8 expression levels are low. WiDr cells were transfected with pCXN2/*FUT8* or pCXN2, and G418-resistant clones were selected as described under "Materials and Methods". The selected Fut8 transfected clones showed elevated enzymatic activities, as 560, 470 and 190 nmol/h/mg protein, respectively. The following experiments were performed with three clones and similar results were observed for all data.

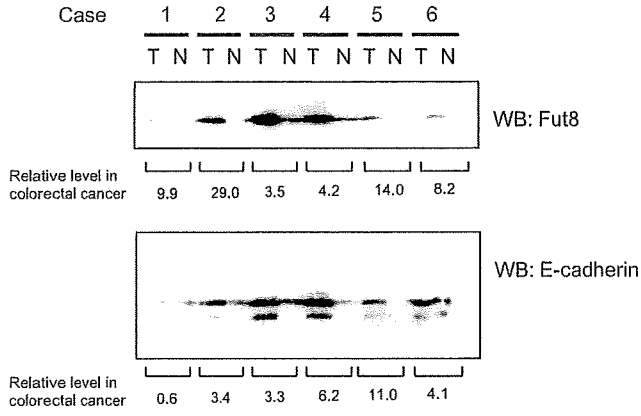

Fig. 1. α1,6-Fucosyltransferase (Fut8) and E-cadherin expression levels were increased in primary colorectal cancer. Total protein lysate was prepared from matched samples of tumor (T) and adjacent non-tumor tissue (N). 100 μg of protein from each pair were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and Fut8 and E-cadherin were detected using an anti-Fut8 antibody and an anti E-cadherin antibody. WB, Western blotting.

Analysis of E-cadherin in the Fut8 transfected WiDr cells. Western blotting showed that the expression level of E-cadherin in *Fut8* transfectants was increased, especially in high-density cultures (~11×10^4 cells/cm^2) when compared to low density cultures (~3×10^4 cells/cm^2) (Fig. 2a). In high-density cultures, a low molecular weight population of E-cadherin appeared in the *Fut8* transfectants, in addition to the band with the same molecular weight as that in mock transfectants. We also established mutated *Fut8* (R365A *Fut8*) which had no enzymatic activity, and examined its transfectants. Western blotting showed that the lower band was not expressed, even in high-density cultures, suggesting that the Fut8 activity was involved in the appearance of the low molecular weight form of E-cadherin (Fig. 2a). It was confirmed that both of the bands in *Fut8* transfectants are expressed at the cell surface of *Fut8* transfectants (Fig. 2b). A lectin blot analysis indicated that both bands in the *Fut8* transfectants reacted with AAL, which binds preferentially to fucose linked α1,6 to GlcNAc although it binds to fucose linked α1,3 to *N*-acetyllactosamine as well (Fig. 2c). The results suggested that the both forms were core-fucosylated. We examined the reactivity toward other lectins such as DSA or ConA, which react with terminal galactose linked β1,6 to GlcNAc residues or mannose, respectively; however, there were no differences between mock and *Fut8* transfectants (data not shown). The mRNA levels of E-cadherin were evaluated by RT-PCR and quantitative real-time PCR. The results indicated that there was no significant difference between the *Fut8* and mock transfectants (data not shown), suggesting that post-translational modification is involved in the increase in E-cadherin expression in the *Fut8* transfectants.

Accumulation of E-cadherin at the cell–cell border in the Fut8 transfected WiDr cells. Morphologically, *Fut8* transfected WiDr cells appeared as clusters with tight cell–cell contacts, whereas mock transfectants had relatively loose contacts. E-cadherin expression was examined immunohistochemically. The *Fut8* transfectants showed more intense fluorescence with condensation at the cell–cell contacts compared to mock transfectants, indicating that the expression of E-cadherin was elevated in *Fut8* transfectants (Fig. 2d).

Increased cell aggregation in the Fut8 transfected WiDr cells. To examine whether increased E-cadherin in Fut8 transfectants functions, we performed a cell aggregation assay. As shown in

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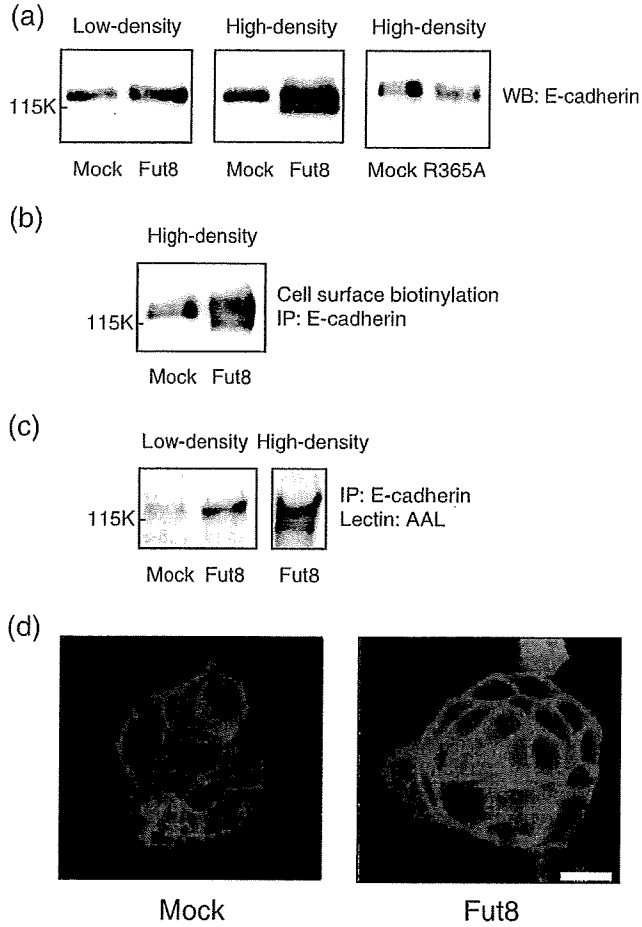

Fig. 2. Effect of α1,6-fucosyltransferase (Fut8) transfection on the characteristics and the expression of E-cadherin in colon carcinoma WiDr cells. (a) Western blotting analysis of E-cadherin in mock, Fut8, and R365A mutated Fut8 transfectants. A whole cell lysate (20 μg) was prepared from low (~3×10^4 cells/cm^2) or high-density (~11×10^4 cells/cm^2) cultures and subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and E-cadherin was detected using an anti-E-cadherin antibody. WB, Western blotting. (b) Cell surface expression of E-cadherin in the mock and Fut8 transfectants. E-cadherin was immunoprecipitated from whole cell lysate of surface biotinylated mock and Fut8 transfectants, and subjected to 8% SDS-PAGE, transferred to nitrocellulose membranes and the biotinylated E-cadherin was visualized using a Vectastain ABC kit and an enhanced chemiluminescence kit. (c) Lectin blot analysis of E-cadherin in the mock and Fut8 transfectants. E-cadherin was immunoprecipitated from 400 μg of whole cell lysate, subjected to 8% SDS-PAGE, and transferred to nitrocellulose membranes, which were probed by *Aleuria aurantia* lectin (AAL). IP, immunoprecipitation. (d) Distribution of E-cadherin in mock and Fut8 transfectants. E-cadherin was detected by a laser scanning confocal microscopy using an anti-E-cadherin antibody, ECCD-2 (scale bar, 10 μm).

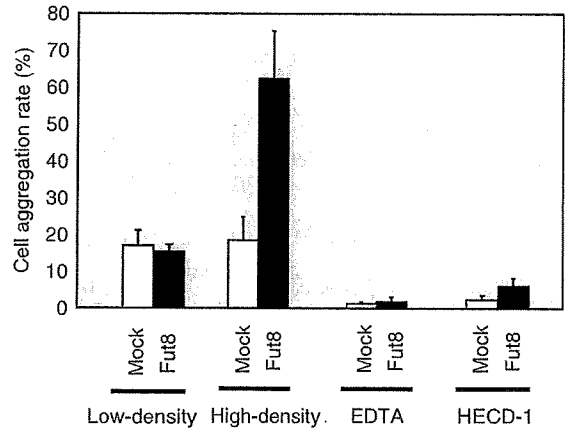

Fig. 3. Effect of α1,6-fucosyltransferase (Fut8) transfection on E-cadherin-dependent cell–cell adhesion in WiDr cells. Cell–cell aggregation was assayed with or without EDTA or the E-cadherin inhibiting antibody, HECD-1, as described in 'Materials and Methods'. Data represent the mean (±SD) of six experiments.

Fig. 3, the cell aggregation rate of the *Fut8* transfectants was significantly higher than that of the mock transfectants under high-density condition (~11×10^4 cells/cm^2), whereas there were no significant differences in aggregation status under low-density condition (~3×10^4 cells/cm^2). This increase in aggregation rate was inhibited in the presence of a calcium chelator, EDTA, or by an anti-E-cadherin monoclonal antibody, indicating that it was dependent on E-cadherin.

Reduction of E-cadherin and cell adhesion in the Fut8 knocked down TGP49 cells. We previously established *Fut8* knocked down mouse pancreatic cancer cells TGP49.[31] The clones showed a low expression of Fut8 and enzymatic activity was not detectable. Lectin blotting confirmed that the two bands of E-cadherin in wild-type cells reacted with AAL lectin, whereas the band in the *Fut8* knocked down cells did not (Fig. 4a). Morphologically, *Fut8* knocked down cells show more loose cell–cell contacts, and the expression levels of E-cadherin at the cell–cell contacts were decreased compared to wild-type cells (Fig. 4b). Consistently, E-cadherin dependent cell aggregation decreased significantly in *Fut8* knocked down cells (Fig. 4c).

Increase in E-cadherin and cell adhesion by restoring Fut8 in Fut8$^{-/-}$ cells. Fut8 deficient kidney epithelial cells were prepared from *Fut8*$^{-/-}$ mice as described previously.[32] The cell surface expression of E-cadherin (Fig. 5a) increased significantly in *Fut8* restored cells compared to *Fut8*$^{-/-}$ cells. Lectin blotting confirmed that the both of the two E-cadherin bands in the *Fut8* restored cells reacted with AAL lectin (Fig. 5b), although the lower band is relatively difficult to be detected. It was considered that the composition of glycosylation of kidney epithelial cells might be different from WiDr colon carcinoma cells. Morphologically, *Fut8*$^{-/-}$ cells show loose cell–cell contacts and *Fut8* restoring rescued them. An immunohistochemical study showed that the expression level of E-cadherin at the cell–cell contacts in *Fut8*$^{-/-}$ cells was also rescued by *Fut8* transfection (Fig. 5c). E-cadherin dependent cell aggregation (Fig. 5d) increased significantly in *Fut8* restored cells compared to *Fut8*$^{-/-}$ cells.

Peptide *N*-glycosidase F (PNGase F) treatment of *N*-glycan of E-cadherin in the Fut8 transfected WiDr cells. To examine the *N*-glycosylation status, *Fut8* and mock transfectants were treated with PNGase F, which cleaves the *N*-glycan between the innermost *N*-acetylglucosamine and asparagines residues. As shown in Fig. 6(a), the upper band of E-cadherin in the *Fut8* transfectant appeared to be *N*-glycosylated to a similar extent as the case of the mock transfectants. On the other hand, no decrease in molecular weight was observed in the PNGase F-treated lower band in the Fut8 transfectants, although the band was reactive with AAL lectin (Fig. 2c), and notably, the molecular weight of the lower band appeared to be even smaller than that of the PNGase-F digest of the upper band in the *Fut8* transfectants. By molecular mass calculation, it was found that the molecular mass of the upper band is around 125 kDa, PNGase F digested upper band is around 110 kDa, and the

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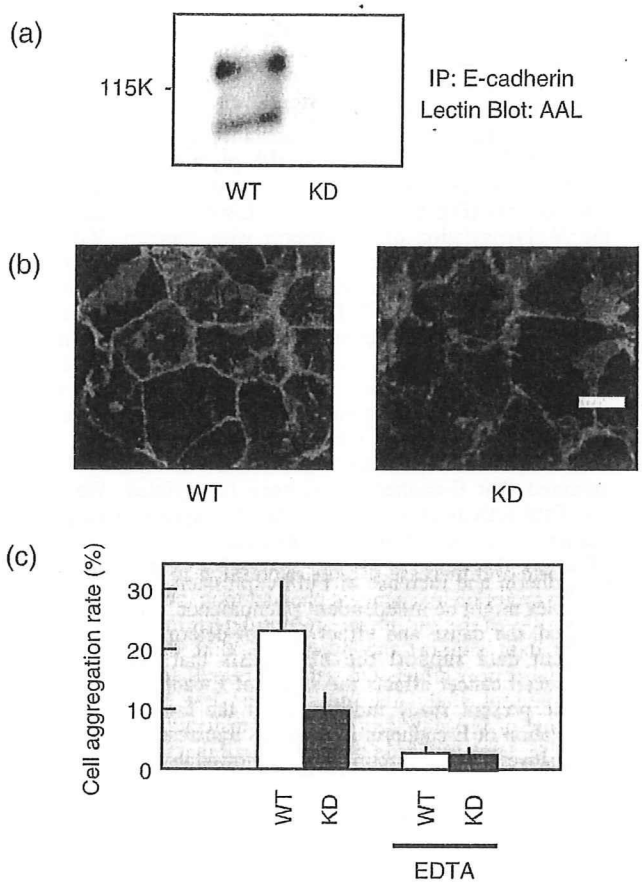

Fig. 4. Changes of E-cadherin expression and E-cadherin-dependent cell–cell adhesion in α1,6-fucosyltransferase (Fut8) knock down cells. (a) Lectin blot analysis of E-cadherin in Fut8 knocked down TGP49 cells and wild-type TGP49 cells. E-cadherin was immunoprecipitated from 400 μg of whole cell lysate, subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes, which were probed by *Aleuria aurantia* lectin (AAL). IP, immunoprecipitation. (b) Distribution of E-cadherin in Fut8 knocked down TGP49 cells and wild-type TGP49 cells. E-cadherin was detected by a laser scanning confocal microscopy using an anti-E-cadherin antibody, ECCD-2. WT, wild-type TGP49 cells; KD, Fut8 knocked down TGP49 cells (scale bar, 10 μm). (c) Cell–cell aggregation was assayed with or without EDTA. Data represent the mean (±SD) of six experiments.

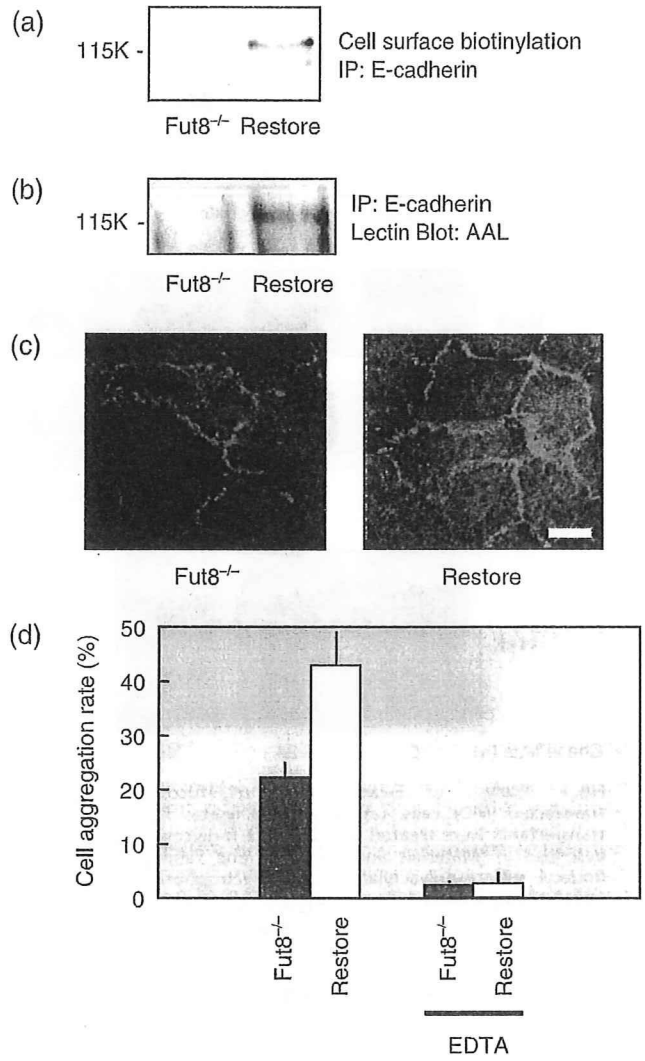

Fig. 5. Changes of E-cadherin expression and E-cadherin-dependent cell–cell adhesion in α1,6-fucosyltransferase (Fut8)$^{-/-}$ cells. (a) Cell surface expression of E-cadherin in the Fut8$^{-/-}$ mouse kidney epithelial cells and Fut8 restored cells. E-cadherin was immunoprecipitated from whole cell lysate of surface biotinylated Fut8$^{-/-}$ cells and Fut8 restored cells, and subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and the biotinylated E-cadherin were visualized using a Vectastain ABC kit and an enhanced chemiluminescence kit. (b) Lectin blot analysis of E-cadherin in the Fut8$^{-/-}$ cells and Fut8 restored cells. E-cadherin was immunoprecipitated from 400 μg of whole cell lysates, subjected to 8% SDS-PAGE and transferred to nitrocellulose membranes, which were probed by *Aleuria aurantia* lectin (AAL). (c) Distribution of E-cadherin on Fut8$^{-/-}$ cells and Fut8 restored cells. E-cadherin was detected by a laser scanning confocal microscopy using an anti-E-cadherin antibody, ECCD-2. Fut8$^{-/-}$, Fut8$^{-/-}$ mouse kidney epithelial cells; Restore, Fut8 restored Fut8$^{-/-}$ cells (scale bar, 10 μm). (d) Cell–cell aggregation was assayed with or without EDTA. Data represent the mean (±SD) of six experiments. IP, immunoprecipitation.

lower band in Fut8 transfectant is around 105 kDa. Together with the lectin blotting results, these results indicate that the E-cadherin corresponding to the lower band contains a form of *N*-glycan that is resistant to PNGase F digestion, such as α1-3 fucosylated *N*-glycan.

Turnover of E-cadherin in the Fut8 transfected WiDr cells. To elucidate the mechanisms by which the low molecular weight population of E-cadherin is produced and the total expression levels of E-cadherin are increased in *Fut8* transfectants, the turnover of E-cadherin was examined in pulse-chase studies. As shown in Fig. 6(b), the turnover rate of E-cadherin in *Fut8* transfectants seemed slightly reduced and the band remained longer time. In this experiment, the upper band and the lower band in the *Fut8* transfectants could not be clearly distinguished. When a long-pulse and long-chase experiment was performed, the low molecular weight form of E-cadherin was detected after 24 h chase but not at 0 h in the *Fut8* transfectants (Fig. 6c), whereas the normal form of E-cadherin appeared 20 min after the pulse (data not shown).

Discussion

Changes in glycosylation status have been implicated in pathological status, especially cancer.[38] We have been studying the functional regulation of signaling molecules by *N*-glycosylation.[36,39–43] E-cadherin plays a central role in cancer

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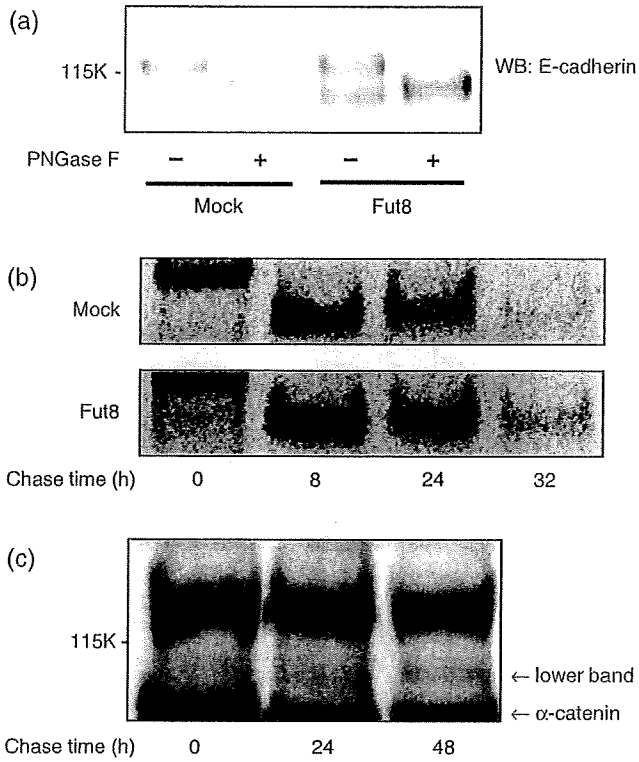

Fig. 6. Analysis of E-cadherin in α1,6-fucosyltransferase (Fut8) transfected WiDr cells. (a) Whole cell lysates from mock and Fut8 transfectants were treated with peptide *N*-glycosidase F (PNGase F) as described in 'Materials and Methods' and subjected to 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane, probed by an anti-E-cadherin antibody. WB, Western blotting. (b) Mock and Fut8 transfectants were radiolabeled with L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min. At 0, 8, 24, and 32 h after pulse labeling, E-cadherin was immunoprecipitated from cell lysates using anti-E-cadherin antibody. After separation of the immunoprecipitated E-cadherin by 6% SDS-PAGE, the gel was dried and autoradiographed for 2 days using imaging plates and a BAS-2500 system. The results were reproducible in three independent experiments. (c) Long-pulse and long-chase study. Fut8 transfectants were radiolabeled with L-[^{35}S]methionine and L-[^{35}S]cysteine for 24 h. At 0, 24, and 48 h after pulse labeling, E-cadherin was immunoprecipitated from cell lysates using anti-E-cadherin antibody. After separation of the immunoprecipitated E-cadherin by 6% SDS-PAGE, the gel was dried and autoradiographed for 2 days using imaging plates and a BAS-2500 system. The results were reproducible in three independent experiments.

metastasis, and it has been reported that its function is also affected by the modification of *N*-glycans.[25,26,44] In this study, we found that Fut8 and E-cadherin expression levels are significantly increased in primary colorectal cancer samples. We established the Fut8 transfectants and examined the E-cadherin. Since Fut8 is widely expressed in human tissues and human cancer cell lines, WiDr cells in which Fut8 expression levels are significantly low, and has malignant potential, were selected as the host cells. We have found that Fut8 activity is involved in the appearance of a low molecular weight population of E-cadherin in high-density culture, and regulates the total amount of cell surface E-cadherin (Fig. 2a,b). E-cadherin expression at cell–cell borders and E-cadherin-dependent cell aggregation were significantly enhanced in the *Fut8* transfectants (Figs 2d and 3). Studies with *Fut8* knocked down cells and *Fut8*$^{-/-}$ cells supported the conclusion that Fut8 activity was closely related to the appearance of low molecular weight population of E-cadherin and total expression levels of E-cadherin (Figs 4 and 5). Since real-time PCR showed that the levels of E-cadherin mRNA were not changed in the *Fut8* transfectants, the expression levels of E-cadherin were most likely up-regulated via a post-translational process. The studies with PNGase F revealed that the glycosylation status was different in the lower band in high-density culture of Fut8 transfectants (Fig. 6a). A study of Liwosz *et al.* indicated that the *N*-glycosylation of E-cadherin with complex *N*-glycans is reduced in dense cultures, and that this change facilitates its association with the actin cytoskeleton, leading to the stabilization of E-cadherin scaffolds.[29] They observed that unstable adherens junctions in sparse cells contained E-cadherin primarily modified with complex *N*-glycans, whereas increased amounts of Triton-insoluble E-cadherin in dense cultures correlated with its modification with high mannose/hybrid oligosaccharides, which are small in size. Since CHO cells and MDCK cells, used as host cells in the report, have quite high Fut8 activity, it can be assumed that E-cadherin was core fucosylated. We propose that Fut8 activity is involved in the glycosylation changes of E-cadherin in dense culture and subsequent alterations in cell–cell adhesion. Appearance of low molecular weight population of E-cadherin and increase of Fut8 expression in colorectal cancer samples might be independent phenomenon, and even if they are related, the cause and effect are not determined. However, the present data support the hypothesis that Fut8 increased in colorectal cancer affects the status of E-cadherin.

The present study indicates that the low molecular weight population of E-cadherin in the Fut8 transfectants was PNGase-F insensitive; however, lectin blotting showed that it was glycosylated and core-fucosylated (Figs 6a and 2c). The results of long-pulse and long-chase studies suggested that the low molecular weight E-cadherin is produced from the normal form (Fig. 6c). Cell–cell adhesions mediated by E-cadherin could activate signaling pathways such as receptor tyrosine kinase signaling or Wnt signaling, and it might be possible that cell–cell adhesion-derived signaling affects the processing of *N*-glycan with core fucose. Changes in glycosylation patterns according to cell density were also observed in our previous work[45] and we consider that cell-cell adhesion-derived signaling is involved in processing of glycoproteins. Thus, it is suggested that the *N*-glycans processing and turnover rate of E-cadherin were altered, which caused the accumulation of low molecular population of E-cadherin and total E-cadherin, in Fut8 transfectants in dense culture. It is also possible that increase in E-cadherin-dependent cell aggregation in Fut8 transfectants is due to enhancement of E-cadherin cell adhesive activity, and we must consider the both possibilities at present.

As seen in Fig. 6a, the PNGase-F treated upper band of E-cadherin seems still larger than the low molecular weight population. It is possible that this occurred as the result of deamidation in the upper band, since SDS-PAGE often reflects amino acid modifications.[46]

We previously reported that E-cadherin turnover is significantly delayed in melanoma cells transfected with *GnT-III*, which is involved in the regulation of branch formation in *N*-glycans.[25] We also showed that the EGFR or Src-mediated tyrosine phosphorylation of β-catenin was decreased in GnT-III transfectants.[26] Guo *et al.* reported that the EGF-induced tyrosine phosphorylation of β-catenin and P120^{ctn} increased in GnT-V transfectants, which led to a reduction in cell–cell adhesion.[27] The fact that GnT-III activity was not changed by *Fut8* transfection suggests that Fut8 activity regulates the expression of E-cadherin independent of GnT-III, and the accumulation of both normal form and the low molecular weight E-cadherin enhances cell–cell adhesion.

It has been revealed that core fucosylation catalyzed by Fut8 is involved in various biological phenomena. *Fut8* transgenic

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mice caused steatosis in the liver and kidney due to a decreased lysosomal acid lipase activity.[47] Core fucose deficient IgG1 showed an improved binding to FcγRIIIA, and consequently, antibody-dependent cellular cytotoxicity activity was up-regulated.[48,49] We developed *Fut8*$^{-/-}$ mice and reported that the mice showed semi lethality, growth retardation, and emphysema-like changes, and the experiments indicated that dysfunction of TGF-β1 receptor[32], impairment in the low-density LRP-1[41] and functional changes of α3β1 integrin[50] are involved in the phenomena. The regulation of the cell surface expression levels of E-cadherin, reported herein, could be involved in the pathology observed in *Fut8* transgenic mice and *Fut8* knock out mice. Metastasis analysis using those animals would indicate the role of Fut8 in cell–cell adhesion *in vivo*.

Acknowledgments

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Plasma levels of C-reactive protein and serum amyloid A and gastric cancer in a nested case–control study: Japan Public Health Center-based prospective study

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Gastric carcinogenesis may be under the combined influence of factors related to the host, *Helicobacter pylori* bacterial virulence and the environment. One possible host-related factor is the inflammatory or immune response. To clarify this point, we investigated the association between plasma levels of C-reactive protein (CRP) and serum amyloid A (SAA) and the subsequent risk of gastric cancer in a population-based nested case–control study. Subjects were observed from 1990 to 2004. Among 36 745 subjects who answered the baseline questionnaire and provided blood samples, 494 gastric cancer cases were identified and matched to 494 controls for our analysis. The overall distribution of CRP and SAA was not apparently associated with the development of gastric cancer. However, a statistically significant increased risk was observed when subjects were categorized dichotomously. The adjusted odds ratio (OR) for the development of gastric cancer for the CRP-positive group (CRP > 0.18 mg/dl) compared with the CRP-negative group was 1.90 [95% confidence interval (CI): 1.19–3.02, $P = 0.007$]. The OR for the SAA-positive group (SAA > 8 μg/ml) compared with the SAA-negative group was 1.93 (95% CI: 1.22–3.07, $P = 0.005$). In conclusion, our results suggest that those who react strongly to inflammation or who have a high host immune response, as reflected by extremely elevated plasma levels of CRP and SAA, are at a high risk to develop gastric cancer.

Introduction

It is well established that cancer arises in chronically inflamed tissue, and one of the classic examples is *Helicobacter pylori*-associated gastric cancer (1). *Helicobacter pylori* persistently colonizes the gastric mucosa, leading to chronic inflammation, atrophic gastritis and, finally, gastric cancer. There are high interindividual differences in the extent of gastric inflammation among *H.pylori*-infected subjects, and only a small proportion of them develop clinical consequences. This indicates that gastric carcinogenesis may be under the combined influence of factors related to the host, bacterial virulence and the environment. One possible host-related factor is the inflammatory or immune response. Many studies have reported an association between serum proinflammatory cytokines [e.g. interleukin (IL)-6, IL-8 and IL-1β] levels (2–4) or polymorphisms (such as IL-1, IL-2 and IL-8) and gastric cancer risk (5–8), but the results are controversial. The lack of consensus may be partly due to the nature of cytokines, which are components of a large, complex signaling network, and difficulties in measuring their levels and interactions. Measurement of cytokines in plasma is difficult because of their short plasma half-lives and the presence of blocking factors (9). Additionally, combinations of cytokines have been found to have additive, inhibitory or synergistic effects. Therefore, more useful or systematic indicators of host inflammatory or immune response are needed.

C-reactive protein (CRP) is a well-established indicator of inflammation in the body (10). It is an acute-phase reactant that reflects low-grade systemic inflammation and has been studied in a variety of cardiovascular diseases. CRP production by the liver is regulated by cytokines, principally IL-6 and tumor necrosis factor α, which is the main trigger for the production of IL-6 by a variety of cells. In fact, strong positive associations between IL-6, tumor necrosis factor α and CRP were observed (11). Serum amyloid A (SAA) is another major acute-phase reactant. It is a putative serum precursor of the amyloid A protein, which constitutes amyloid fibrils in secondary amyloidosis and is an apolipoprotein associated with the high density lipoprotein 3 fraction of serum (12). In most studies, a parallel increase of SAA and CRP has been observed, although some studies have delineated acute-phase SAA as the more sensitive parameter (13,14). Therefore, to indicate the host inflammatory or immune response systematically, CRP and SAA may be useful markers.

In this large-scale nested case–control study, we aimed to examine whether the host inflammatory or immune response has any association with the development of gastric cancer. To clarify this point, we explored the relation of plasma levels of CRP and SAA to risk of developing gastric cancer. As far as we know, this is the first study to prospectively seek this association in a population.

Materials and methods

Study population

The Japan Public Health Center-based prospective study (JPHC Study) is an ongoing cohort study to investigate cancer, cardiovascular disease and other lifestyle-related diseases. The first group (Cohort I) of the JPHC Study was started in 1990 and the second group (Cohort II) in 1993 (15). The JPHC Study included 140 420 subjects (68 722 men and 71 698 women), defined as all inhabitants in the study areas [27 cities, towns or villages served by 11 public health centers (PHCs)] who were 40–59 years old (Cohort I) or 40–69 years old (Cohort II). Among the study subjects, those registered at one PHC area in Cohort I were excluded from the present analysis because data on cancer incidence were not available. Additionally, one subcohort in Cohort II was excluded because the selection of subjects differed from that of other cohort subjects, i.e. random sampling of residents from a municipality population registry for one city, stratified by 10 year age–gender groups. We thus defined 123 576 subjects (61 009 men and 62 567 women) for the present study. The JPHC Study was approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

Baseline survey

In 1990 for Cohort I and in 1993–1994 for Cohort II, subjects were asked to reply to a lifestyle questionnaire that covered sociodemographic characteristics, medical history, smoking and drinking habits, diet and so on. Details of the food frequency questionnaire included in the baseline survey have been described previously (16). A total of 99 808 (81%) subjects—47 525 men and 52 283 women—responded to the questionnaires.

We excluded subjects who self-reported cancer at baseline ($n = 2136$), those who were not Japanese ($n = 18$) and those who were later discovered to have moved away at baseline ($n = 11$). This left 97 644 eligible subjects (46 803 men and 50 841 women). Among them, 36 745 subjects (38%; 13 467 men and 23 278 women) donated blood samples at health checkups conducted by the PHC in each area. Each subject voluntarily provided 10 ml of blood during the health checkups. As customary, subjects were asked to avoid having a meal later than 21:00 on the day before the examination. The last time of either consuming a meal or drinking water or tea was recorded. The plasma and buffy layer were divided into four tubes, with each tube holding 1.0 ml (3 tubes for plasma and 1 for the buffy layer) and stored at 80°C. Blood was collected from 1990 to 1992 in Cohort I and from 1993 to 1995 in Cohort II.

Follow-up and identification of gastric cancer

In Japan, at the time the study was conducted, a PHC played a role as an organization that provided primary health care, including health checkups,

Abbreviations: BMI, body mass index; CagA, cytotoxin-associated gene A; CI, confidence interval; CRP, C-reactive protein; ICD-O, International Classification of Diseases for Oncology; Ig, immunoglobulin; IL, interleukin; JPHC, Japan Public Health Center; OR, odds ratio; PG, pepsinogen; PHC, public health center; SAA, serum amyloid A.

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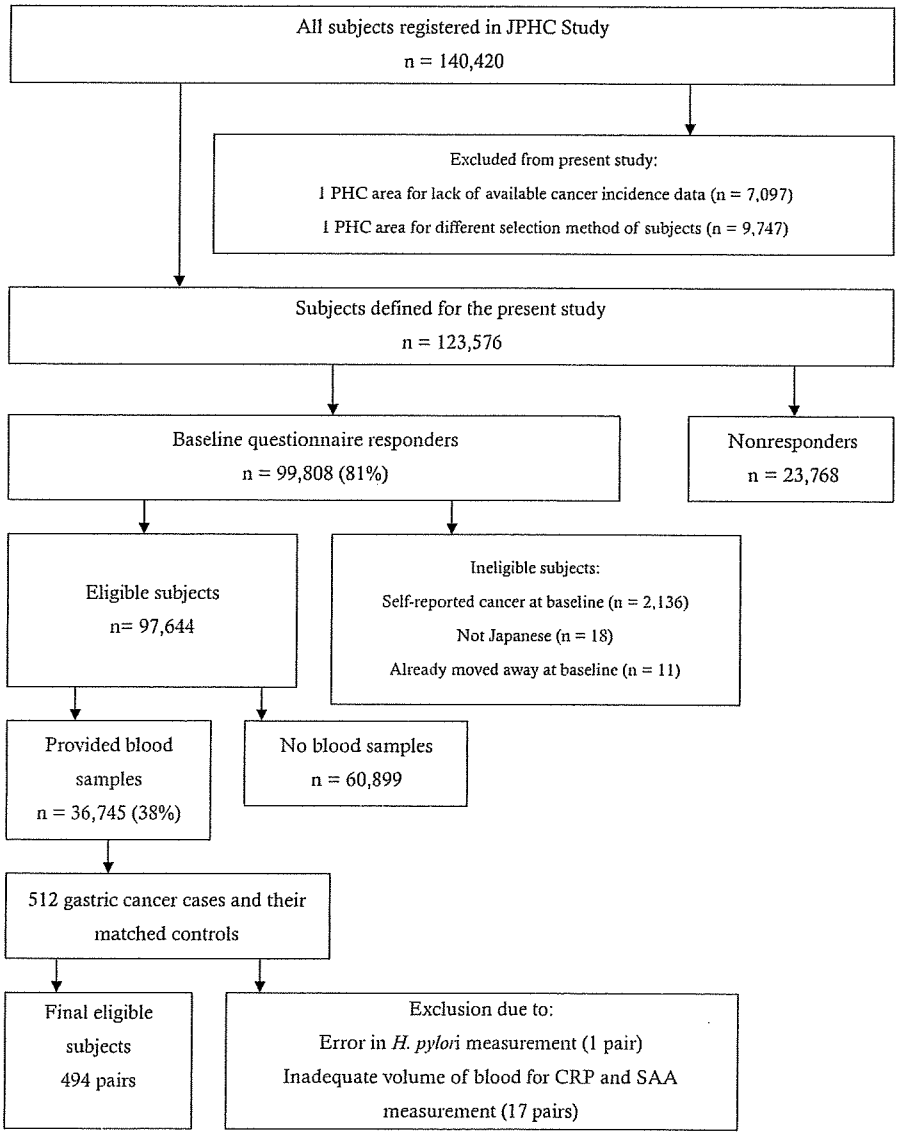

Fig. 1. Flow of study population.

or other health promotion activities for all inhabitants of the municipalities supervised by the PHC. In this study, the main role of the PHC was to collect and report data on mortality, relocation and cancer cases.

Death and relocation

We observed study subjects until 31 December 2004. The changes in residency status, including death, were identified annually through the residential registry in each area. To confirm causes of death, we used mortality data from the Ministry of Health, Labour and Welfare. Residence and death registration are required by law in Japan, and the registries are believed to be complete. Among 36 745 study subjects, 1423 (3.9%) moved away from the study area, 1610 (4.4%) died and 11 (0.03%) were lost to follow-up within the study period.

Cancer registry for JPHC Study

Data on newly diagnosed cases of cancer were collected from two sources: active patient notification from the local major hospitals in the study area and data linkage with population-based registries (usually prefecture-wide). Death certificate information was used as a supplementary information source. In our cancer registry system, the proportion of cases of gastric cancer for which information was based on death certificate notification was 7.6% and on information available from death certificates only was 2.1%. This level of quality for the information was considered satisfactory for the present study.

Identification of gastric cancer and selection of control subjects

Cases of gastric cancer were extracted from the cancer registry for the JPHC Study on the basis of site [International Classification of Diseases for Oncology (ICD-O) code C160–169] (17). Up to the end of the study period, 512 new gastric cancer cases were identified. Until quite recently in Japan, the upper third of the stomach has been called the 'cardia' on the basis of the guidelines for gastric cancer classification (18). Because it seemed difficult to distinguish the cardia, which is mainly located in the esophagogastric junction, from the upper third of the stomach, we combined tumors at these sites into one group for analysis (ICD-O code C160–161). A tumor located on the lower side of the stomach was classified as distal gastric cancer (ICD-O code C162–167). Subsites that could not be classified because of a diffuse lesion (ICD-O code C168) or those with no information (ICD-O code C169) were categorized as an unclassified subsite. Histologic classification was based on one author's (S.S.) review, in consultation with a pathologist, of the record reported by each hospital. The subdivisions were made on the basis of a classification derived by Lauren (19). For each case, one control was selected from subjects who had no history of gastric cancer and who lived in the study area when the case was

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diagnosed. Each control was matched to a case for gender, age (±3 years), PHC area, blood donation date (±2 months) and fasting time at blood donation (±5 h). Because of a technical error in measurement of *H.pylori* and inadequate volume of blood available for CRP and SAA measurements, 1 case with its matched control and another 17 pairs (8 cases with their matched controls and 10 controls with their matched cases) were excluded. Finally, we had 494 sets each of cases and controls for use in the present analysis. A flowchart of the study subjects is provided in Figure 1.

Laboratory analysis

CRP and SAA concentrations were determined by the latex agglutination nephelometric immunoassay test (LZ test 'Eiken' CRP-HG; Eiken Kagaku Co. Ltd, Tokyo, Japan; and LZ test 'Eiken' SAA; Eiken Kagaku Co. Ltd, respectively). For the CRP test, based on 10 replicated measurements of three concentrations of blood samples (0.07, 0.50 and 4.41 mg/dl) at the time of analyses, the coefficients of variation were 1.69%, 0.59% and 0.76%, respectively. For SAA, 10 replicated measurements of two concentrations of blood samples (22 μg/ml and 110 μg/ml) yielded a coefficient of variation of reproducibility values of 1.53% and 1.17%. Normal values for the examined parameters were <0.18 mg/dl for CRP and <8 μg/ml for SAA according to the kit's protocol. Both cutoff values were based on data from reports for the same kit. The cutoff value of CRP was set by the iterative truncation method among 478 health checkup samples (20). In brief, after repeated deletion of outliers, mean ± 1.96 SD was considered the normal range. For SAA, after being converted to a logarithm, the value was set as the upper 95th percentile of the distribution of 1056 normal subjects (0–70 years old) (21).

Immunoglobulin (Ig) G antibodies to *H.pylori* were measured with a direct enzyme-linked immunosorbent assay kit (E Plate 'Eiken' *H.pylori* Antibody; Eiken Kagaku Co. Ltd). Levels of IgG were categorized as seropositive and seronegative for *H.pylori* according to a selective cutoff value (≤10 or >10). The cutoff value was based on the results of sensitivity and specificity calculated with the urea test, which is the gold standard (report by company). Assays of cytotoxin-associated gene A (CagA) were performed with the use of an enzyme-linked immunosorbent assay kit, in which horseradish peroxidase was used as the enzyme tracer (CagA IgG EIA; Sceti Co. Ltd, Rome, Italy). According to the manufacturer's protocol, samples with IgG values ≤10 RU/ml must be considered non-reactive for anti-CagA IgG antibodies; samples with IgG values within 10–15 RU/ml must be considered weakly reactive and samples with IgG values >15 RU/ml must be considered reactive for anti-CagA IgG antibodies. With regard to interpretation of these results, reactive and/or questionable samples are considered positive for anti-CagA IgG antibodies, i.e. values >10 are regarded as CagA positive. Serum levels of pepsinogen I and II (PGI and PGII, respectively) were measured by commercial kits based on a two-step enzyme immunoassay (E Plate 'Eiken' Pepsinogen I; Eiken Kagaku Co. Ltd; and E Plate 'Eiken' Pepsinogen II; Eiken Kagaku Co. Ltd). Results were defined as 'atrophic' when the criteria of both PGI level ≤70 ng/ml and PGI : PGII ratio ≤3.0 were fulfilled. Comparing the PG levels between gastric cancer cases and healthy controls retrospectively, Miki (22) reported that applying a PGI level ≤70 ng/ml and a PGI : PGII ratio ≤3.0 as cutoff values was most effective in distinguishing cases from controls. Using these criteria, other authors have showed an extremely high correlation (r = 0.999) between atrophy and age-adjusted gastric cancer mortality among inhabitants of five areas in Japan (23). Among atrophic cases, more severe cases with a PGI level ≤30 ng/ml and PGI : PGII ratio ≤2.0 were defined as severe atrophy.

All measurements were conducted by a person blinded to the case–control situation.

Statistical analysis

Statistical analysis included chi-square test, analysis of variance, analysis of covariance and conditional logistic model. Multiple conditional logistic regression analyses were conducted to control for potential confounding factors. For cardia cancer, smoking status, alcohol consumption (for SAA analysis), intake of salt, body mass index (BMI), family history of gastric cancer, history of infectious or inflammatory disease (i.e. cardiovascular disease, ischemic heart disease, liver disease and kidney disease) and current use of analgesics for lumbago, neuralgia, common cold, arthrosis and joint pain were controlled. For all gastric cancer, all non-cardia cancer, differentiated-type non-cardia cancer and undifferentiated-type non-cardia cancer further adjustment was applied for *H.pylori* infection, atrophy and CagA seropositivity. Smoking status was divided into four groups: never smoker, past smoker, current smoker with <20 cigarettes per day and current smoker with ≥20 cigarettes per day). Alcohol consumption was defined as drinker (>1 day/week) and non-drinker (<1 day/week). BMI was categorized into three groups so that each category included an approximately equal number of controls. Salt was treated as a continuous variable. Family history of gastric cancer was regarded as positive if at least one parent or sibling had gastric cancer. CRP and SAA status (positive/negative) were determined according to the protocol's normal value. Additionally, the non-linear continuous models of the association between CRP and SAA and gastric cancer risk were tested by PROC GAM. Odds ratios (ORs) were calculated relative to the cutoff points of CRP and SAA. Because the distribution was skewed, log transformation was conducted for CRP, SAA, *H.pylori* titer, CagA titer, PGI level and PGII and PGI : PGII ratio, which altered the distribution close to normal in comparisons of the mean values between groups.

Reported P-values were two sided, and all statistical analyses were done with SAS software version 9.1 (SAS Institute Inc., Cary, NC).

Results

Baseline characteristics of cases and controls are shown in Table I. Among listed factors, predominance of *H.pylori* positivity, CagA status, atrophy and family history of gastric cancer were apparent in cases compared with controls.

Table II summarizes the distribution of lifestyle factors and plasma biomarkers according to the CRP and SAA status among controls. Forty-seven (9.5%) and 63 (12.8%) subjects met the criteria for being positive for plasma CRP and SAA, respectively. For CRP status, no factors were differently distributed other than SAA levels; the mean value of SAA among CRP-positive subjects was >10 times that of CRP-negative subjects ($P < 0.0001$). Plasma CRP level among SAA-positive subjects was 13 times that among SAA-negative subjects ($P < 0.0001$). Correlation of the log-transformed CRP and SAA was 0.55 ($P < 0.0001$). Mean daily salt intake was higher in SAA-negative subjects compared with SAA-positive subjects. This may be due to the predominance of male gender and alcohol consumption among SAA-negative subjects, which contribute to high salt intake. When gender and alcohol consumption were adjusted (analysis of covariance), the difference in salt intake was no longer significant ($P = 0.40$). Compared with positive subjects, SAA-negative subjects had a significantly higher *H.pylori* titer against IgG antibody and more frequent distribution of male gender, alcohol consumption, *H.pylori* positivity and atrophy.

Table I. Baseline characteristics of cases and controls

	Case	Control	P-value[a]
n	494	494	
Age	57.3 (0.3)	57.3 (0.3)	Matching value
Men (%)	329 (66.6%)	329 (66.6%)	Matching value
Cigarette smoking			
Never smoker (%)	228 (46.2%)	245 (49.6%)	
Past smoker (%)	91 (18.4%)	98 (19.8%)	
Current smoker with <20 cigarettes per day (%)	133 (26.9%)	109 (22.1%)	
Current smoker with ≥20 cigarettes per day (%)	42 (8.5%)	42 (8.5%)	0.35
Alcohol consumption			
Never or occasional (%)	245 (49.6%)	244 (49.4%)	
≥1 day, <300 g/week (%)	187 (37.9%)	203 (41.1%)	
≥1 day, ≥300 g/week (%)	62 (12.6%)	47 (9.5%)	0.26
BMI			
<25	396 (80.2%)	369 (74.7%)	
25–29.9	89 (18.0%)	113 (22.9%)	
≥30	9 (1.8%)	12 (2.4%)	0.12
Family history of gastric cancer (%)	60 (12.2%)	40 (8.1%)	0.03
Salt (g/day)	5.3 (0.1)	5.1 (0.1)	0.40
Helicobacter pylori positive (%)[b]	463 (93.7%)	371 (75.1%)	<0.0001
Helicobacter pylori positive (%)[c]	489 (99.0%)	445 (90.1%)	<0.0001
CagA (+) (%)	375 (75.9%)	346 (70.0%)	0.04
Atrophy (%)	406 (82.2%)	285 (57.7%)	<0.0001

Values are mean (SE) except where specified otherwise.
[a]Based on chi-square test or analysis of variance.
[b]Based on IgG antibody.
[c]Based on CagA positive and/or *Helicobacter pylori* IgG antibody positive.

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Table II. Distribution of lifestyle factors and plasma biomarkers according to CRP and SAA status among control

	CRP status			SAA status		
	Negative (CRP ≤ 0.18 mg/dl)	Positive (CRP > 0.18 mg/dl)	*P*-value[a]	Negative (SAA ≤ 8 μg/ml)	Positive (SAA > 8 μg/ml)	*P*-value[a]
n	447	47		431	63	
Age	57.1 (0.3)	58.6 (1.1)	0.20	57.2 (0.3)	58.1 (0.9)	0.35
Men (%)	296 (66.2%)	33 (70.2%)	0.58	296 (68.7%)	33 (52.4%)	0.01
BMI						
<25	336 (75.2%)	33 (70.2%)		327 (75.9%)	42 (66.7%)	
25–29.9	100 (22.4%)	13 (27.7%)		93 (21.6%)	20 (31.8%)	
≥30	11 (2.5%)	1 (2.1%)	0.71	11 (2.6%)	1 (1.6%)	0.19
Cigarette smoking						
Never smoker (%)	225 (50.3%)	20 (42.6%)		207 (48.0%)	38 (60.3%)	
Past smoker (%)	91 (20.4%)	7 (14.9%)		90 (20.9%)	8 (12.7%)	
Current smoker with <20 cigarettes per day (%)	97 (21.7%)	12 (25.5%)		98 (22.7%)	11 (17.5%)	
Current smoker with ≥20 cigarettes per day (%)	34 (7.6%)	8 (17.0%)	0.12	36 (8.4%)	6 (9.5%)	0.23
Alcohol consumption						
Never or occasional (%)	218 (48.8%)	26 (55.3%)		204 (47.3%)	40 (63.5%)	
≥1 day, <300 g/week (%)	184 (41.2%)	19 (40.4%)		185 (42.9%)	18 (28.6%)	
≥1 day, ≥300 g/week (%)	45 (10.1%)	2 (4.3%)	0.39	42 (9.7%)	5 (7.9%)	0.05
Family history of gastric cancer (%)	36 (8.1%)	4 (8.5%)	0.91	36 (8.4%)	4 (6.4%)	0.85
Salt (g/day)	5.2 (0.1)	4.9 (0.3)	0.43	5.2 (0.1)	4.6 (0.3)	0.04
CRP (mg/dl)/SAA (μg/ml)[b]	3.6 (1.8)	38.6 (5.6)	<0.0001[a]	0.05 (0.03)	0.65 (0.07)	<0.0001[a]
Helicobacter pylori positive (%)[c]	338 (75.6%)	33 (70.2%)	0.42	332 (77.0%)	39 (61.9%)	0.01
Helicobacter pylori positive (%)[d]	403 (90.2%)	42 (89.4%)	0.86	390 (90.5%)	55 (87.3%)	0.43
Helicobacter pylori titer	43.9 (2.3)	36.1 (7.1)	0.31[e]	44.0 (2.3)	37.1 (6.1)	0.02[e]
CagA (+) (%)	314 (70.3%)	32 (68.1%)	0.76	302 (70.1%)	44 (69.8%)	0.97
CagA titer	85.1 (4.2)	74.7 (12.9)	0.72[e]	84.6 (4.3)	80.8 (11.1)	0.82[e]
PGI	28.6 (0.8)	29.7 (2.5)	0.55[e]	28.5 (0.8)	30.1 (2.1)	0.52[e]
PGII	11.2 (0.3)	10.8 (1.0)	0.60[e]	11.2 (0.3)	11.0 (0.8)	0.71[e]
PGI : PGII	3.5 (0.6)	2.9 (1.8)	0.83[e]	3.5 (0.6)	3.2 (1.6)	0.28[e]
Atrophy (%)	260 (58.2%)	25 (53.2%)	0.51	256 (59.4%)	29 (46.0%)	0.04
Severe atrophy (%)	122 (27.3%)	9 (19.2%)	0.23	119 (27.6%)	12 (19.1%)	0.15

Values are mean (SE) except where specified otherwise.
[a]Based on chi-square test or analysis of variance.
[b]Mean plasma CRP level for SAA status and mean plasma SAA level for CRP status.
[c]Based on IgG antibody.
[d]Based on CagA positive and/or *Helicobacter pylori* IgG antibody positive.
[e]Based on analysis of variance of log biomarkers.

In Table III, ORs and 95% confidence intervals (CIs) of CRP positivity for development of gastric cancer are presented by tumor subsite and histologic types. CRP ranged from 0 to 19.1 mg/dl (mean: 0.14 mg/dl, median: 0.033 mg/dl) among cases and from 0 to 9.3 mg/dl (mean: 0.13 mg/dl, median: 0.032 mg/dl) among controls. The risk of developing gastric cancer increased by ~36% among those who were CRP positive; the crude OR equaled 1.36 (95% CI: 0.91–2.02, $P = 0.13$), although with no significance. After being adjusted for potential confounding variables, the point estimate altered substantially and reached the level of statistical significance; the adjusted OR equals 1.90 (95% CI: 1.19–3.02, $P = 0.007$). Among the adjusted covariates, *H.pylori* infection contributed the most to the elevation of risk; adding only *H.pylori* infection to the model elevated the OR to 1.67, which was much higher than the OR for adding CagA seropositivity (adjusted OR = 1.39), atrophy (adjusted OR = 1.48) or even all other lifestyle factors [i.e. cigarette smoking, BMI, family history, history of infectious or inflammatory disease, current drug use of analgesics and salt intake (adjusted OR = 1.47)]. When the cancers were stratified by tumor location and histologic type, the largest OR was demonstrated for cardia cancers, but it failed to reach statistical significance; adjusted OR equaled 3.14 (95% CI: 0.51–19.39, $P = 0.22$). Among non-cardia cancers, the association did not differ much by histologic type. When the analyses were repeated with subjects divided into quartiles according to control distribution of the CRP level (<0.012, 0.012–0.032, 0.032–0.081 and ≥0.081 mg/dl), no apparent association was observed. Compared with the lowest (reference) group, the adjusted ORs (95% CIs) for development of gastric cancer for the second, the third and the highest group were 0.85 (0.56–1.29), 0.96 (0.62–1.47) and 1.35 (0.88–2.07), respectively (P for trend = 0.0496). When CRP was treated as a continuous measure, the adjusted OR for development of gastric cancer was 1.06 (0.87–1.28), for 1 mg/dl increase of log-transformed CRP. Furthermore, non-linear continuous models did not reveal any evidence of dose response.

SAA among cases and controls ranged from 0 to 319.7 μg/ml (mean: 5.9 μg/ml, median: 2.6 μg/ml) and from 0 to 847.5 μg/ml (mean: 7.0 μg/ml, median: 2.5 μg/ml), respectively. For SAA positivity, about a 2-fold increased risk was observed for total gastric cancer and non-cardia cancer; the adjusted ORs (95% CIs) were 1.93 (1.22–3.07, $P = 0.005$) and 2.13 (1.14–3.98, $P = 0.02$), respectively (Table IV). Among adjusted covariates, atrophy as well as *H.pylori* infection contributed most of the elevation of risk. Among non-cardia cancers, no difference was observed by histologic type. The largest OR was demonstrated for cardia cancers, although it failed to reach the level of statistical significance; the adjusted OR equaled 3.84 (95% CI: 0.82–17.99, $P = 0.09$). When results for SAA status were shown separately for men and women, there was no material difference; the adjusted ORs for developing total gastric cancer were 1.95 and 2.15 for men and women, respectively. The adjusted OR for cardia cancer among women could not be calculated because of the small sample size; therefore, all analyses were conducted for men and women combined. No apparent association was observed when SAA

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Table III. ORs and 95% CIs of CRP positivity (CRP > 0.18 mg/dl) for development of gastric cancer by tumor subsite and histologic type

	No. of CRP-positive cases/ controls	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)[a]	*P*-value
All (494 pairs)	62/47	1.36 (0.91–2.02)	0.13	1.90 (1.19–3.02)	0.007
Cardia (39 pairs)	7/2	3.50 (0.73–16.85)	0.12	3.14 (0.51–19.39)	0.22
Non-cardia (355 pairs)	44/33	1.36 (0.85–2.16)	0.20	2.18 (1.24–3.84)	0.007
Differentiated type (232 pairs)	30/23	1.32 (0.76–2.29)	0.33	1.77 (0.89–3.52)	0.10
Undifferentiated type (107 pairs)	9/8	1.14 (0.41–3.15)	0.80	2.01 (0.53–7.62)	0.30

[a]Cardia cancers, adjusted for cigarette smoking, BMI, family history of gastric cancer, history of infectious or inflammatory disease, current drug use of analgesics and salt intake. All gastric cancers, all non-cardia cancers, differentiated-type non-cardia cancer and undifferentiated-type non-cardia cancer, further adjusted for *Helicobacter pylori* infection, CagA positivity and atrophy.

Table IV. ORs and 95% CIs of SAA positivity (SAA > 8 μg/ml) for development of gastric cancer by tumor subsite and histologic type

	No. of SAA-positive cases/ controls	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)[a]	*P*-value
All (494 pairs)	75/63	1.26 (0.86–1.86)	0.24	1.93 (1.22–3.07)	0.005
Cardia (39 pairs)	11/5	3.00 (0.81–11.08)	0.10	3.84 (0.82–17.99)	0.09
Non-cardia (355 pairs)	45/39	1.21 (0.74–2.00)	0.45	2.13 (1.14–3.98)	0.02
Differentiated type (232 pairs)	27/25	1.11 (0.59–2.06)	0.75	1.73 (0.81–3.72)	0.16
Undifferentiated type (107 pairs)	12/11	1.14 (0.41–3.15)	0.80	1.80 (0.41–7.92)	0.44

[a]Cardia cancers, adjusted for cigarette smoking, alcohol consumption, BMI, family history of gastric cancer, history of infectious or inflammatory disease, current drug use of analgesics and salt intake. All gastric cancers, all non-cardia cancers, differentiated-type non-cardia cancer and undifferentiated-type non-cardia cancer, further adjusted for *Helicobacter pylori* infection, CagA positivity and atrophy.

level was divided into quartiles (<1.3, 1.3–2.5, 2.5–5.1 and ≥5.1 μg/ml). Compared with the lowest (reference) group, the adjusted ORs (95% CIs) for development of gastric cancer for the second, the third and the highest group were 0.81 (0.53–1.24), 1.06 (0.70–1.61) and 1.19 (0.77–1.85), respectively ($P = 0.20$). When SAA was treated as a continuous measure, the adjusted OR for development of gastric cancer was 1.00 (0.995–1.00) for 1 mg/dl increase of log-transformed SAA. Similar to the analysis of CRP, non-linear continuous models did not reveal any evidence of dose response.

Because of the high correlation between CRP and SAA, we included only the values for the marker being analyzed (Tables III and IV). When CRP and SAA were included in the model simultaneously, the OR was attenuated and was no longer significant for CRP, but was still significant for SAA (data not shown). This may not contradict previous reports that suggest overlapping of the roles of the two markers and delineation of SAA as the more sensitive parameter (13,14).

The observed association did not differ for stratification by smoking status (never/past + current) for SAA; however, for CRP, the association was clearer among never smokers [2.50 (1.13–5.53)] compared with past and current smokers [1.15 (0.56–2.33)]. Using the World Health Organization category to adjust BMI did not alter the results essentially. When the interactions between each covariate in the model and CRP and SAA status were tested, no significant interaction was observed.

When all analyses were repeated in only those who were *H.pylori* positive (seropositive for IgG antibody and/or CagA), the associations were slightly attenuated, although they did not differ essentially; the adjusted ORs (95% CIs) for developing total gastric cancer were 1.72 (1.07–2.78, $P = 0.03$) for CRP-positive status and 1.82 (1.13–2.94, $P = 0.01$) for SAA-positive status, respectively.

Discussion

In this study, the overall distributions of CRP and SAA were not apparently associated with the development of gastric cancer. However, when subjects were divided on the basis of dichotomous categorization of positive versus negative, an increased risk was observed for positive subjects. The association was statistically significant even after adjustment for *H.pylori* infection, CagA status, atrophy and lifestyle factors. Elevated levels of CRP and SAA reflect a generalized host reaction that is either localized or systematic with regard to the initial event. Mechanisms of inflammation-associated tumor development are well described. These include stimulation of cellular proliferation (e.g. in cellular proto-oncogenes, DNA and cellular repair), inhibition of apoptosis, cellular adhesion, stimulation of angiogenesis and cellular transformation (1). In our data set, under the conditions that most subjects were infected with *H.pylori*, only those who reacted strongly to inflammation or had a high host immune response, as reflected by extremely elevated plasma levels of CRP and SAA, showed an elevated risk of developing the malignancy. The proportions of those who were categorized as positive were small; therefore, the findings should be interpreted with caution. However, this may be one of the explanations for why only a small proportion of *H.pylori*-infected subjects develop clinical consequences. CRP and SAA were useful markers to detect these high-risk groups.

Several clinical studies have shown that, compared with controls, gastric cancer patients have elevated CRP levels (24–26). Previous studies have even revealed that CRP has an impact on gastric cancer prognosis (24,27). It has been observed in previous studies that the SAA level increases in patients with stomach, lung, renal, colorectal, breast and other forms of cancers (28–35). With regard to gastric cancer, Chan *et al.* (28) demonstrated that patients with gastric cancer have higher SAA concentrations than do patients with gastric ulcers and healthy subjects and that levels of SAA correlate with tumor status, prognosis and recurrence. In our study, the average duration between blood donation and cancer diagnosis among cases was 5.4 years. When subjects who developed gastric cancer within 2 years of blood donation and their matched controls were excluded, the observed associations were strengthened; the adjusted ORs (95% CIs) for the association between development of gastric cancer and CRP and SAA positivity were 2.25 (1.31–3.85, $P = 0.003$) and 2.29 (1.32–3.95, $P = 0.003$), respectively. Furthermore, when subjects were stratified by the median duration between blood donation and

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diagnosis (5.12 years), the adjusted ORs (95% CIs) for the association between development of gastric cancer CRP and SAA positivity within 5.12 years were 1.38 (0.69–2.73, $P = 0.36$) and 1.59 (0.82–3.09, $P = 0.17$), respectively. The values for CRP and SAA diagnosed after 5.12 years were 2.42 (1.23–4.77, $P = 0.01$) and 2.25 (1.12–4.52, $P = 0.02$), respectively. Therefore, our findings cannot be explained by the effect of preclinical samples among cases. Rather, our findings suggest that CRP and SAA may be useful markers for predicting the malignancy.

In our study, *H.pylori* seropositivity, *H.pylori* titer and atrophy were not distributed differently according to CRP status. Surprisingly, *H.pylori* seropositivity and atrophy were more frequent, and higher *H.pylori* titer was observed among SAA-negative subjects than among SAA-positive subjects. When the values were compared on the basis of tumor location, CRP did not show any difference; mean value (SE) was 0.09 (0.16) for cardia and 0.15 (0.05) for non-cardia cancer, respectively ($P = 0.75$). The value for SAA was 6.77 (1.87) for cardia, which was higher than that for non-cardia, 5.12 (0.62) ($P = 0.03$). High SAA level with an upper tumor site compared with a middle or a lower site was also observed by Chan *et al.* (28). Furthermore, the largest OR was observed for cardia cancer for both CRP and SAA. It is well known that *H.pylori* infection is related to non-cardia gastric cancer. As the majority of our subjects were infected with *H.pylori*, we were unable to show the results among *H.pylori*-seronegative subjects. Therefore, we cannot clarify whether the observed phenomenon was independent of *H.pylori*. We can state only that the observed elevated risk of gastric cancer with high levels of CRP and SAA is probably a phenomenon that cannot be totally explained by *H.pylori*; this conclusion is in line with that of previous studies (26,36). Comparing 153 preoperative gastric cancer patients with 19 healthy subjects, Tsavaris *et al.* (26) observed high serum levels of CRP, ceruloplasmin and a1-acid glycoprotein in cancer patients; however, among cancer patients, CRP level did not differ by status of *H.pylori* infection. Also, Delanghe *et al.* (36) showed that neither SAA nor other acute-phase proteins, including CRP, correlated with *Chlamydia pneumoniae* IgG, *H.pylori* IgG and IgA and cytomegalovirus IgG. On the other hand, the reason for the large OR observed in the cardia for both CRP and SAA positivity is unknown. One recent study reported that plasma CRP levels were associated with high BMI and other indicators of obesity (37). On the other hand, some studies, but not all, have proposed that elevated body weight may increase the risk of gastroesophageal reflux, which has been associated with adenocarcinomas of the gastroesophageal junction (38). Therefore, it is possible that elevated CRP and SAA were strongly associated with cardia cancer because of BMI status. However, in our data set, BMI did not differ by either CRP status or SAA status. The observed high OR in cardia cancer may be due to factors other than BMI or may be a mere chance finding.

On the basis of self-reported information, we adjusted for any condition that might alter the plasma levels of CRP or SAA. When these subjects were deleted (61 pairs; corresponds to 12% of total subjects), the overall findings did not change essentially, except when CRP values were divided into quartiles; the P for trend then became not significant ($P = 0.44$). Alternatively, when subjects with an extremely high level of CRP (>0.5 mg/dl) or SAA (>16.5 μg/ml) were excluded (55 pairs; corresponds to 11% of total subjects), the observed ORs became slightly higher, although the overall findings did not change essentially. To ensure the generalizability of findings and statistical power, we retained these subjects in the analyses.

Our study has several limitations. First, among 97 644 eligible subjects of the JPHC Study cohort, 36 745 (38%) men and women participated in the survey and provided blood samples. As reported previously, compared with non-participants, participants in the health checkup survey, especially women, had a different socioeconomic status and a favorable lifestyle profile, such as less smoking and alcohol consumption, greater participation in physical exercise and greater consumption of fruits or green vegetables (39). These findings mean that caution is needed in generalizing or interpreting the results in this report. Second, because of the relatively small sample size, further studies are needed to test our findings in analyses conducted by tumor location and histologic subtype.

The advantage of this study is its population-based prospective design and analysis of prediagnosed blood samples. Also, detailed information including *H.pylori* infection, CagA status, atrophy and environmental factors contributed to the detection of the relationships independent of these factors. Other strengths include negligible loss to follow-up and the satisfactory quality of our cancer registry system during the study period.

In conclusion, the overall distribution of CRP and SAA was not apparently associated with the development of gastric cancer. However, it was suggested that those who react strongly to inflammation or who have high host immune response, as reflected by extremely elevated plasma levels of CRP and SAA, were at high risk to develop gastric cancer.

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Appendix

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Grading system for lymph vessel tumor emboli: significant outcome predictor for patients with invasive ductal carcinoma of the breast who received neoadjuvant therapy

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The purpose of this study was to confirm that the grades of lymph vessel tumor emboli in biopsy specimens obtained before neoadjuvant therapy and in the surgical specimens obtained after neoadjuvant therapy according to the grading system we devised are significant histological outcome predictor for invasive ductal carcinoma (IDC) patients who received neoadjuvant therapy. The subjects of this study were the 318 consecutive IDC patients who had received neoadjuvant therapy in our institution. The lymph vessel tumor embolus grades in the biopsy specimens and in the surgical specimens were significantly associated with the increases in mean number of nodal metastases. Multivariate analyses with well-known prognostic factors and p53 expression in tumor-stromal fibroblasts clearly showed that the lymph vessel tumor embolus grade based on the biopsy specimens and based on the surgical specimens significantly increased the hazard rates for tumor recurrence and tumor-related death in all the IDC patients as a whole, in the IDC patients who did not have nodal metastasis, and in the IDC patients who had nodal metastasis, and the outcome-predictive power of the lymph vessel tumor embolus grades based on the surgical specimens was superior to that of the lymph vessel tumor embolus grades based on the biopsy specimens. The grades in the grading system for lymph vessel tumor emboli were significantly associated with nodal metastasis, and the histological grading system is an excellent system for accurately predicting the outcome of patients with IDC of the breast who have received neoadjuvant therapy.
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Lymphatic invasion in breast cancer patients with invasive ductal carcinoma (IDC) has been reported to have prognostic significance.[1–5] We have already reported that the grading system for lymph vessel tumor emboli that we devised is a very useful histological grading system for accurately predicting the outcome of patients with IDC who did not receive neoadjuvant therapy, and that the grading system can be used to classify IDC patients with lymph vessel invasion into a low-, intermediate-, and high-risk groups for outcome.[6] Furthermore, we have recently reported finding that the lymph vessel tumor embolus grades based on the biopsy specimens and based on the surgical specimens are also

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an important outcome-predictive factor for IDC patients who received neoadjuvant chemotherapy and had nodal metastasis.[7]

The purpose of this study was to confirm that the grading system for lymph vessel tumor embolus is a significant outcome predictor for IDC patients who received neoadjuvant therapy according to nodal status, by multivariate analysis with well-known clinicopathological factors and p53 protein expression in tumor-stromal fibroblasts in IDCs.[8] p53 protein expression in tumor-stromal fibroblasts, but not in tumor cells, in IDCs has been recently demonstrated to be a very important outcome predictor for IDC patients who received neoadjuvant therapy.[8] The results of this study clearly showed that the grading system for lymph vessel tumor emboli is an excellent histological outcome predictive of the histological grading system for IDC patients who received neoadjuvant therapy independent of nodal metastasis.

Materials and methods

Cases

The subjects of this study were the 318 consecutive patients with IDC of the breast who received neoadjuvant therapy and were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (the same series of patients as in an earlier study we conducted[8] and 88 patients of the 318 patients in this series were included among the subjects of our previous study[7]). Their IDCs were diagnosed preoperatively by needle biopsy, aspiration cytology, mammography, or ultrasonography. Clinical information was obtained from the patients' medical records after complete histological examination of all IDCs. All patients were Japanese women, and they ranged in age from 23 to 77 years old (median, 55 years). All had a solitary lesion; 127 patients were premenopausal, and 191 were postmenopausal. Partial mastectomy had been performed in 152, and modified radical mastectomy in 166. Level I and Level II axillary lymph node dissection had been performed in all patients, and Level III axillary lymph node dissection had been performed in some of the IDC patients.

Of the 318 subjects, 37 (12%) had shown a pathological complete response to neoadjuvant therapy (34, no residual tumor and no nodal metastasis; 3, residual ductal carcinoma *in situ* and no nodal metastasis).

The neoadjuvant therapy consisted of chemotherapy in 235 patients, endocrine therapy in 43 patients, and chemoendocrine therapy in 3 patients, and 214 out of the 281 patients who had received neoadjuvant therapy had also received adjuvant therapy, which consisted of chemotherapy in 47 patients, endocrine therapy in 116 patients, and chemoendocrine therapy in 51 patients. The chemotherapy regimens used were anthracycline-based with or without taxane and non-anthracycline-based, and the endocrine therapy regimens consisted of tamoxifen with or without a gonadotropin-releasing-hormone agonist, tamoxifen with or without an aromatase inhibitor, an aromatase inhibitor alone, or a gonadotropin-releasing-hormone agonist alone. There were no cases of inflammatory breast cancer in this series. All tumors were classified according to the pathological UICC-TNM (pTNM) classification system.[9] The protocol of this study (20–112) was reviewed by the institutional review board of the National Cancer Center, and all patients provided written informed consent.

For the pathological examination, biopsy specimens obtained before neoadjuvant therapy and surgically resected specimens obtained after neoadjuvant therapy were fixed in 10% formalin and subsequently examined. The size and gross appearance of the surgically resected tumor specimens were recorded as the residual invasive tumor size. The tumor size of the surgically resected specimens was confirmed by comparison with the tumor size on histological slides; if more than one invasive focus was present, the size of the largest invasive focus was recorded as the residual invasive tumor size in this study.

Histological Examination

Serial sections of the biopsy specimens obtained before neoadjuvant chemotherapy and of the tumor area in the surgically resected specimens obtained after neoadjuvant therapy were cut from paraffin wax blocks. One section of each biopsy specimen and surgical specimen was stained with hematoxylin and eosin and examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following eight histological features of the primary-invasive tumors were evaluated in the biopsy specimens obtained before neoadjuvant therapy and the surgical specimens obtained after neoadjuvant therapy: (1) residual tumor size (no residual tumor or residual ductal carcinoma *in situ*, residual tumor ≤ 20 mm, >20 to ≤ 50 mm, >50 mm), (2) histological grade (1, 2, 3),[10] (3) tumor necrosis (absent, present),[11] (4) fibrotic focus (biopsy specimen: absent, present; surgical specimen: absent, fibrotic focus diameter <8 mm, fibrotic focus diameter >8 mm),[12,13] (5) blood vessel invasion (absent, present), (6) adipose tissue invasion (absent, present), (7) skin invasion (absent, present), and (8) muscle invasion (absent, present). We also evaluated the outcome-predictive power of Fisher's neoadjuvant therapy effect classification for surgical specimens obtained after neoadjuvant therapy.[14,15]

Grading System for Lymph Vessel Tumor Emboli in IDCs

We have already stated the histological criteria of the grading system for lymph vessel tumor emboli in