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Molecular Biological Characteristics of Differentiated Early Gastric Cancer on the Basis of Mucin Expression

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Background: It is clear that the biologic characteristics of gastric cancer are different on the basis of mucin phenotypes. However, there are unabated controversies on the exact biologic differences of mucin expression in gastric cancer. **Methods:** We analyzed various protein expressions and microsatellite instability (MSI) status based on mucin expression in 130 differentiated early gastric adenocarcinoma cases. Furthermore, we evaluated the genomic alternation in 10 selected differentiated early gastric adenocarcinoma cases using array based comparative genomic hybridization (aCGH). **Results:** Intestinal mucin predominant subtype showed significantly elevated p53 protein and caudal-related homeobox 2 expression, and delocalization of beta catenin expressions compared to the gastric mucin predominant subtype. On MSI status, the gastric mucin predominant subtype more frequently showed unstable status than the intestinal mucin predominant subtype. CGH study showed more frequent chromosomal gain and loss in the intestinal mucin predominant subtype than the gastric mucin predominant subtype, albeit without statistical significance. Interestingly, there were significant differences in chromosomal alternation between four mucin phenotypes. **Conclusions:** Study results suggest possible different points of biologic behaviors in early differentiated gastric adenocarcinomas by mucin expression type.

Key Words: Stomach neoplasms; Tumor suppressor protein p53; beta catenin; Microsatellite instability; Comparative genomic hybridization; Mucins

Gastric cancer remains one of the most common malignancies globally. More than 90% of gastric cancer are adenocarcinomas, which are divided into two histological subtypes: differentiated and undifferentiated, or intestinal and diffuse, based on gland formation tendency.[1] Pathogenesis of the differentiated gastric adenocarcinoma (DC) associated with changes such as chronic atrophic gastritis, intestinal metaplasia, and adenoma. On the contrary, the diffuse subtype lacks well-recognized precursor lesions and these two subtypes result in different genetic carcinogenetic mechanisms.[2] On the basis of mucin phenotypes, DCs mostly show intestinal type mucin, but some DCs have been shown to have gastric type mucins.[3,4] Furthermore, it has become clear that the biologic characteristics of DCs are different by mucin phenotypes.[5] That is, DCs with gastric mucin have increased malignant potential, compared to intestinal subtype mucin. It has been reported that DCs of the gastric phenotype are more likely to transform into undifferentiated carcinoma and show infiltrative growth into the deeper layers or invasion into the surrounding structures.[5,6] Regarding the clinicopathologic significance of mucin expression of gastric carcinoma, Koseki *et al*.[5] reported that gastric phenotype is associated with lymph node metastasis. However, some investigators suggested that human gastric cancer at an early stage mainly consists of gastric phenotypic cells, while advanced gastric cancer retains more intestinal phenotype irrespective of histologic subtypes in accordance of progression.[7-9] Although studies have de-

monstrated correlation between the mucin phenotype and gastric cancer prognosis, controversies remain unabated among investigators on the exact clinicopathologic significance of mucin expression in gastric cancer.[6,10-13] Therefore, we attenpted to elucidate the biologic significance of mucin expression in early DCs. In the present study, we analyzed expression of p53 protein, caudal-related homeobox 2 (CDX2), beta catenin, E-cadherin and SMAD family member 4 (SMAD4), and microsatellite instability status based on mucin expression in 130 early DC cases. Furthermore, we evaluated for genomic alternation in 10 selected early DC cases using array based comparative genomic hybridization (aCGH).

MATERIALS AND METHODS

Tumor samples

This study includes formalin-fixed and paraffin embedded specimens (provided by National Biobank of Korea, Pusan National University Hospital) of 130 patients dignosed with early DCs based on the Lauren classification after gastrectomy with lymph node dissection at the Pusan National University Hospital between 2002 and 2003. The patients were 103 males and 27 females, with a mean age of 59.0 years (range, 42 to 75 years). No preoperative radiotherapy and/or chemotherapy were administered.

Immunohistochemistry for mucin phenotypes

Sections were de-waxed and rehydrated according to standard procedure, and washed with phosphate buffered saline (PBS). For immunohistochemical stain, sections were heated in a microwave oven at 600 W for 2×5 minutes in 0.01 M citrate buffer at pH 6.0. Sections were immersed in 3% H_2O_2 to quench endogenous peroxidase activity, and unspecified binding was blocked in 5% normal goat serum (0.1% bovine serum albumin in PBS). Immunohistochemical staining was performed by the avidin-biotin peroxidase complex method with aminoethylcarbazole as a chromogen using the Vetastain ABC elite kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin solution. We used primary antibodies for mucin-5AC (MUC-5AC; 1:500, mouse monoclonal antibody CLH2, Novocastra, New Castle, UK), mucin-6 (MUC-6; 1:500, mouse monoclonal antibody CLH5, Novocastra), mucin-2 (MUC-2; 1:500, mouse monoclonal antibody Ccp58, Novocastra), and CD10 (1:100, mouse monoclonal antibody 56C6, Novocastra). MUC-5AC and MUC-6 are markers of gastric phenotypes, whereas MUC-2 and CD10 are typical of intestinal phenotypes. Gastric cancers with more than 10% of positive area for each mucin were deemed as positive phenotypes. One hundred and thiry (n = 130) cases of early DCs were further subdivided into gastric mucin predominant DCs (DCGPs), intestinal mucin predominant DCs (DCIPs), gastrointestinal mucin phenotypes (gastric = intestinal) (DCBPs) and null phenotypes, based on combination of predominant patterns of MUC-5AC, MUC-2, MUC-6 and CD10 staining.

Immunohistochemistry for p53 protein, CDX2, beta catenin, E-cadherin and SMAD4

We used the same immunohistochemistry method that had been used for the mucin phenotypes previously. We used primary antibodies for p53 protein (1:100, mouse monoclonal antibody DO7, Dako, Carpinteria, CA, USA), beta catenin (1:100, mouse monoclonal antibody 14, Transduction Laboratories, Lexington, KY, USA), E-cadherin (1:100, mouse monoclonal antibody 36, Transduction Laboratories), CDX2 (1:100, mouse monoclonal antibody CDX2-88, BioGenex, Fremont, CA, USA), and SMAD4 (1:100, goat polyclonal antibody C-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For statistical analysis of p53 protein expression, specimens with over 10% positive nuclear staining in the tumor cells were considered positive. CDX2 expression was estimated as positive when nuclear immunostaining for CDX2 was seen in over 10% of tumor cells. The expression of SMAD4 and membranous expression of E-cadherin and beta catenin in tumor cells were compared to that of normal epithelial cells in the same sample. Tumor cells stained as strongly as the adjacent normal epithelial cells were deemed positive. When over 90% of tumor cells for membranous expression of E-cadherin and beta catenin and over 50% of tumor cells for SMAD4 were respectively stained, the case was considered preserved while other cases were regarded as reduced expression. When beta catenin stained clearly in the cytoplasm or nuclei in more than 10% of tumor cells, the expression was considered positive for cytoplasmic or nuclear expression of beta catenin.

Microsatellite analysis

The DNA of cancerous tissue and the corresponding normal gastric mucosa was obtained from formalin-fixed, paraffin-em-

bedded surgical blocks. The DNA was extracted by proteinase K digestion and the phenol-chloroform procedure. Extracted DNA was amplified by polymerase chain reaction (PCR) with fluorescent dye labeled primers on 5 microsatellite loci: BAT25, BAT26, D5S346, D2S123, and D17S250. DNA was detected by a temperature-controlled DNA sequencer (PRISM 377, PerkinElmer, Foster City, CA, USA), and fragment analyses were conducted with the Genscan software (PerkinElmer). Microsatellite instability (MSI) status was determined by size variation and the presence of additional bands in the PCR product from tumor DNA and not observed in the DNA of normal tissues from the same patient. High-MSI (MSI-H) was defined as instability in at least 2 out of 5 microsatellite loci, low-MSI (MSI-L) as a shift in only one locus, and microsatellite stability (MSS), as when none of the loci were shifted, according to the National Cancer Institute criteria.

Array based CGH

Case selection and DNA extraction

Ten cases of DCs (4 cases of DCIP, 6 cases of DCGP) were selected for aCGH. The DNA of cancerous tissue was obtained from formalin-fixed, paraffin-embedded surgical blocks. The DNA was extracted by proteinase K digestion and the phenol-chloroform procedure.

Construction of bacterial artificial chromosome library

Bacterial artificial chromosome (BAC) clones were selected from Macrogen's proprietary BAC library (http://www.macrogen.com, Seoul, Korea). Briefly, pECBAC1 was restricted with *HindIII*, and size selected *HindIII* digested pooled male DNA was used to generate a BAC library. The vectors for this library were transformed and grown in DH10B.

Construction of BAC-mediated array CGH microarray

The clones were first selected bioinformatically to give an average genomic coverage of 2 Mb resolution. All the clones were two end-sequenced using the Applied Biosystems 3700 sequencers (Applied Biosystems, Foster City, CA, USA), and their sequences were blasted and mapped according to their positions in the UCSC human genome database (http://genome.ucsc.edu). Confirmation of locus specificity of the chosen clones was performed by removing multiple loci binding clones, and by examining individually under standard fluorescence *in situ* hybridization condition. These clones were prepared by conventional alkaline lysis method to obtain BAC DNA. DNA was sonicated to generate 3 kb fragments before mixing with 50% dimethyl sulfoxide spotting buffer. The arrays were manufactured by Molecular Dynamics, Generation III using the 12-pin format (Molecular Dynamics, Sunnyvale, CA, USA). Each BAC clones were represented on an array as triplicated spots, and each array was pre-scanned using an Axon scanner for proper spot morphology. The array used in this study consisted of 1,440 human BACs, spaced approximately 2.3 Mb across the whole genome.

DNA labeling for array CGH

The labeling and hybridization protocols were used.[14] Briefly, 2 μg of tumor DNA and reference DNA were digested with *Dpn* II overnight. After purification, 21 μL of solution containing 500 ng of normal DNA (reference) or tumor DNA, 20 μL of BioPrime® aCGH Labeling System random primers solution (Invitrogen, Carlsbad, CA, USA) and water were combined and incubated for 5 minutes at 95℃, and subsequently cooled on ice. After adding 5 μL of 10× dNTPs labeling mix (0.6 mM dCTP, 1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP), 3 μL of 1 mM Cy3-dCTP or Cy5-dCTP (PerkinElmer, Boston, MA, USA) and 40 U of BioPrime® aCGH Labeling System Klenow fragment (Invitrogen), the mixture was gently mixed and incubated for 16 hours at 37℃. The addition of 5 μL of BioPrime® aCGH Labeling System Stop Buffer (Invitrogen) ended the reaction. After labeling, unincorporated fluorescent nucleotides were removed by BioPrime® aCGH Purification Module (Invitrogen). In one tube, Cy3-labeled sample and Cy5-labeled reference DNAs were mixed together, and 70 μg of human *Cot* I DNA (Invitrogen), 20 μL of 3 M sodium acetate and 600 μL of cold 100% ethanol were precipitated.

Array hybridization, imaging, and data analysis

The pellet was resuspended in 40 μL of a hybridization solution containing 50% formamide, 10% dextran sulfate, 2× saline sodium sulfate, 4% sodium dodecyl sulfate and 200 μg yeast tRNA. The hybridization solution was denatured for 10 minutes at 70℃, and was incubated subsequently for 1 hour at 37℃, to allow blocking of repetitive sequence. Hybridization was performed in slide chambers for 48 hours at 37℃. After post hybridization washes, arrays were rinsed, dried with spin, and scanned into two 16-bit TIFF image files using GenePix-4200A two-color fluorescent scanner (Axon Instruments, Foster City, CA, USA) and quantitated using GenePix software (Axon Instruments). The median ratio of the three replicate spots for each clone was calculated. Clones with >70% missing or poor

values were excluded, a total of 1,294 different BAC clones were included in the final analysis. Log-transformed fluorescent ratios were calculated by intensity with the median backround subtracted. These ratios were used to perform LOWESS normalization before performing copy number calculations. R and G indicate background-corrected Cy5 and Cy3 intensities for each spot, respectively. Normalization is usually applied to the log-ratios of Cy3 and Cy5, which will then be written as M = logR-logG. The log-intensity of each spot was written as A = (logR+logG)/2. The M value is normalized by the corresponding value of the print-tip group LOWESS curve. Normalized log-ratios N are the residuals from the print-tip group LOWESS regression, i.e., N = M-lowess (A) where lowess (A) is the LOWESS curve as a function of A for the print-tip group. Each LOWESS curve was constructed by performing a series of local regression, one local regression for each point in the scatter plot. Chromosomal aberrations were categorized as a gain when the normalized log2 transformed fluorescent ratio was higher than 0.25 and as a loss when this ratio was below -0.25. These two threshold values were chosen empirically by selecting a 3×SD value calculated from 30 normal male to normal female hybridization experiments. Macrogen's MAC viewer (Macrogen, Seoul, Korea), aCGH analysis software (Macrogen), MS Excel VBA (Microsoft, Redmond, WA, USA), and avadis3.3 Prophetic software (Strand Genomics, Bangalore, India) were used for graphical illustration and image analysis of aCGH data.

Statistical analysis

The data were analyzed by student's t-test, Fischer's exact test or χ^2 test for differences between the groups. A p-value of less than 0.05 was considered statistically significant. Statistical calculations were performed with SPSS ver.10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Mucin phenotypes and relationship between clinicopathologic features in early DCs

Of the 130 cases of early DCs, 56.5% (74 cases), 46.6% (61 cases), 24.4% (32 cases), and 25.2% (33 cases) were positive for MUC-2, MUC-5AC, MUC-6 and CD10, respectively. One hundred and thirty cases of early DCs were further subdivided into DCGPs, DCIPs, DCBPs and null phenotypes based on combination of predominant patterns of MUC-5AC, MUC-2, MUC-6 and CD10 staining. In total, there were 53 cases (40.8%), 61 cases (46.9%), 6 cases (4.6%) and 10 cases (7.7%) of DCGPs, DCIPs, DCBPs, null phenotypes, respectively. There was no significant difference in clinicopathologic features between DCGPs and DCIPs (Table 1).

p53 protein, SMAD4, CDX2, E-cadherin and beta catenin expression in early DCs

To elucidate different biological characteristics of differentiated early gastric cancer on basis of mucin phenotypes, we examined the expression of p53 protein, SMAD4, CDX2, E-cadherin and beta catenin. Of the 130 cases of early DCs, DCIPs (42/61 cases, 68.9%) showed significantly increased p53 protein expression than DCGPs (20/53 cases, 37.7%). DCIPs (53/61 cases, 86.9%) showed more CDX2 expression than DCGPs (30/53 cases, 56.6%). Also, increased cytoplasmic and nuclear beta catenin expressions (delocalization) were found to be significantly higher in DCIPs than DCGPs (Table 2, Fig. 1).

Table 1. Clinicopathologic characteristics and mucin phenotypes of differentiated type early gastric cancer

	No. of cases	Mucin phenotype		p-value
		DCGP	DCIP	
Age (yr)				
≤50	30	16	14	0.381
>50	84	37	47	
Gender				
Male	91	41	50	0.543
Female	23	12	11	
Tumor size (cm)				
≤2	57	27	30	0.851
>2	57	26	31	
Location				
Upper	32	15	17	0.959
Middle and lower	82	38	44	
Invasion depth				
Mucosa	54	27	27	0.476
Submucosa	60	26	34	
Gross type				
Elevated/Flat	55	30	25	0.096
Depressed	59	23	36	
Lymphovascular emboli				
Negative	91	40	51	0.280
Positive	23	13	10	
Lymph node metastasis				
Negative	100	46	54	0.779
Positive	14	7	7	

DCGP, gastric mucin predominant differentiated type early gastric cancers; DCIP, intestinal mucin predominant differentiated type early gastric cancers.

MSI status in early DCs

Of the 91 cases of early DCs, 39 cases (42.9%) were MSI-H and 52 cases (57.1%) were MSS or MSI-L (Fig. 2). There was significant correlation between MSI status and mucin phenotypes of differentiated early gastric cancer. That is, DCGPs (32/47 cases, 68.1%) showed more MSI-H than DCIPs (7/44 cases, 15.9%) (Table 3).

Table 2. p53, SMAD4, CDX2, E-cadherin, beta-catenin expression in differentiated type early gastric cancer

	No. of cases	Mucin phenotype		p-value
		DCGP	DCIP	
p53				
Negative	52	33	19	0.001
Positive	62	20	42	
SMAD4				
Preserved	67	33	34	0.480
Reduced	47	20	27	
CDX2				
Negative	31	23	8	0.000
Positive	83	30	53	
E-cadherin				
Preserved	49	22	27	0.767
Reduced	65	31	34	
Beta catenin (membranous expression)				
Preserved	48	26	22	0.161
Reduced	66	27	39	
Beta catenin (cytoplasmic expression)				
Negative	39	12	27	0.015
Positive	75	41	34	
Beta catenin (nuclear expression)				
Negative	78	41	37	0.056
Positive	36	12	24	

SMAD4, SMAD family member 4; CDX2, caudal type homeobox 2; DCGP, gastric mucin predominant differentiated type early gastric cancers; DCIP, intestinal mucin predominant differentiated type early gastric cancers.

Genomic alterations identified by array based CGH in differentiated early gastric cancer

We have selected 10 cases of early DCs (DCIP, 4 cases; DCGP, 6 cases) for aCGH study to elucidate genomic alternation based on mucin expression. A total of 1,440 qualified array BAC clone were applied for the purpose of data analysis, and all of our cases showed chromosomal alternations (gain or loss) in more than one BAC clones (Fig. 3). The mean number of gained and lost clones were respectively 133 (range, 39-312) and 351 (range, 117-555). There was a difference of mean number in the gained clone (173 vs 107) and lose clones (419 vs 305) between DCIP and DCGP. There were more frequent chromosomal loss and

Table 3. MSI status in differentiated type early gastric cancer

	No. of cases	Mucin phenotype		p-value
		DCGP	DCIP	
MSI status				
MSI-H	39	32	7	0.001
MSI-L, MSS	52	15	37	

MSI-H, high-microsatellite instability; MSI-L, low-MSI; MSS, microsatellite stability; DCGP, gastric mucin predominant differentiated type early gastric cancers; DCIP, intestinal mucin predominant differentiated type early gastric cancers.

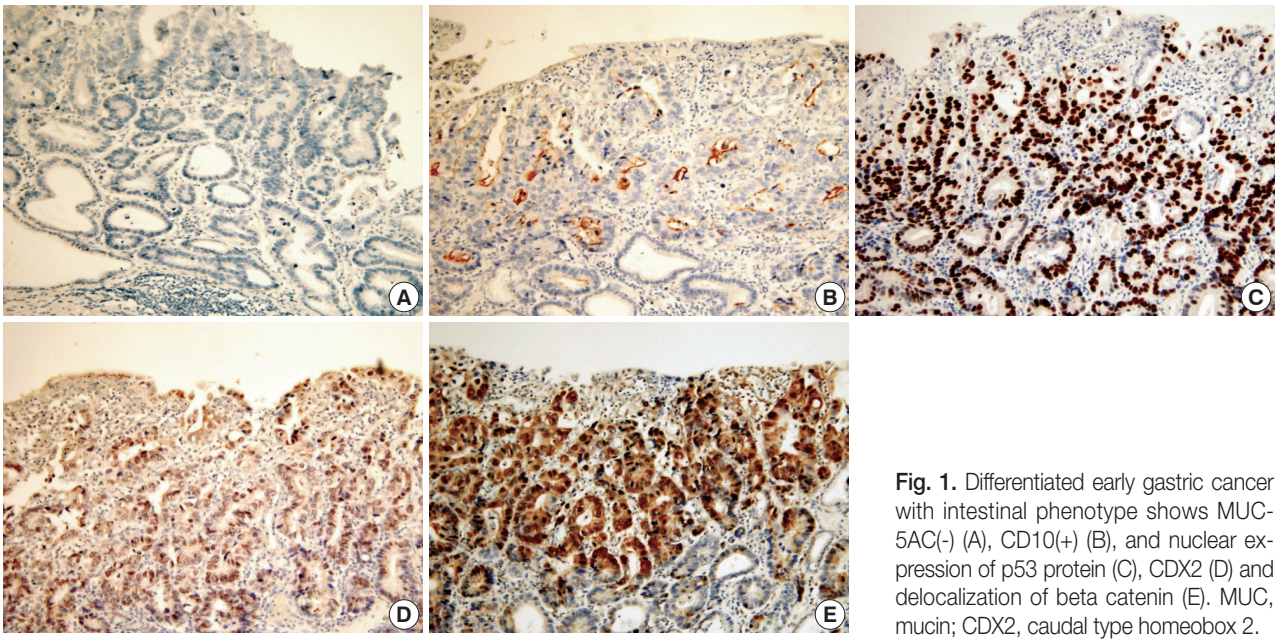

Fig. 1. Differentiated early gastric cancer with intestinal phenotype shows MUC-5AC(-) (A), CD10(+) (B), and nuclear expression of p53 protein (C), CDX2 (D) and delocalization of beta catenin (E). MUC, mucin; CDX2, caudal type homeobox 2.

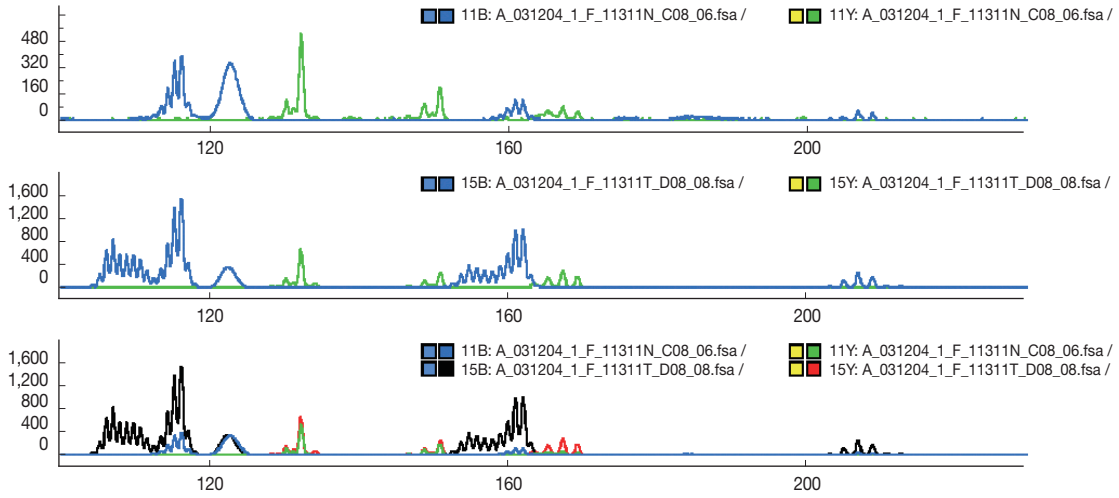

Fig. 2. MSI status of differentiated early gastric cancer with gastric phenotype showing MSI-H (microsatellite instable for BAT25, BAT26, D2S123). MSI-H, high-microsatellite instability.

gain in DCIP than DCGP, but it was not statistically significant (p = 0.197, p = 0.097, respectively). The most frequent chromosomal gain were seen at 19p12-p13.3, 19q12-q13.33, 19q13.2, 22q13.33, 16p13.3, and chromosomal loss at 2q21.1-q24.2, 4q21.21-q34.3, 6p11.1-p12.3, 6q11.1-q23.3, 12p11.23, 2q11.2-q14.3, 4p12-p15.2, 4q12-q25, 4q23-q25, 5p12-p15.1, 5q11.2-q21.1, 6q24.1-q25.2, 9p21.1-p24.3, 20p12.2 (Table 4). There was difference in chromosomal alternations in early DCs based on mucin expression. DCIP showed more chromosomal loss at 1p12-1p13.3, 3q21.1-q26.1, 5q31.1, 5q33.3,q35.1-q35.2, 7q22.1-q35, 7q31.1-q33, 8p12-23.3, 8q13.2-13.3, 8q21.12-q23.1 and chromosomal gain at 16q12, 16q21, 16q22.1, 16q-22.3, 16q23.1, 16q24.3, 17p11.2, 17p13.1-3 than DCGP (Table 5).

Table 4. Most frequent chromosomal changes in 10 differentiated type early gastric cancers detected by array based CGH

Loss of copy No.		Gain of copy No.	
Chromosome band	No. of cases	Chromosome band	No. of cases
2q21.1-q24.2	10	19p12-p13.3	10
4q21.21-q34.3	10	19q12-q13.33	10
6p11.1-p12.3	10	19q13.2	9
6q11.1-q23.3	10	22q13.33	9
12p11.23	10	16p13.3	9
2q11.2-q14.3	9	17q12-q21.2	8
4p12-p15.2	9	19q13.33-q13.42	8
4q12-q25	9	9q34.11-q34.3	7
4q23-q25	9	17q12	7
5p12-p15.1	9	22q11.21-q13.31	7
5q11.2-q21.1	9		
6q24.1-q25.2	9		
9p21.1-p24.3	9		
20p12.2	9		
6q27	8		
12q24.32	8		
1p22.3	8		
1p31.1-p32.3(1p32.1)	8		
2q11.2-q33.1	8		
5q21.2-q23.2	8		
2p11.2-2p14(2p13.2)	8		
1p21.1-p22.3	7		
2q33.2-q34	7		
3p12.1-p14.2	7		
3q11.2-q13.33	7		
5p13.1	7		
6p21.1	7		
9p24.3	7		
10q23.1-q23.31	7		

CGH, comparative genomic hybridization.

DISCUSSION

In this study, we found that DCIP exhibited more p53 protein, increased CDX2, cytoplasmic and nuclear beta catenin expression compared to DCGP. DCGP showed more unstable MIS status than DCIP. Also, aCGH revealed difference in genomic alternation between DCIP and DCGP.

In regard to various protein expression associated with the biologic behavior of gastric cancer, DCIPs (42/61 cases, 68.9%) showed significantly increased p53 protein expression compared to DCGPs (20/53 cases, 37.7%). DCIPs (53/61 cases, 86.9%) showed more CDX2 expression than DCGPs (30/53 cases, 56.6%). p53 protein over-expression is associated with aggressive biological behavior of gastric cancer and poor gastric carcinoma prognosis.[15,16] In regard to mucin phenotypes, p53 protein over-expression appears to be mostly associated with intes-

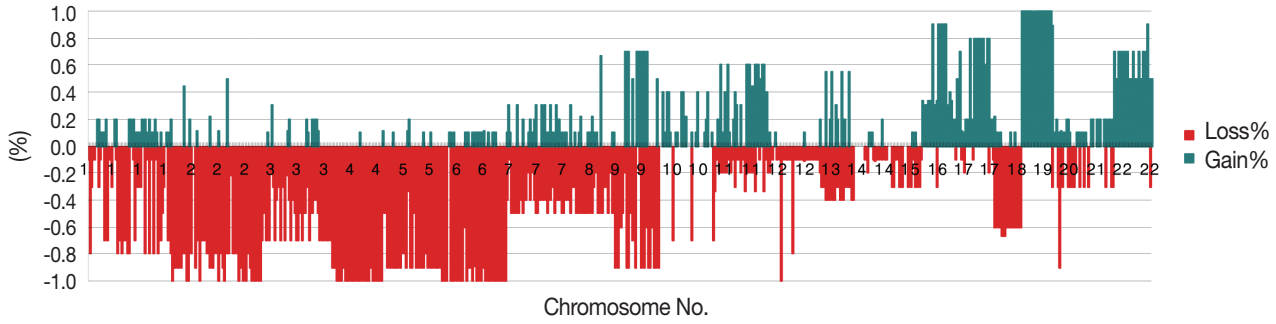

Fig. 3. Frequency of chromosomal copy number changes in 10 differentiated early gastric cancer cells.

Table 5. Differences in the chromosomal changes between two mucin phenotypes in 10 differentiated types of early gastric cancers detected by array based CGH, and the relevant genes

Chromosome band	Mucin phenotype		Genes[a]
	DCIP	DCGP	
Loss			
1p12-1p13.3	2 (50.0)	1 (16.7)	*ZNF697, PHGDH, C1orf137, CD2, HIPK1, OLFML3, CHIA, C1orf88, OVGP1, MRP63P1, WNT2B, ST7L, PROK1, KCNA10, GNAI3, GNAT2, AMPD2, GSTM4, GSTM2, GSTM1, OR11I1P, CD53, C1orf103, CSF1, AHCYL1*
3q21.1-q26.1	2 (50.0)	1 (16.7)	*KALRN, ACAD9, TF, ACPP, EPHB1, EPHB1, NPM1P17, ZBTB38, RASA2, PCOLCE2, PAQR9, CLSTN2, EIF2A, C3orf44, GPR149, MME, VEPH1, IFT80, SMC4L1*
5q31.1,q33.3,q35.1-q35.2	2 (50.0)	1 (16.7)	*IRF1, IL5, TIMD4, KCNIP1, SLIT3, STC2, CPLX2LEPROTL1, DCTN6*
7q22.1-q35	3 (75.0)	1 (16.7)	*LHFPL3, PIK3CG, DLD, LAMB1, LAMB4, GRM8, ATP6V1F, CRIM2, IRF5, UBE2H, CHCHD3, CNOT4, AKR1B1, TRIM24, TRBV6-6, TRBV7-5, TRBV5-5, TRBV6-7, TRBV7-6, TRBV5-6, TRBV6-8, TRBV7-7, TRBV5-7, TRBV6-9, TRBV7-8, TRBV5-8, TRBV7-9, TRBV13, TRBV10-3, TRBV11-3, TRBV12-3, TRBV12-4, OR2F2, OR2F1, OR2Q1P*
7q31.1-q33	3 (75.0)	2 (33.3)	*FOXP2, ST7, ST7OT2, MET, LSM8, PTPRZ1, EXOC4*
8p12-23.3	2 (50.0)	0 (0.0)	*LEPROTL1, DCTN6, LETM2, FGFR1, EXTL3, EXTL3, DOCK5, GNRH1, KCTD9, CDCA2, DOK2, XPO7, INTS10, PDGFRL, OACT4, MTUS1, CLDN23, MFHAS1, GATA4, C8orf49, NEIL2, FDFT1, CTSB, FBXO25, C8orf42, FBXO25*
8q13.2-13.3, q21.12-q23.1	3 (75.0)	2 (34.0)	*C8orf34, SULF1, IL7, PMP2, FABP4, NBN, DECR1, GRHL2, RSPO2*
Gain			
16q12,q21,q22.1,q22.3, q23.1,q24.3	2 (50.0)	1 (16.7)	*GPR114, GPR56, GPR97, GNAO1, AMFR, GNAO1, AMFR, TK2, CKLF, CMTM1, NFAT5, NQO1, PSKH1, CTRL, PSMB10, LCAT, SLC12A4, DPEP3, DPEP2, TERF2, CYB5B, ATBF1, WWOX, CRISPLD2, ZDHHC7, ZFPM1*
17p11.2,p13.1-3	2 (50.0)	0 (0.0)	*FLCN, COPS3, FBXW10, FAM18B, MAPK7, MFAP4, ZNF179, ALDH3A2, ALDH3A1, MYH3, SCO1, C17orf48, FXR2, SAT2, SHBG, ATP1B2, TP53, NDEL1, MYH10, ASGR1, DLG4, ACADVL, DVL2, PHF23, GABARAP, DULLARD, C17orf81, CLDN7, SLC2A4, YBX2, EIF5A, GPS2, GLP2R, RCV1, GAS7, ATP2A3, ZZEF1, ALOX12P2, SPATA22, ASPA, TRPV3*

Values are presented as number (%) for each mucin phenotype.
[a]Symbol of genes are based one the HEGO Gene Nomenclature Committee.
CGH, comparative genomic hybridization; DCIP, intestinal mucin predominant differentiated type early gastric cancers; DCGP, gastric mucin predominant differentiated type early gastric cancers.

tinal mucin in differentiated-type of early gastric cancer.[17] The outcomes of our study are compatible with that of previous studies. In regard to CDX2 expression in gastric cancer, increased CDX2 expression is associated with less invasiveness and intestinal mucin phenotypes in gastric cancer.[18,19] Our results are partially in agreement with these reports.

Beta catenin is an important mediator of the Wnt signaling pathway and cell to cell adhesion.[20,21] Genetic alteration of adenomatous polyposis coli, beta catenin and other components of the Wnt signaling pathway results in impaired degradation of beta catenin and increased cellular and nuclear beta catenin accumulation (delocalization), which can induce neoplastic transformation.[22,23] There exist published reports on beta catenin expression in gastric cancers. Miyazawa *et al*.[24] showed nuclear expression of beta catenin in 12% of gastric cancer, all of which were intestinal (differentiated) type adenocarcinoma, and suggested that nuclear accumulation of beta catenin is associated with early tumor invasion in intestinal-type gastric carcinoma.

In our study, nuclear and cytoplasmic beta catenin expressions (delocalization) are higher in intestinal mucin phenotype than gastric mucin phenotypes in differentiated early gastric cancer. There are few reports on beta catenin expression on the basis of mucin phenotypes in gastric cancer. Aihara *et al*.[25] showed that mixed gastro-intestinal mucin phenotype was associated with nuclear expression in early undifferentiated gastric carcinoma. In conjunction with our data and previous reports, we hypothesize that delocalization of beta catenin in differentiated early gastric cancer exists more commonly in intestinal mucin phenotype than in gastric mucin phenotype, and has different biological significance based on mucin expression.

In regard to MSI status in gastric cancer, we demonstrated that DCGP exhibited more MSI-H pattern than DCIP. MSI-H gastric cancers are associated with older age, antral location, lower prevalence of lymph node metastasis and lower pathologic tumor-node-metastasis stage. In additions, patient survival is poorer in advanced gastric cancer.[26] In regard to mucin expression and MSI status, MSI-H is significantly associated with MUC-6 mucin expression (gastric mucin phenotypes).[6] In differentiated type of early gastric cancer, foveolar-type (featuring gastric foveolar mucin expression) showed higher rate of MSI-H type than other types.[17] These previous results are compatible with that of our study.

To demonstrate different genomic alternation in differentiated early gastric cancer based on mucin expression, we performed aCGH which detects small amplification and deletion that appear to contain specific oncogenes and tumor suppressor genes with resolution as precise as 0.2 to 0.4 Mb.[14]

Previous studies have shown many chromosomal alterations in gastric cancer.[27] In this study, we identified consistent results, including loss of 1p, 2q, 4p, 4q, 5p, 5q, 9p, and gain of 16p, 17q, 19p, 19q (Table 4). The most frequent chromosomal alterations detected in this study were 2q21-q24, 4q21-q34, and 6q11-q23, which showed copy number reduction at 100% frequency, and 19p12-p13, which showed copy number elevation at 100% frequency.

Several novel genes showing high frequency of DNA copy number changes were also identified in this study, including loss of 12p11 (100% of frequency), 20p12 (90%), 3p12-p14 (70%), 3q11-q13 (70%), 10q (70%), and gain of 22q13 (90%), 9q34 (70%). Further integrative genomic study is needed to confirm these findings.

Regarding intestinal type gastric cancer, previous studies reported intestinal type dominant alteration, including gains at 17q12-21 and 20q, and losses at 8p and 9p.[28] Regarding histologic type-specific chromosomal alteration, this study showed two contentious findings on chromosome 6p and 20q. In this study, 6p loss was detected in all cases of intestinal type gastric adenocarcinoma. On the other hand, diffuse type gastric cancer showed 6p11.1-p12.3 copy number gain in a previous study.[28] Therefore, we hypothesize that 6p11.1-p12.3 plays a key role in differentiation of histologic type. Meanwhile, copy number loss of 20q was not identified in this study. According to the result of a previous study, 20q loss was identified in 44% of intestinal type gastric cancer,[29] and the chromosomal change was more frequently present in poorly differentiated intestinal gastric cancer than in well to moderately differentiated intestinal subtype.[29] Those findings suggest that 20q loss may be more commonly associated with poorly DC than well to moderately differentiated intestinal gastric adenocarcinoma. Chromosome loss of 9p and 8q were consistent with the results of previous studies.[28] Particularly, 8q loss was more frequently presented in the tumors of gastric mucin phenotype than in intestinal mucin phenotype (Table 5).

There was a study which reported chromosomal alternations based on mucin expression that identifed gains of 5p15.2, 7p21 and 13q33-34 in intestinal mucin phenotype tumors compared to gastric and gastrointestinal phenotypes.[30] We also identified additional differences about the type and frequency of chromosomal alternations based on mucin phenotype in differentiated type early gastric cancer. DCIP showed more chromosomal losses at 1p12-1p13.3, 3q21.1-q26.1, 5q31.1, 5q33.3, q35.1-q35.2, 7q22.1-q35, 7q31.1-q33, 8p12-23.3, 8q13.2-13.3, 8q21.12-q23.1 and chromosomal gains at 16q12, 16q21, 16q22.1, 16q22.3, 16q23.1, 16q24.3, 17p11.2, 17p13.1-3, compared to DCGP. There was no dominant copy number alteration in DCGP compared to DCIP. The result of the study of Morohara *et al*.[30] also showed that losses of 1p, 5q, 7q, 8p, 8q and gain of 17p were more frequent in the tumors of intestinal mucin phenotype than other mucin phenotype tumors, albeit without statistical significance.

Taken together the results of immunohistochemical and molecular studies, it is clear that the biologic behavior of differentiated early gastric cancer is associated with mucin expression in early DCs. It has been suggested that the pathologic behavior is more variable in intestinal mucin phenotype gastric cancer than that of gastric mucin phenotype. More integrative genetic studies are required to validate these results and reveal candidate genes responsible for the molecular pathologic behavior of mucin in early DCs.

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