change represents the first hit and was present in differentiated blood cells, and the corresponding pre-leukemic clones were simultaneously eradicated together with the leukemic clones by chemotherapy.

On the other hand, as shown in Supplementary Tables S1 and S2, a heterozygous mutation for NRAS(G12S) was found only in the $CD34^+$ fraction, but not in the $CD4^+$ fraction of the patient ID JM16. Conventional chemotherapy for this patient eradicated the leukemic blasts carrying the mutation (Supplementary Figure S7), also confirming that a successful treatment results in the disappearance of cells with a (possible) 'second hit'.

Our hypothesis of the stepwise leukemogenesis is also consistent with the previous detection of the *RUNX1-CBFA2T1* oncogene in differentiated blood cells (Kwong *et al.*, 1996; Miyamoto *et al.*, 1996, 2000).

Discussion

Our large-scale genomic resequencing of human leukemia specimens with DNA microarrays has identified recurrent nucleotide changes responsible for the generation of JAK3 and DNMT3A mutants. Whereas JAK3 mutants were unexpectedly found in adult AML, their transforming ability, and possibly their contribution to leukemogenesis, varied substantially. However, our bone marrow transplantation experiments showed that at least one of these JAK3 mutants (M511I) directly participates in the development of leukemia. Identification of the M511I mutation of JAK3 in the leukemic fraction but not in the control fraction of patient ID JM07 suggests that this mutation may be the second hit triggering AML. Given that the blasts of this patient had a normal karyotype, it is likely that the first hit is present in the genome of both fractions. Karyotyping of other patients with *JAK3* mutations showed a total of three cases with a normal karyotype, one case with t(8;21), and one case with a numerical anomaly of several chromosomes (Supplementary Table S3), suggesting that *JAK3* mutations may be preferentially associated with leukemia with a normal karyotype.

Although JAK3(M511I) was identified in AML, our bone marrow transplantation experiments with hematopoietic stem cells expressing this mutant yielded T-cell acute lymphoblastic leukemia. In contrast to human leukemia, in which JAK3 changes may constitute a second hit (probably in progenitor cells), JAK3(M511I) may have been expressed in all hematopoietic cells of the recipient mice. JAK3(M511I) thus likely triggered leukemia within a T-cell fraction the intracellular context of which is optimized for JAK3 signaling.

It has been frequently observed that transgenic mouse or bone marrow transplantation experiments for leukemic oncogenes do not accurately recapitulate the original leukemia subtypes (Wong and Witte, 2001). Transgenic mice expressing $p210^{BCR\text{-}ABL1}$, for instance, usually develop T-cell lymphoma or acute lymphoblastic leukemia, not chronic myeloid leukemia. Furthermore, bone marrow transplantation with hematopoietic progenitor cells expressing $p210^{BCR\text{-}ABL1}$ often leads to development of lymphoma, AML, acute lymphoblastic leukemia or macrophage tumors. Generation of malignancy in such systems may, thus, be elaborately influenced by mouse strains, promoter fragments for artificial expression and/or cell types to be used for gene transduction.

Our detection of recurrent DNMT3A hypomorphic mutations in leukemia clones may indicate the presence of an abnormal methylation profile in the genome of such blasts. However, given the limited amount of the specimens available, we were able to investigate microsatellite stability only at certain loci (Koinuma *et al.*, 2005), revealing no apparent microsatellite instability (data not shown). We also generated BA/F3 cells expressing wild-type or R882H forms of DNMT3A to compare the methylation status of some CpG islands in the genome; again, we detected no discernable differences between the two cell preparations (data not shown). However, given that BA/F3 cells contained two copies of wild-type *Dnmt3a* in addition to multiple copies of mutant *DNMT3A*, whereas the leukemic blasts likely harbor one copy each of the wild-type and mutant *DNMT3A* alleles, the clinical relevance of the R882 mutant requires further examination under the latter condition. Cell proliferation/differentiation is indeed influenced substantially by the copy number of *DNMT3* genes (Okano *et al.*, 1999; Ehrlich, 2003).

Our observations indicate the importance of preparing paired normal fractions in large-scale resequencing projects, but they also reveal a difficulty in the preparation of *bona fide* 'normal' fractions in the case of leukemic disorders. Our data thus indicate that nonleukemic blood cells may harbor early genomic hits, rendering them inappropriate as controls. Furthermore, a substantial proportion of fingernail DNA was recently shown to be derived from donor cells among recipients of allogeneic stem cell transplants (Imanishi *et al.*, 2007), indicating that nonblood cells may contain DNA derived from transplanted cells. Therefore, it is possible that buccal, fingernail or even hair cells may not be suitable as normal cell controls. In contrast to solid tumors, for which blood cells are appropriate as paired normal fractions, leukemic disorders require that caution be taken to discriminate somatic nucleotide changes from germline polymorphisms.

Materials and methods

Wafer sequencing

$CD34^+$ and $CD4^+$ fractions were isolated from leukemic individuals using CD34microbeads and CD4microbeads, respectively, and a MidiMACS separator (Miltenyi Biotec, Gladbach, Germany). All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both the Jichi Medical University and the Nagasaki University. DNA sequencing wafers were designed and processed at Perlegen Sciences. Genes to be interrogated on the wafers were selected from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)

by searching with various keywords characteristic to each subcategory (such as DNA repair, regulation of chromatin structure, etc.), followed by manual inspection. The final gene list for the wafers is shown in Supplementary Table S6. Construction of the wafers, quality control analysis and data processing are described in Supplementary Text.

JAK3 analysis

Complementary DNAs for JAK3 mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and ligated into the pMX retroviral vector (Onishi *et al.*, 1996). Ecotropic recombinant retroviruses encoding each mutant were produced in BOSC23 cells transfected with the corresponding pMX-based plasmid and were used to infect BA/F3 or 32D cells as described previously (Choi *et al.*, 2007). Both types of cell were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Life Technologies, Carlsbad, CA, USA) and mouse IL-3 (Sigma, St Loius, MO, USA) at 10 Units/ml; differentiation of 32D cells was induced by culture in the presence of serum and mouse granulocyte colony-stimulating factor (Sigma) at 0.5 ng/ml. A concentrated preparation of a retrovirus with a VSV-G envelope and encoding both JAK3(M511I) and enhanced green fluorescent protein was used to infect $CD34^-$ c-Kit^+ $Sca\text{-}1^+$ Lineage-$marker^-$ ($CD34^-$KSL) hematopoietic stem cells isolated from the bone marrow of C57BL/6 mice, and the infected cells were transplanted into lethally irradiated mice congenic for the *Ly5* locus (Iwama *et al.*, 2004). *CD4*, *JAK2* and *JAK3* mRNAs were quantitated by reverse transcription and real-time PCR analysis using an ABI7900HT system (Life Technologies) and with the primers 5′-CTGGAATCCAACATCAAGGTTCTG-3′ and 5′-AATTGTAGAGGAGGCGAACAGGAG-3′ for CD4, 5′-CTCCAGAATCACTGACAGAGAGCA-3′ and 5′-CCAC TCGAAGAGCTAGATCCCTAA-3′ for *JAK2* and 5′-GAGC TCTTCACCTACTGCGACAAA-3′ and 5′-AGCTATGAAA AGGACAGGGAGTGG-3′ for JAK3; the cDNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also amplified with the primers 5′-GTCAGTGGTGGACC TGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. The relative abundance of the cDNAs of interest was calculated from the threshold cycle (C_T) for each cDNA and that for *GAPDH* cDNA.

DNMT3A analysis

Recombinant His_6-tagged DNMT3A or DNMT3A(R882H) was expressed in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA), and each protein was purified by stepwise column chromatography as described previously (Suetake *et al.*, 2003). The enzymatic activity of each protein was assayed with -*S*-adenosyl-L-methionine (GE Healthcare, Waukesha, WI, USA) and dIdC or dGdC as substrates (Suetake *et al.*, 2003). The association between Myc epitope-tagged human DNMT3L and wild-type or R882H forms of human DNMT3A in transfected HEK293 cells was examined by immunoprecipitation and immunoblot analyses.

Conflict of interest

The authors declare no conflict of interest.

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Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers

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Identification of the molecular characteristics of intramucosal (IMCs) and submucosal cancers (SMCs) is essential to our understanding of early gastric carcinogenesis. However, little is known regarding the differences between the 2 lesions. One hundred and forty-eight patients with primary early gastric cancer [IMC, 106; SMC, 42] were characterized for expression of cell cycle-related proteins and loss of heterozygosity (LOH). We also examined microsatellite instability (MSI) and methylation status. For LOH and methylation studies, we used a panel of 17 microsatellite markers (3p, 4p, 5q, 9p. 13q, 17p, 18q and 22q) and promoter regions of 9 genes (*MLH-1*, *RUNX3*, *p16*, *HPP1*, *RASSF2A*, *SFRP1*, *DKK-1*, *ZFP64* and *SALL4*) that are frequently altered or methylated in gastric cancers. Overexpression of p53 and cyclin D1 was observed in SMC. In addition, low expression of p27 was more frequent in SMC than in IMC. Frequencies of 4p, 9p, 13q and 22q were significantly higher in SMC than in IMC. The *SALL4* gene was frequently methylated in SMC compared with IMC. However, other gene methylations were common in both IMC and SMC. The frequency of LOH-high status/methylation-low status was significantly higher in SMC than in IMC. However, LOH-low status/methylation-high status in SMC was more frequently found in IMC. Our data confirm that methylation of cancer-related genes plays a major role in the development of IMCs. Importantly, the results also show that gastric submucosal progression is characterized by the accumulation of specific genetic alterations. In addition, changes of cell cycle-related proteins are associated with cancer progression.

Gastric adenocarcinoma is one of the most commonly diagnosed malignancies worldwide and is a leading cause of cancer mortality in Japan, Korea and South America. Although human tumor development has been analyzed at the molecular level,[1,2] the underlying molecular alterations that drive the neoplastic process in gastric cancers are not understood. In human tumors, impairment of the cell cycle is likely a critical mechanism underlying tumor development. G1 cyclins (cyclins D1 and A) and cyclin-dependent kinase (CDK) complexes play important roles in the transition through the G1 phase of the cell cycle, and their overexpression is implicated in neoplasia.[3–6] CDK inhibitors, including p27 and p21, negatively regulate G1 progression by binding to G1 cyclins/CDK complexes, thus inhibiting their activity and thereby preventing entry into the cell cycle.[3–5] In addition, nuclear accumulation of β-catenin plays an essential role in cell cycle progression as it induces cyclin D1, c-myc and MMP7.[7,8,9] Analysis of cell cycle-related protein expression is important for early gastric cancers (EGCs) to elucidate the mechanisms behind early gastric carcinogenesis.[3,4]

Many laboratories have studied genomic instability and the resulting allelic imbalance in gastric carcinomas through analyses of loss of heterozygosity (LOH) and comparative genomic hybridization.[10,11] Alterations most commonly found in gastric carcinogenesis include LOHs at 3p, 5q, 9p, 13q, 17p, 18q and 22q.[12–14] Thus, LOHs are useful markers for defining tumor aggressiveness. Accumulation of tumor LOHs (LOH-high status) is closely associated with tumor progression or tumor-invasive ability. Epigenetic aberrations are mechanistically important in human carcinogenesis.[15] A number of tumor suppressor genes are silenced by promoter methylation during gastric cancer development. Some gastric cancers undergo promoter methylation, which is referred to as the CpG island methylator phenotype (CIMP, methylation status).[15,16] In general, methylation-high (CIMP-high) tumors have distinct features, such as favorable tumor location (proximal location), greater predilection for females and specific genetic alterations (BRAF mutation and low incidence of

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Table 1. Clinicopathological findings of early gastric cancers

	Intramucosal cancer (%)	Submucosal cancer (%)
Total	106	42
Sex (man/woman)	70/36	32/10
Age (mean)	49–86 (71.6)	61–90 (73.6)
Size (mm, mean)	4–80 (17.1)	6–59 (21.8)
Location (P/D)	12/94	11/31
Macroscopic type		
Elevated type	73 (68.9)	18 (42.8)
Mixed type	7 (6.6)	12 (28.6)
Depressed type	26 (24.5)	12 (28.6)
Histological type		
WDA	94 (88.7)	28 (66.7)
MDA	9 (8.5)	13 (31.0)
PA	3 (2.8)	1 (2.3)
Histological grade		
Low	11 (10.3)	0 (0)
Intermediate	60 (56.7)	12 (28.6)
High	35 (33.0)	30 (71.4)

p53 alteration).[17] In addition, microsatellite instability (MSI) defines a novel molecular subtype of tumors.[17,18] In previous studies, MSI detected different changes compared with those observed in LOH-high status tissue. MSI overlaps with CIMP status (methylation-high status) in terms of clinicopathological and molecular features.[17–19] A molecular classification based on LOH status, methylation status and MSI is increasingly important in gastric carcinogenesis, because those alterations reflect global genomic or epigenetic aberrations.[15,17,18]

EGC is defined as a tumor that may invade into but is confined to the submucosa, irrespective of the presence of lymph node metastases.[19] Analysis of molecular alterations in EGC is important for understanding initial events in early gastric carcinogenesis. EGC is subclassified into intramucosal (IMCs) and submucosal (SMCs) cancers.[19] SMC could be regarded as an intermediate stage between IMC and advanced cancer. We hypothesize that submucosal tumors possess distinct molecular alterations compared with IMCs and that those alterations are critical for tumor progression. Thus, identifying the molecular differences between IMCs and SMCs is essential for understanding gastric carcinogenesis. In the present study, we attempted to identify these molecular differences.

Material and Methods

Patients

Detailed clinicopathological data derived from 106 IMCs and 42 SMCs are summarized in Table 1. The criteria used to diagnose IMC were based on Japanese histological criteria,[20] which differ from those used by western pathologists.[21,22]

Table 2. List of antibodies that we used

Antibody	Clone	Supplier	Dilution
p53	DO-7	Novocastra	1:100
p21	SX118	Dako	1:25
p27	SX53G8	Dako	1:50
β-Catenin	BD	Transduction Laboratories	1:200
Cyclin D1	SP4	Nichirei	1:100
Cyclin A	6E6	Novocastra	1:100
MLH-1	G168-15	BD Pharmingen	1:20
MMP7	141-7B2	Daiichi Finechemical	1:100
Ki-67	MIB1	Dako	1:100
MUC2	Ccp58	Novocastra	1:100
CD10	56C6	Novocastra	1:100
MUC5AC	CLH2	Novocastra	1:100
MUC6	CLH5	Novocastra	1:100

The nuclear grade was determined according to published criteria.[14]

For molecular investigations, tumor tissue was isolated from the resected stomach [samples from endoscopic submucosal dissection (ESD): 106 IMCs and 9 SMCs; samples from gastrectomy: 33 SMCs] using biopsy forceps within 30 min of resection. Most SMCs were obtained from gastrectomy, given that those SMCs were not an indication for ESD. The most distant normal gastric mucosa from the neoplasm was removed from the submucosa using scissors as a control for molecular analysis. Tissue for clinicopathological analysis was obtained from a region of the resected stomach adjacent to the region used for genetic analysis. All of the noncontrol tissue samples contained cancer tissue. Only tumor samples where the neoplastic cells accounted for at least 50% of the tissue cell population were selected (13 of 161 tumors were omitted due to failure to meet these criteria). In the SMCs, only tumor samples obtained from submucosal lesions were used for molecular analysis.

Immunohistochemical procedure

EGC specimens were fixed in buffered formalin and embedded in paraffin, according to routine procedures. For this study, 3-μm sections were prepared, dried, deparaffinized and rehydrated before microwave treatment (H2500, Microwave Processor, Bio Rad) in citrate buffer (pH 6.0) for 5 min. An automatic staining machine (DAKO Envision+ system) was used for the immunohistochemical procedure.[14] The slides were counterstained in hematoxylin, dehydrated and mounted. The antibody sources used in this study are shown in Table 2.

Immunohistochemical assessment

Nuclear immunostaining data for cyclin D1, cyclin A, p21, p27, p53, β-catenin, MLH-1 and ki-67 were expressed as the percentage of positive epithelial cells in relation to the total

number of cells encountered in at least 5–10 representative high-power fields (500–1,000 epithelial cells). The immunoreactivity was measured by means of light microscopic examination and evaluated independently by 2 experienced pathologists. (T.S. and N.U., not included in this manuscript). Differences in interpretation were reconciled by reviewing the slides separately. No necrosis was found in the tissue examined. Only nuclear staining was regarded as significant, and tumor cells showed a range of staining intensities. Cytoplasmic staining was disregarded. Tumor cells with weak staining were not considered positive.

Cutoffs for positive expression of p21 and p27 were defined as >10%, while for cyclin D1 and cyclin A, positivity was >30% as conducted by previous studies.[6,23,24] In the evaluation of p53 overexpression, samples showing >10% staining were considered positive.[14] More than 20% of cells showing β-catenin nuclear expression were regarded as positive.[25] Expression of MLH-1 was interpreted and compared with the surrounding tissue. If expression of MLH-1 in the tumor cell was considered to be low compared with its surrounding nonneoplastic tissue, the tumor tissue was regarded as low/negative staining. Finally, ki-67 staining >30% was judged as a highly proliferative tumor.

Mucin phenotypes of tumor cells were subclassified as follows: gastric, intestinal, mixed and unclassified, according to previous criteria.[14] In brief, the gastric phenotype included tumors having predominantly intracytoplasmic expression of gastric mucin, as determined by immunostaining of human gastric mucin (MUC5AC) or (and) pyloric gland mucin (MUC6), but with no MUC-2 positive cells. The intestinal phenotype was constituted by MUC2-positive cells or (and) CD10-positive cells (along with a brush border). The mixed-type contained immunostaining features of both gastric type as well as intestinal type. Finally, tumors lacking gastric or intestinal staining patterns were termed "unclassified." Immunopositivity of >5% of the tumor cells was regarded as positive according to the guidelines established by a previous report.[26]

PCR analysis

PCR reactions were performed using a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer, CA) with 50–100 ng of genomic DNA as template, 25 pM of each primer, 0.2 mM deoxynucleotide triphosphate (dNTP), 1× reaction buffer containing 1.5 mM $MgCl_2$, and 1.5 u Taq polymerase (Boehringer Mannheim Co., Germany) in a final reaction volume of 25 μL. Samples were processed for 25–30 cycles, with each cycle consisting of 30 sec at 94°C, 1 min at 55–58°C and 2 min at 72°C, followed by a final 10-min extension at 72°C. For quantitative detection of the allelic loss at each locus, PCR-LOH analysis and MSI were performed as described previously.[27,28] A 1-μL aliquot of the PCR product was added to 3 μL formamide and a 0.5 μL TAMRA 500 size standard (Applied Biosystems, CA), loaded on a 6% polyacrylamide-8 M urea gel, and run for 2–6 hr in a 373A Automated Sequencer (Applied Biosystems) at a constant power of 30 W.

Assessment of LOH by polymerase chain reaction

Allelic losses on chromosomes 3p, 4p, 5q, 9p, 13q, 17p, 18p and 22q were examined in paired tumor and normal DNA samples obtained from 148 EGC patients using 17 highly pleomorphic microsatellite markers (D3S2402, D3S1234, D4S2639, D4S1601, D5S107, D5S346, D5S299, D5S82, D9S171, D9S1118, D13S162, TP53, D18S487, D18S34, D22S274, D22S1140 and D22S1168). These microsatellite markers have been used frequently in studies of gastric carcinomas.[10,12–14] In addition, a variable number of tandem repeat polymorphisms at the DCC locus were tested. Microsatellite sequences were obtained from specific primers reported in the Genome Database (http://gdbwww.gdb.org/gdb/).

Determination of LOH

The peaks produced by the normal DNA sample were used to determine whether the cancerous sample was homozygous (1 peak) or heterozygous (2 peaks). The allelic ratio was calculated as described by Habano *et al.*[27] A tumor was considered to have allelic loss if the allele peak ratio was less than or equal to 0.60, representing an allelic signal reduction of at least 40%. We interpreted this allelic imbalance as allelic loss (LOH) with the provision that, in some cases, the changes in the allele peak ratio may have resulted from allelic amplification. Tumors exhibiting MSI at a given locus were not evaluated for allelic loss. The data were collected automatically and analyzed using GeneScan software (Applied Biosystems) to determine the allele score and to assess the possibility of allelic loss.

Scoring of LOH

LOH status was scored according to the following criteria. A tumor sample was considered to be LOH-high if 3 or more of the markers showed allelic loss. When data showed that 1 or 2 markers were lost, the tumor was designated as LOH-low.[19]

Assessment of MSI

Six different loci were considered for MSI assessment, including all those recommended by the Bethesda panel for colon cancer (BAT25, BAT26, D5S346, D2S123 and D17S250).[29] A tumor was defined as MSI-positive when a novel, abnormal-sized band occurred in the tumor sample compared with the corresponding normal DNA sample. MSI-positive colorectal carcinomas were used as controls in the study and were divided into 2 groups, those with high-level instability (*i.e.*, MSI at [dbmtequ]33% of loci) and those with low-level instability (*i.e.*, MSI at [dbltequ]17% of loci), as described previously. However, tumors with only 1 alteration of the marker examined using the above criteria and those previously

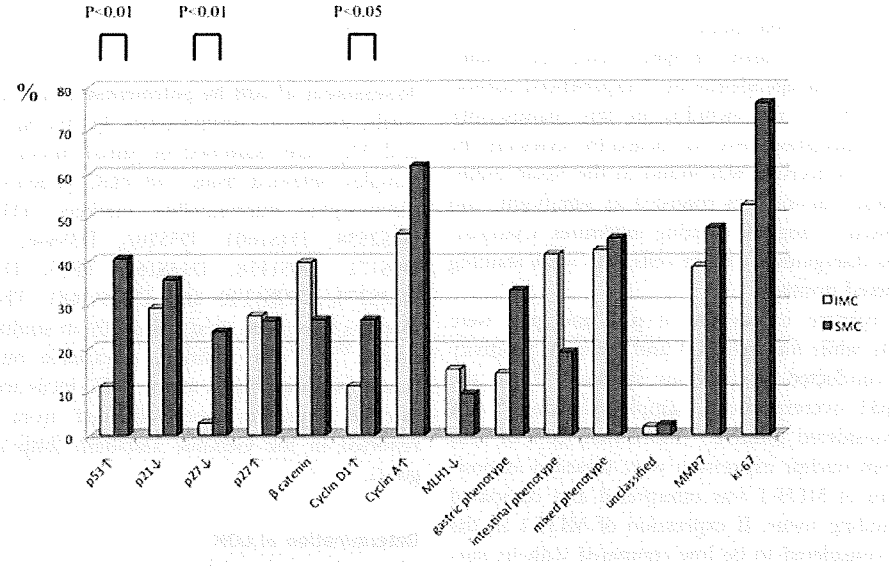

Figure 1. Frequencies of expression of cell cycle-related proteins and mucins in IMCs and SMCs. Sample numbers are as follows: IMC, 106 and SMC, 42 for all parameters.

categorized as MSI-low were considered MSI-negative tumors in this study.

Confirmation of methylation

Bisulfite treatment of genomic DNA was carried out as described previously.[30] For examination of methylation status, we used a combined bisulphate restriction analysis (COBRA) as described previously.[29,30] The COBRA of *MLH-1*, *RUNX3*, *p16*, *HPP1*, *RASSF2A*, *SFRP1*, *DKK-1*, *ZFP64* and *SALL4* genes were determined using primers, restriction enzyme and conditions as previously described.[39–37]

The colon cancer cell lines RKO and SW48 (American Tissue Culture, Manassas, VA) and water were used as positive and negative controls, respectively. After amplification, the PCR products were digested with restriction enzymes and electrophoresed on 3% agarose gels. The gels were stained with ethidium bromide, and the proportion of methylated alleles was visually compared with unmethylated alleles. Cleavage fragments were quantified by densitometry.

Tumors were classified as methylation-negative/low (methylation-low) if 1 or 2 loci were methylated and methylation-high if 3 or more were methylated.

Statistical analysis

The data were analyzed using a chi-squared test with the aid of StatView-IV software (Abacus Concepts, Berkeley, CA). Samples were determined to be significantly different when the p value was less than 0.05.

Results

Expression of cell cycle-related proteins in gastric IMCs and SMCs

The results of the survey are depicted in Figure 1. Overexpression of the p53 gene product was significantly more frequent in SMCs (40.5%) than in IMCs (11.3%; $p < 0.01$). Conversely, the frequency of low expression of the p27 product was statistically higher in SMCs (23.8%) than in IMCs (2.8%; $p < 0.01$). In addition, cyclin D1 overexpression was more frequently found in SMCs (26.5%) compared with IMCs (11.3%; $p < 0.05$). The Ki-67 positivity rate for SMCs (76.2%) was higher than that of IMCs (52.8%; $p < 0.05$). There were no significant differences in low expression of p21 and MLH-1 between IMCs (29.2 and 15.1%, respectively) and SMCs (35.7 and 9.5%, respectively). Overexpression of cyclin A and nuclear accumulation of β-catenin were commonly observed in IMCs (46.2 and 39.6%, respectively) and SMCs (61.9 and 26.5%, respectively). Although MMP7 was primarily expressed at the invasive front of the submucosal lesion, no significant difference of MMP7 was observed between IMCs (38.7%) and SMCs (47.6%). As for mucin phenotype, the gastric phenotype was found significantly more often in SMCs (33.3%) than in IMCs (14.2%) ($p < 0.05$). By contrast, the intestinal phenotype was expressed more in IMCs (41.5%) than in SMCs (19%).

Analysis of LOHs at multiple chromosomal loci in IMCs and SMCs

LOH data are summarized in Figure 2. Allelic loss of 5q was a common alteration in IMCs (38.5%) and SMCs (39.5%).

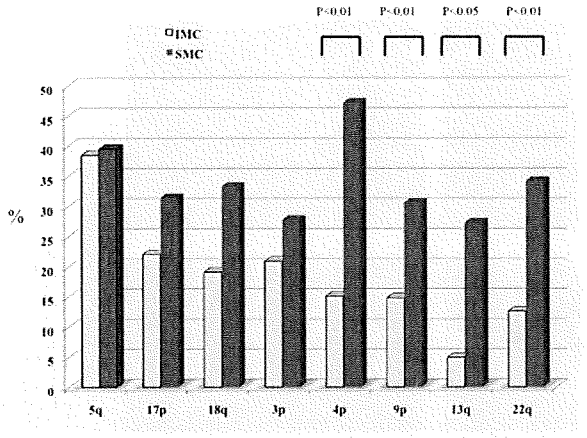

Figure 2. Frequencies of LOH at multiple cancer-related chromosomal loci in IMCs and SMCs. IMC, 5q, 35/91, 38.5%; 17p, 18/82, 22%; 18q, 17/89, 19.1%; 3p, 19/91, 20.8%; 4p, 14/93, 15.1%; 9p, 13/88, 14.8%, and 22q, 11/87, 12.6%. SMC, 5q, 15/38, 39.5%; 17p, 11/35, 31.4%; 18q, 12/36, 3p, 33.3%; 10/36, 27.8%; 4p, 17/36, 47.2%, 9p, 11/36, 30.6% and 22q, 13/38, 34.2%.

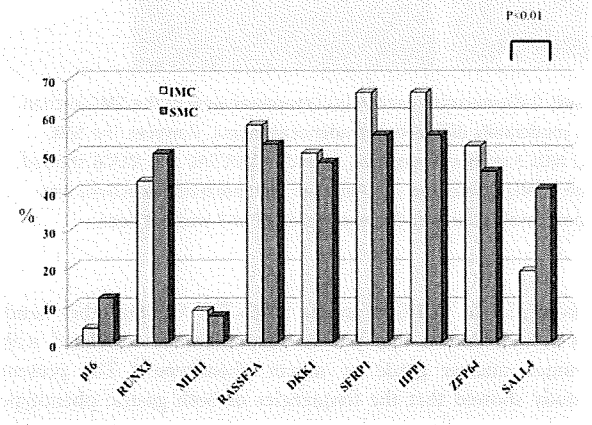

Figure 3. Frequencies of multiple gene promoter methylations in IMCs and SMCs. Sample numbers are as follows: IMC, 106 and SMC, 42 for all genes.

Although allelic losses of 17p, 18q and 3p were more frequent in SMCs (31.4, 33.3 and 27.8%, respectively) than in IMCs (22, 19.1 and 20.8%, respectively), no significant differences were found. The frequencies of 4p, 9p and 22q allelic losses were significantly higher in SMCs (47.2, 30.6, and 34.2%, respectively) than in IMCs (15.1, 14.8 and 12.6%, respectively) ($p < 0.01$). Finally, allelic loss at 13q was more frequently found in SMCs (27.3%) than in IMCs (4.9%) ($p < 0.05$).

Analysis of methylation in IMCs and SMCs

The associations between the 2 lesions are shown in Figure 3. High frequencies of methylation of *RUNX-3*, *RASSF2A*,

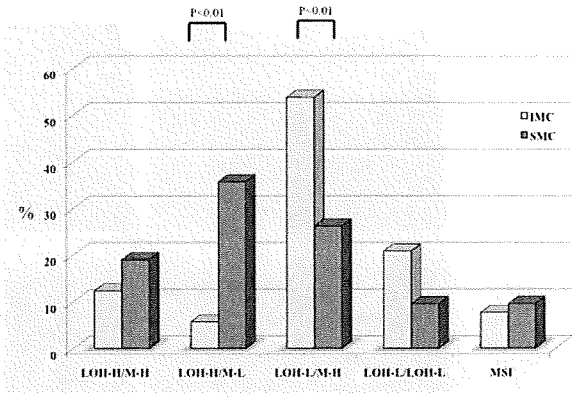

Figure 4. Frequencies of LOH and methylation status in IMCs and SMCs. Sample numbers are as follows: IMC, 106 and SMC, 42.

DKK1, *SFRP1*, *HPP1* and *ZFP64* were commonly observed in both IMCs (42.7, 57.5, 50, 66, 66 and 51.9%, respectively) and SMCs (50, 52.4, 47.6, 54.8, 54.8, and 45.2%, respectively). However, only *SALL4* methylation was significantly more frequent in SMCs (40.5%) compared with IMCs (18.9%) ($p < 0.01$). Although p16 was frequently methylated in SMCs (11.9%), when compared with IMCs (3.8%), no significant difference was detected between them. The frequencies of *MLH-1* methylation were low in IMCs (8.5%) and SMCs (7.1%).

Analysis of molecular status of IMCs and SMCs

The molecular status of intramucosal and submucosal tumors is displayed in Figure 4. The frequency of LOH-high/methylation-low status was significantly higher in SMCs (35.7%) than in IMCs (5.7%; $p < 0.01$). On the other hand, LOH-L/methylation-H status was more frequently found in IMCs (53.8%) than in SMCs (26.2%; $p < 0.01$) Although LOH-L/methylation-L status was found more frequently in IMCs (20.8%) compared with SMCs (9.5%), the difference did not reach a statistically significant level. There were no significant differences of LOH-H/methylation-H status between IMCs and SMCs. The frequencies of MSI in IMCs and SMCs were 7.6 and 9.5%, respectively. Finally, the frequency of p53 overexpression of LOH-high status cancers (47.6%) was significantly higher than that of LOH-L status (9.6%; $p < 0.01$).

Representative histological and molecular examples of SMC are shown in Figures 5 and 6.

Discussion

This study was conducted to examine the differences in molecular alterations between IMCs and SMCs in early gastric carcinogenesis. Identifying the molecular differences between the 2 lesions is important in understanding carcinogenetic mechanisms involving SMCs. This study successfully documented a number of differences in the molecular status of IMCs and SMCs.

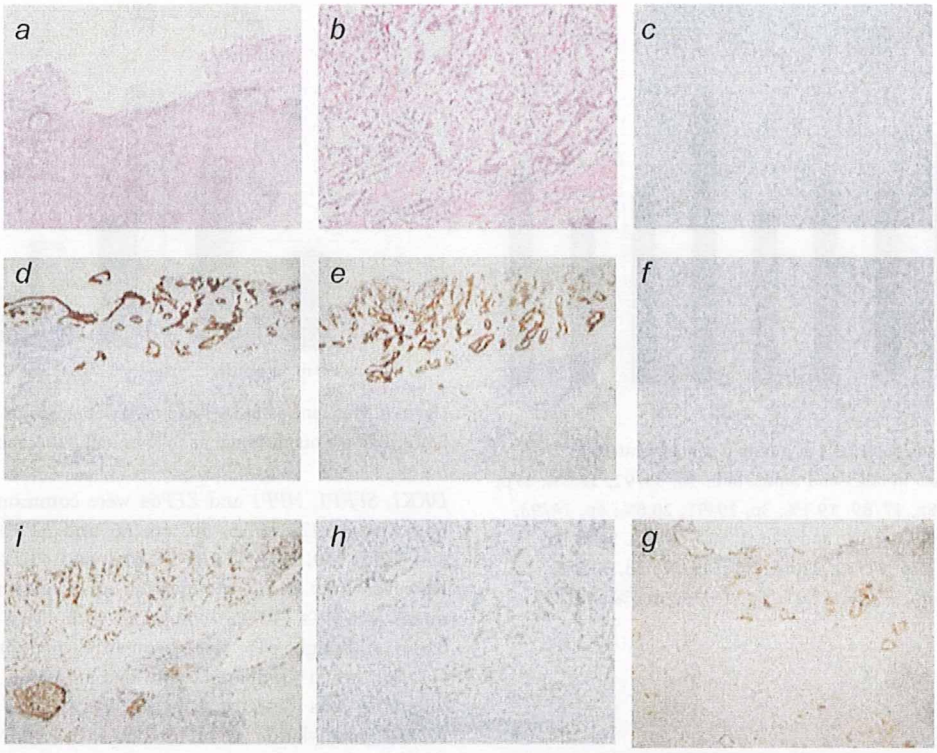

Figure 5. Representative example of the immunohistochemical study of early (intramucosal) cancer. (*a*) Low-power view of the SMC section. (*b*) High-power view of the cancer section. The tumor was diagnosed as a moderately differentiated adenocarcinoma. However, the SMC was poorly differentiated and also had massive lymphocytic infiltrates. (*c*) MUC2 stain was negative. (*d* and *e*) MUC5AC and MUC6 showed positive staining in the primary mucosal lesion, respectively. (*f*) Expression of MLH-1 was low. (*g*) Although high proliferative activity was seen in the mucosal lesion, low expression of ki-67 was found in the submucosal lesion. (*h*) Overexpression of p53 was not seen. (*i*) MMP-7 is positive in cancer tissue.

Recent studies have shown that mucin expression by tumor cells is closely associated with gastric tumorigenesis.[38] In fact, the mucin phenotype of tumor cells correlates with clinicopathological findings and molecular alterations.[14,38] In the present study, although the intestinal phenotype was primarily found in IMCs, the gastric phenotype was frequently detected in SMCs. On the other hand, Wakatsuki *et al.* indicated that tumors with the intestinal phenotype had the worst prognosis.[39] While this finding seems to conflict with our data, patient prognosis is often determined by a multitude of factors. Our finding suggests that tumor cells with a gastric phenotype are more likely to invade into the submucosa in gastric cancers.

In the present study, p53 overexpression was more frequently found in SMCs than in IMCs. This finding suggests that p53 overexpression plays a major role in submucosal invasion of IMC and represents a novel predictive marker for SMC. In addition, this finding implies that p53 overexpression is not associated with the mucosal onset of gastric carcinogenesis. In our data, the frequency of p53 overexpression by SMC was ~40%, consistent with previous reports ranging from 35.7 to 57.1% in gastric SMCs.[40,41] On the other hand, it is well known that p53 overexpression is correlated with tumor nuclear grade or mucin phenotype.[22] The present study showed that the frequencies of both gastric phenotype and tumor cells of high nuclear grade were greater in SMCs than in IMCs. These findings suggest that both gastric mucin phenotype and tumor nuclear grade reflect p53 overexpression in gastric SMCs.

Previous studies have shown that cyclins are useful immunohistochemical markers when evaluating the aggressiveness of tumor cells.[4,6] In the present study, cyclin D1 was upregulated in SMCs compared with IMCs. *Cyclin D1*, a target gene of Wnt signal transduction, is an essential molecule in human tumorigenesis. Cyclin D1 induces significant changes in gene expression in human tumors, such as phosphorylated Rb, myc and cell adhesion-related genes.[8,42] This finding suggests that *cyclin D1* overexpression may play an important role in the progression from IMC to SMC. With regard to cyclin A, however, no difference was found between IMCs and SMCs, suggesting that upregulation of cyclin A is an early event in the development of differentiated-type gastric cancers.

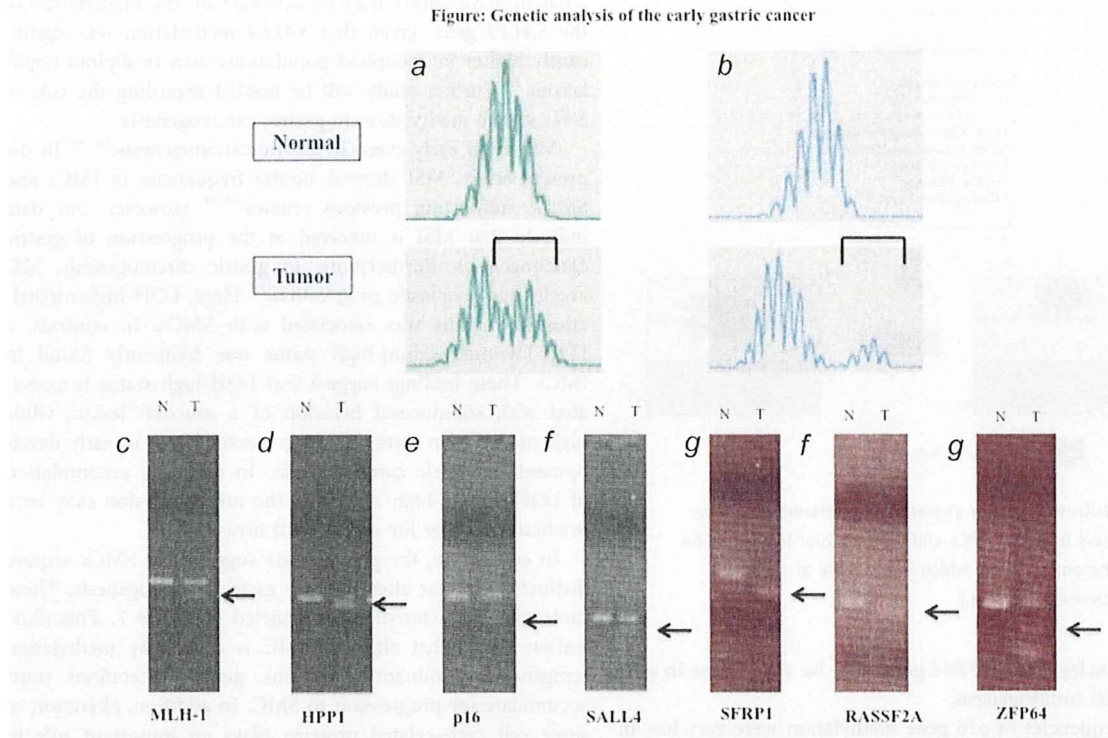

Figure 6. Representative example of the molecular study of early (intramucosal) cancer. Additional peaks of BAT 25 and BAT 26 were positive. Multiple methylations were seen in c to g. Brackets and arrow heads indicate MSI and methylated bands, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Low or nonexistent expression of p27 was associated with patient outcome and prediction of tumor aggressiveness,[43] although opposing data have been reported.[44] However, the point at which low p27 expression occurs in the progression of gastric cancers remains unknown. We found that low expression of p27 was associated with submucosal invasion in differentiated-type gastric cancers. However, Oya *et al.* indicated that reduced expression of p27 occurred more frequently in carcinoma than in adenoma.[45] Although the reason for these differences remains unknown, they could be due to the number of samples examined, differences in cutoff values for the examined proteins, the sources of antibodies, or the tumor grade examined in the study. In addition, our survey demonstrated that the reduction of p21 was a common alteration in both IMCs and SMCs. This finding suggests that p21 reduction is closely associated with early tumorigenesis of gastric cancers.

Numerous publications have reported regions of LOH that occurred frequently in gastric carcinogenesis, particularly those at 5q, 17p, 18q, 3p, 4p, 9p, 13q and 22q, including *APC* and *p53* genes.[10–14] One previous study showed that aggressive tumor cells tend to acquire LOH and thus LOH, in some cases, proves to be a novel marker for predicting tumor-invasive or aggressive activities.[11] In the present study, 5q allelic loss was a common alteration in both IMCs and SMCs. In contrast, the frequencies of 4p, 9p, 13q and 22q allelic losses were significantly higher in SMCs than in IMCs. These findings suggest that although 5q allelic loss plays a major role in early gastric carcinogenesis, 4p, 9p, 13q and 22q allelic losses are associated with submucosal invasion from mucosal lesions. The frequencies of 17p, 18q and 3p allelic losses in IMCs were low in the present study. We suggest that those allelic losses also contribute to the early development of gastric carcinogenesis, because the frequencies of those allelic losses differed among mucin phenotypes of the differentiated EGCs. That is, the frequencies of those allelic losses were low in intestinal phenotype cancers, but high in gastric phenotype cancers.

We demonstrated that gastric cancer cells are heavily methylated in the early stages of gastric tumorigenesis. This finding suggests that a progressive increase in methylation of most of those genes does not occur during the progression of EGCs. Rather, the data indicate that methylation is an early and essential event in gastric carcinogenesis. In addition, we found that methylation of *RUNX3*, *HPP1*, *RASSF2A*, *SFRP1*, *DKK-1* and *ZFP64* gene promoters plays an important role in early gastric carcinogenesis. In particular, methylation of the *ZFP64* gene was described in the first report identifying frequent alterations in gastric cancers, although its function

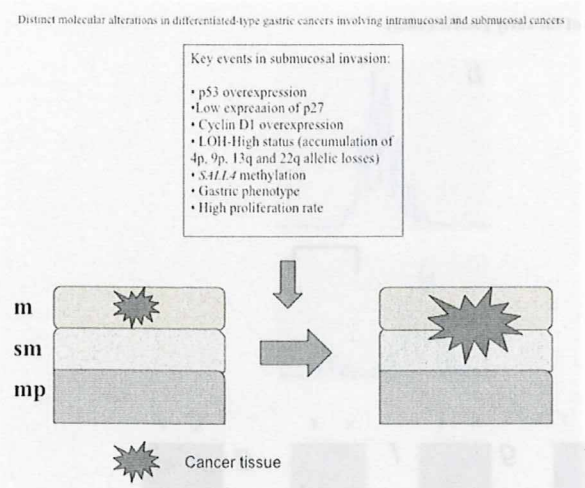

Figure 7. Distinct molecular alterations in differentiated-type gastric cancers involving IMCs and SMCs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

remains unclear. The *ZFP64* gene may be a key gene in gastrointestinal tumorigenesis.

The frequencies of *p16* gene methylation were very low in both lesions. This suggests that inactivation of the p16 gene plays a minor role in EGC. According to our data, only *SALL4* methylation frequency was higher in SMCs than in IMCs. *SALL4* methylation may therefore be associated with submucosal invasion of IMC. Although the function of the *SALL4* gene remains unknown,[46] its expression is linked to Wnt signal transduction.[47] Our finding implies that submucosal invasive ability may be activated by the methylation of the *SALL4* gene, given that *SALL4* methylation was significantly higher in aneuploid populations than in diploid populations.[36] Further study will be needed regarding the role of *SALL4* gene methylation in gastric carcinogenesis.

MSI is an early event in gastric carcinogenesis.[46–48] In the present study, MSI showed similar frequencies in IMCs and SMCs, supporting previous studies.[48,49] However, our data indicate that MSI is involved in the progression of gastric carcinogenesis. Furthermore, in gastric carcinogenesis, MSI accelerates neoplastic progression.[50] Here, LOH-high/methylation-low status was associated with SMCs. In contrast, a LOH-low/methylation-high status was frequently found in IMCs. These findings suggest that LOH-high status is associated with submucosal invasion of a mucosal lesion, while high methylation status plays an essential role in early development of gastric carcinogenesis. In addition, accumulation of LOHs (LOH-high status) in the mucosal lesion may be a predictive marker for submucosal invasion.

In conclusion, the present data suggest that SMCs acquire distinct molecular alterations in gastric carcinogenesis. These molecular characteristics are depicted in Figure 7. This illustration shows that although IMC is caused by methylation (epigenetic) dominant alterations, genetic alterations must accumulate for progression to SMC. In addition, alteration of some cell cycle-related proteins plays an important role in cancer evolution. Further studies will be required to clarify the origins of human early gastric carcinogenesis.

Acknowledgements

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Molecular analysis of single isolated glands in gastric cancers and their surrounding gastric intestinal metaplastic mucosa

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Abstract. The biological properties and underlying genetics of gastric cancer and gastric intestinal metaplasia evolve with neoplastic progression from the genetics of the original gland cell. PCR assay with crypt isolation was used in tumors from 20 patients to examine microsatellite alterations (allelic imbalance at 17p, 5q, 18q, 3p, 4p, and 9p, and microsatellite instability) in glands from each tumor and from intestinal metaplastic lesions. Tumor specimens were processed as either pooled-gland samples or single-gland samples. Pooled gland sample was composed of 10-20 tumor glands, intestinal metaplastic glands, or nonmetaplastic glands. Single gland sample was 10 tumor glands from tumor and single gland sample was 5 gastric intestinal metaplastic and 5 nonmetaplastic glands from its surrounding metaplastic mucosa. Multiple genetic alterations were found in individual tumor glands, with various subclonal expansions seen within the same tumor. Although microsatellite instability was found in 2 of 20 tumor single-gland samples, none was detected in metaplastic single-gland samples. Most cancers appear to have a heterogeneous composition. On the other hand, microsatellite alterations were also detected within the nonmetaplastic as well as intestinal metaplastic single-gland samples. In conclusion, the present data on tumor and corresponding intestinal metaplastic and nonmetaplastic glands suggest that genetic alterations already occur within the surrounding of the noncancerous mucosa.

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Key words: crypt isolation, gastric cancer, intestinal metaplasia, loss of heterozygosity, microsatellite instability

Introduction

It has long been established that gastric cancers develop from a single gland cell that undergoes an accumulation of genetic changes (1), and which eventually results in a malignant tumor with monoclonal character (2,3). Recent molecular genetic evidence also supports this concept (1). Despite the monoclonal origin of such cancerous tumors, any single tumor consists of numerous tumor glands, which may have various genetic alterations relative to the cell of origin. According to established theory, these accumulated genetic alterations can be identified by loss of heterozygosity (LOH) at many chromosomal loci within the tumor (4). It is also true that differences between tumor glands are thought to cause genetic heterogeneity within the same tumor (1-3), and some studies stated that genetic heterogeneity is frequently found in individual gastric cancers (5-7). Such genetic heterogeneity within the same tumor complicates the development of a tumor-treatment strategy based on tumor pathogenesis (8,9). Analyzing the accumulation of multiple genetic alterations in a single tumor gland is thought to be useful for assessing genetic differences among the cells of a single tumor (2,3).

Gastric intestinal metaplasia (GIM) is frequently found in the mucosa surrounding a gastric cancer (10), and is commonly thought to be a precancerous condition for gastric cancer, especially differentiated-type gastric cancers (11,12). However, an alternative hypothesis, the 'paracancerous' condition, as distinct from the 'precancerous' condition, has been described, especially in Japan (13). The paracancerous hypothesis derives from the fact that, based on routine pathological examination of surgical specimens, no pathologist has yet been able to identify the feature proving that differentiated-type gastric carcinomas arise directly from intestinal metaplastic glands. Accordingly, it remains unclear whether GIM is truly a precancerous lesion. In addition, GIM is not a single entity but rather a heterogeneous group of metaplastic glands (14). Previous studies suggested that GIM by itself is associated with carcinogenesis of differentiated-type gastric cancer and that GIM has a heterogeneous composition with different

biological characteristics (14-17). Identification of 1 or more biomarkers to reliably differentiate GIM types associated with gastric cancer and the molecular alterations associated with various types of GIM may be very valuable clinically as a tool for identifying patients who may be at higher risk for gastric cancer. However, molecular alterations contributing to the development or neoplastic progression of GIM have not been clarified (18). In addition, a non-neoplastic gland which is seen in the gastric intestinal metaplastic mucosa is not necessarily an intestinal metaplastic gland. Nonmetaplastic glands are often observed in gastric metaplastic mucosa. It is likely that the nonmetaplastic glands are also associated with gastric carcinogenesis. However, the molecular alterations of nonmetaplastic glands within the gastric intestinal metaplastic mucosa are still not identified.

For genetic evaluations of an individual gland taken from tissue of a single gastric cancer and the surrounding GIM and nonmetaplastic glands (intestinal metaplastic and non-intestinal glands), it is necessary to isolate single glands from the larger tissue mass. The crypt isolation method can be used to obtain individual tumor and non-neoplastic glands from a given tumor or surrounding tissue (19,20). In the current study, we used crypt isolation methodology to examine molecular alterations of single tumor glands from a gastric cancer and single glands from surrounding GIM tissue. The aim of this study was to verify the role of intratumoral molecular differences in sporadic gastric cancer and to further our understanding of molecular alterations in GIM tissue that result in gastric tumorigenesis.

Materials and methods

Tissue samples. Tissues were obtained from 20 patients with sporadic primary gastric adenocarcinoma of differentiated type, who had undergone gastrectomy. Of the 20 patients, there were 17 men and 3 women (mean age: 64.2 years). Tumor histological type and stage were classified according to the Japanese Research Society criteria for cancer of the stomach (21). The location of the gastric cancer was determined and the tumors were subclassified into 2 groups: proximal or distal. Clinicopathological data for the 20 patients in our study are shown in Table I. In addition, in order to clarify genetic alterations in non-neoplastic glands not demonstrating intestinal metaplasia (nonmetaplastic gland), nonmetaplastic glands associated with gastric adenocarcinomas were analyzed in the 10 tumors in which they were available.

Fresh tumor specimens and adjacent tissue were obtained from resected gastric cancers. Gastric mucosa most distal to the tumor that was regarded as normal was removed from the submucosa with scissors and discarded. In contrast, intestinal metaplastic mucosa was obtained from antral mucosa. Tissue tumor samples were obtained primarily from the central area of the tumor.

Crypt isolation technique. Isolation of tumor and mucosal glands was performed as previously described (22). Briefly, fresh tumor and non-neoplastic mucosa were separated from the underlying tissue layer and cut with a razor into minute pieces, and then incubated at 37°C for 30 min in Hanks' balanced salt solution (HBSS), which is calcium- and magnesium-free, containing 30 mmol/l ethylenediaminetetraacetic acid (EDTA). Specimens were stirred in HBSS for 30-40 min to allow isolation of cancerous and normal glands from the lamina propria mucosa or fibrous stroma. Isolated specimens were immediately fixed in 70% ethanol and stored at 4°C until analysis.

Table I. Clinicopathological findings of gastric cancers examined.

Total	20
Gender (male/female)	17/3
Age (mean)	32-80 (62.7)
Locus	
Proximal	2
Distal	18
Histological type	
WDA	8
MDA	12
Stage	
I	3
II	4
III	10
IV	3

WDA, well differentiated adenocarcinoma; MDA, moderately differentiated adenocarcinoma.

Representative cancerous glands are shown in Fig. 1a-f. To obtain intestinal metaplastic glands, nonmetaplastic glands were isolated from antral mucosa [stained with Alcian blue (pH 2.5).

Identification of isolated gastric intestinal metaplastic glands. In the present study, GIM was recognized by the presence of goblet cells stained by Alcian blue. Detailed histological identification of intestinal metaplasia was performed on sections of paraffin-embedded tissue located adjacent to the tissue used for crypt isolation. Most tissue sections showed incomplete intestinal metaplasia.

Identification of isolated gastric intestinal nonmetaplastic glands. Nonmetaplastic glands which were not stained by Alcian blue were isolated from antral mucosa. These glands could be easily distinguished from metaplastic glands. However, nonmetaplastic glands were obtainable in only 10 of 20 cases.

Identification of isolated normal gastric glands. Normal mucosal tissue was defined as glands obtained from Alcian blue-negative fundic mucosa, and was confirmed by histological examination. Normal mucosa was used to examine microsatellite alterations in normal (negative) controls.

DNA extraction. Isolated tumor glands were handled as follows: Ten to 20 isolated glands (tumor, intestinal metaplastic, and nonmetaplastic glands) were obtained from each

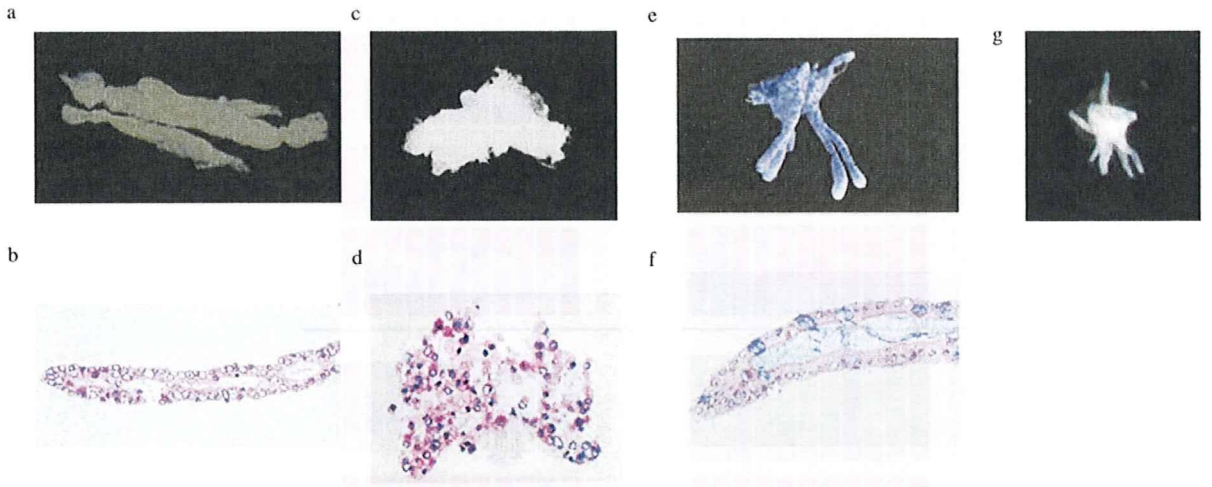

Figure 1. Well-differentiated adenocarcinoma. (a) As seen under a dissecting microscope and (b) with hematoxylin and eosin staining (H&E). A single moderately differentiated tumor gland (c) under a dissecting microscope and (d) as seen with H&E staining. Single intestinal metaplastic gland (e) as stained by Alcian blue (pH 2.5) under a dissecting microscope and visualized with H&E staining (f). Single non-intestinal metaplastic gland (g).

Table II. Frequencies of allelic imbalances at each locus in tumor pooled-gland samples.

	Informative cases	Pooled-gland sample (%)
17p	15	8 (53.3)
5q	18	10 (55.6)
18q	18	8 (44.4)
3p	18	8 (44.4)
4p	18	10 (55.6)
9p	14	13 (92.6)

tissue sample. DNA extraction was performed on these glands to create the pooled-gland samples (3). Separately, 10 tumor glands and 5 intestinal metaplastic glands or nonmetaplastic glands were obtained from each tumor examined and its surrounding antral mucosa. DNA was extracted from each individual tumor, intestinal metaplastic, and nonmetaplastic gland, respectively, to create the single-gland samples, using techniques described previously (2,3). The DNA concentration of single tumor glands was estimated by the TagMan real-time PCR method using the ribosomal protein P0 (3684) gene as a reference (3). In addition, the amount of DNA content that we examined varied from 98.6 to 1034.8 ng (mean: 410.1 ng). On the other hand, that of DNA content examined in single intestinal metaplastic glands varied from 112.6 to 253.4 ng (mean: 198.7 ng).

Microsatellite analysis. Microsatellite analysis included 15 microsatellite markers (2 mono- and 13 dinucleotide repeats). These markers were selected either because of their location at chromosomal sites in or near genes known to be involved in gastric carcinogenesis (3p, 4p, 5q, 9p, 17p, and 18q), or because they are very sensitive markers for determination of microsatellite instability (MSI) (BAT25 and 26). The markers used for analysis of LOH in this study were: 3p (D3S2402, D3S1234), 4p (D4S2639, D4S1601), 5q (D5S107, D5S346, D5S299, D5S82), 9q (D9S171, D9S1118), 17p (TP53), and 18q (D18S487, D18S34).

PCR reactions were performed as described previously (23,24). One of the primers used for amplification was fluorescently labeled. PCR products were separated and detected with an automated sequencing system as previously reported (19,23).

Scoring of allelic imbalance. Allelic imbalance (AI) was determined using a calculation method described previously (25). A tumor was considered to have AI if the allelic peak ratio was <0.6, representing an allelic signal reduction of at least 40%. We interpreted this allelic imbalance as LOH with the provision that, in some cases, the change in allele peak ratio may have resulted from allelic amplification. When AI was observed in at least 1 locus of the markers examined, imbalances of the examined loci were confirmed. Finally, tumors exhibiting MSI at a given locus were not evaluated for LOH.

The overall extent of LOH for each single-gland sample (tumor, intestinal metaplastic, or nonmetaplastic single-gland sample) was calculated as follows: the number of single glands showing LOH divided by the number of informative (excluding uninformative cases, samples showing MSI, and cases that were not examined) single glands for each tumor or each intestinal metaplastic gland sample.

Scoring of microsatellite instability. An additional peak in tumor DNA compared with nontumor tissue was classified as instability for the marker examined. Instability in BAT 25 or 26 was defined as MSI positive (26).

Results

In the present study, PCR analysis was performed reproducibly and there were no PCR failures, as has been described elsewhere (3). The frequencies of allelic imbalance at the

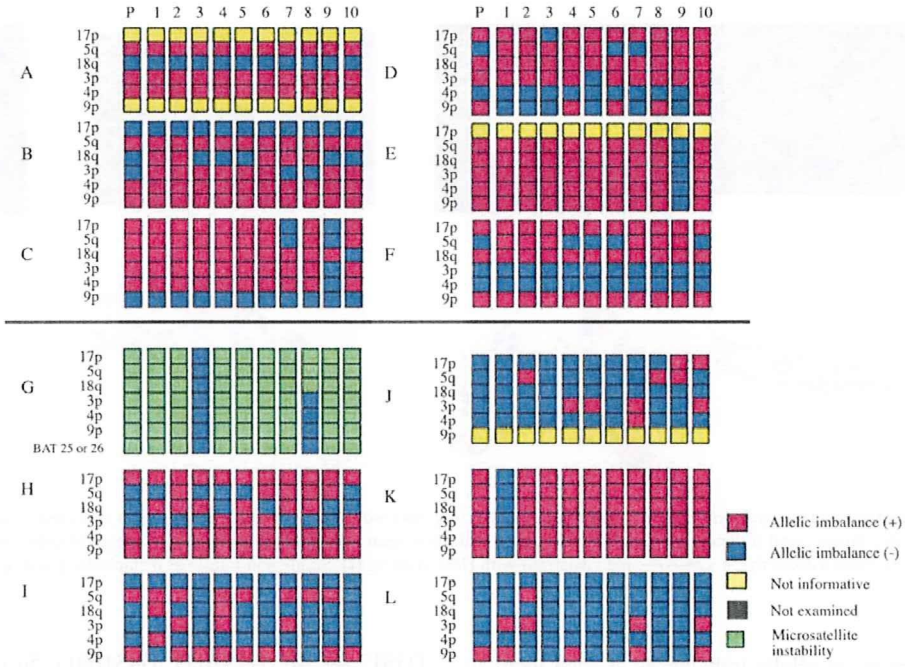

Figure 2. A detailed view of allelic imbalances on 5q, 17p, 18q, 3p, 4p, 9p, and microsatellite instability in pooled-tumor samples and in corresponding tumor single-gland samples (cases: A-L). P, pooled gland sample; LOH, loss of heterozygosity; N, negative; NI, not informative; NA, not amplified; MSI, microsatellite instability.

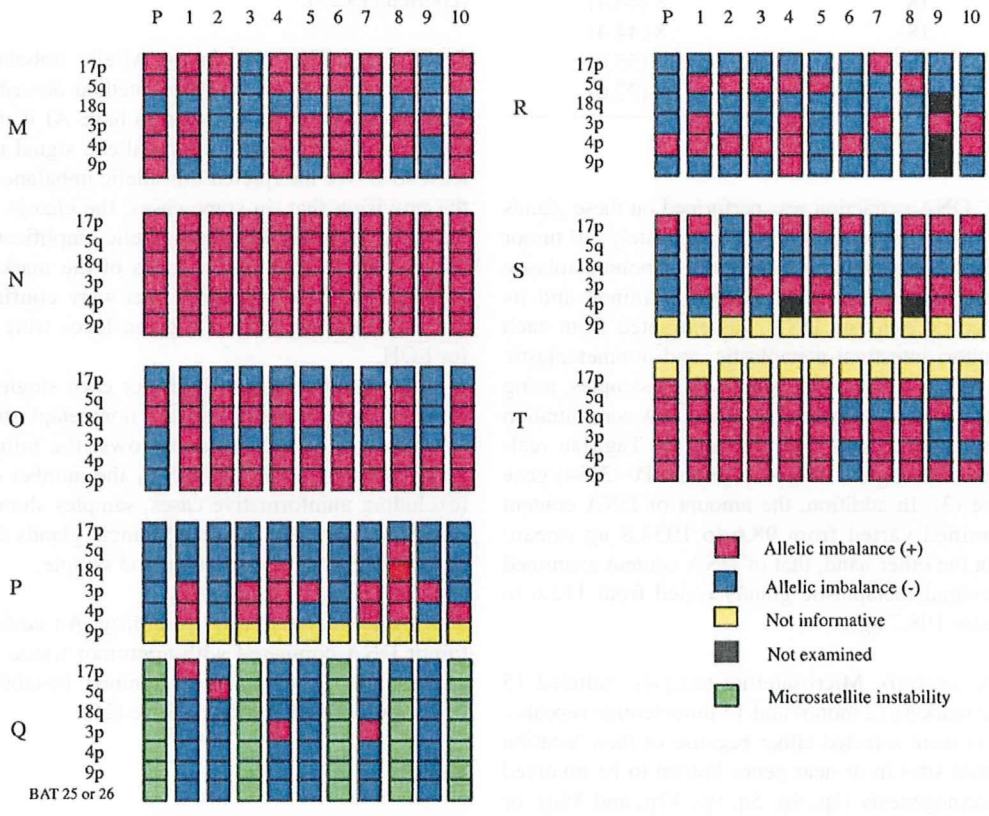

Figure 3. A detailed view of allelic imbalances on 5q, 17p, 18q, 3p, 4p, 9p, and microsatellite instability (MSI) in pooled-tumor samples and in corresponding tumor single-gland samples (cases: M-T). P, pooled gland sample; LOH, loss of heterozygosity; N, negative; NI, not informative; NA, not amplified; MSI, microsatellite instability.

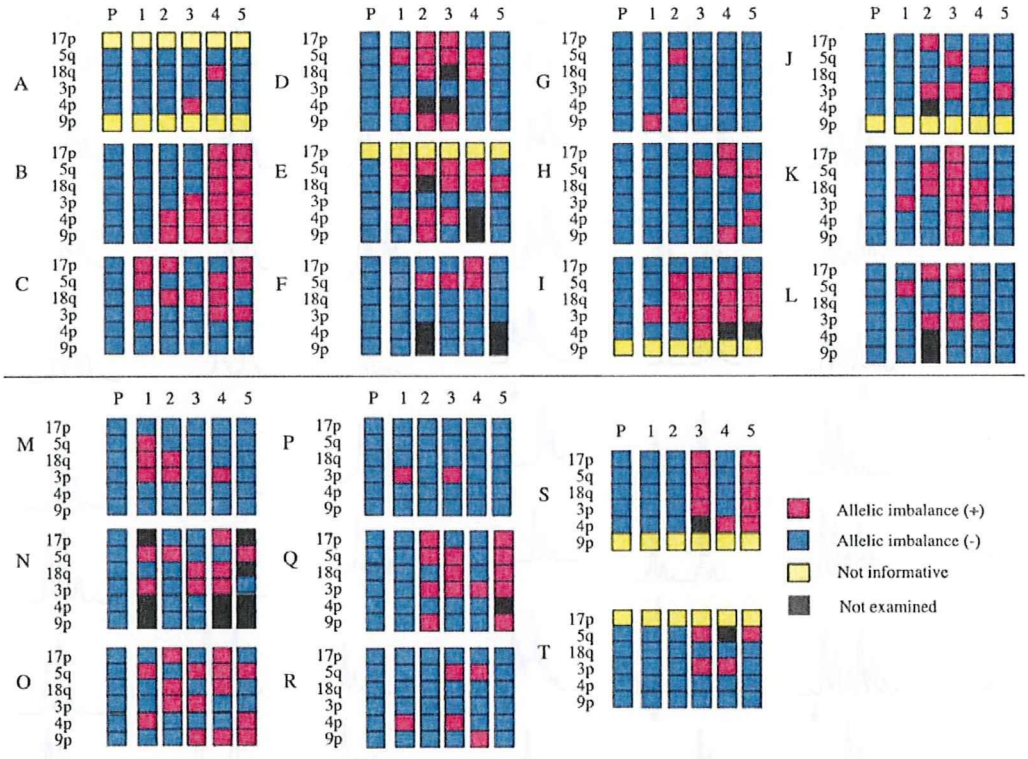

Figure 4. A detailed view of allelic imbalances on 5q, 17p, 18q, 3p, 4p, 9p, and microsatellite instability in intestinal metaplastic pooled-gland samples and in corresponding intestinal metaplastic single-gland samples (samples: A-T). Multiple genetic alterations were seen in the single intestinal metaplastic gland samples. P, pooled gland sample; LOH, loss of heterozygosity; N, negative; NI, not informative; NA, not amplified.

chromosomal loci that were studied in the tumor pooled-gland samples are listed in Table II. We defined genotype as the AI pattern of each examined sample.

Concordance of genotype of pooled-gland samples and single-gland samples. We examined the genotype concordance of pooled-gland samples and the predominant genotype in corresponding single-gland samples. In 4 cases there were different AI patterns seen in the tumor pooled-gland samples and in those of the corresponding tumor single-gland samples (samples B, H, O, and S; Figs. 2 and 3). In addition, in another 7 cases (samples D, F, I, J, L, P, and R; Figs. 2 and 3), the predominant genotypes in tumor single-gland samples were different from those of the corresponding pooled-gland samples. Alternatively, in the other 9 cases the genotypes of the tumor pooled-gland samples were consistent with those of the corresponding tumor single-gland samples (Figs. 2 and 3).

In the samples of intestinal metaplastic glands, although no genetic alterations were found in the pooled-gland samples, multiple alterations were frequently detected in single-gland samples (Fig. 4). A representative example of allelic imbalance at each chromosomal locus in a pooled-gland sample and in corresponding intestinal metaplastic single-gland samples is illustrated in Fig. 5 (case C).

In the samples of nonmetaplastic glands, although no genetic alterations were found in the pooled-gland samples, alterations were observed in corresponding single-gland samples (Fig. 6).

Heterogeneous genotypes in individual glands within the same sample. A total of 18 carcinomas (18/20, 90%) consisted of heterogeneous single genotypes (>1 different genotype) within the same tumor. Single glands within the same sample having the same genotype were interpreted as an occurrence of the same subclone. The number of subclones within the same sample was determined and is shown in Table III. Many subclones were identified within the same sample. No more than 7 different genotypes (subclones) in single-tumor glands from the same sample were observed. In addition, multiple subclones within the same sample of not only intestinal metaplastic single glands but also nonmetaplastic single glands were found. The mean numbers of genetic alterations per tumor, intestinal metaplastic, and nonmetaplastic single-gland samples were 4.15, 3.75 and 4.5, respectively.

Two samples (10%) had homogeneous single genotypes within the same tumor (A and N, Figs. 2 and 3). In contrast, MSI did not occur in a homogeneous pattern within the same tumor (G and Q, Figs. 2 and 3). There were no homogeneous patterns observed among either metaplastic or nonmetaplastic single-gland samples (Figs. 4 and 6).

Genotypic pattern of individual glands within the same sample. As shown in our previous study on colorectal carcinoma (3), the genotype of each single-tumor gland was classified as either a major-altered or a minor-altered genotype. The first group, the major-altered genotype, was defined as a single-tumor gland with >1 genetic alteration. In contrast, the minor-altered genotype was defined as a single-tumor gland with none or

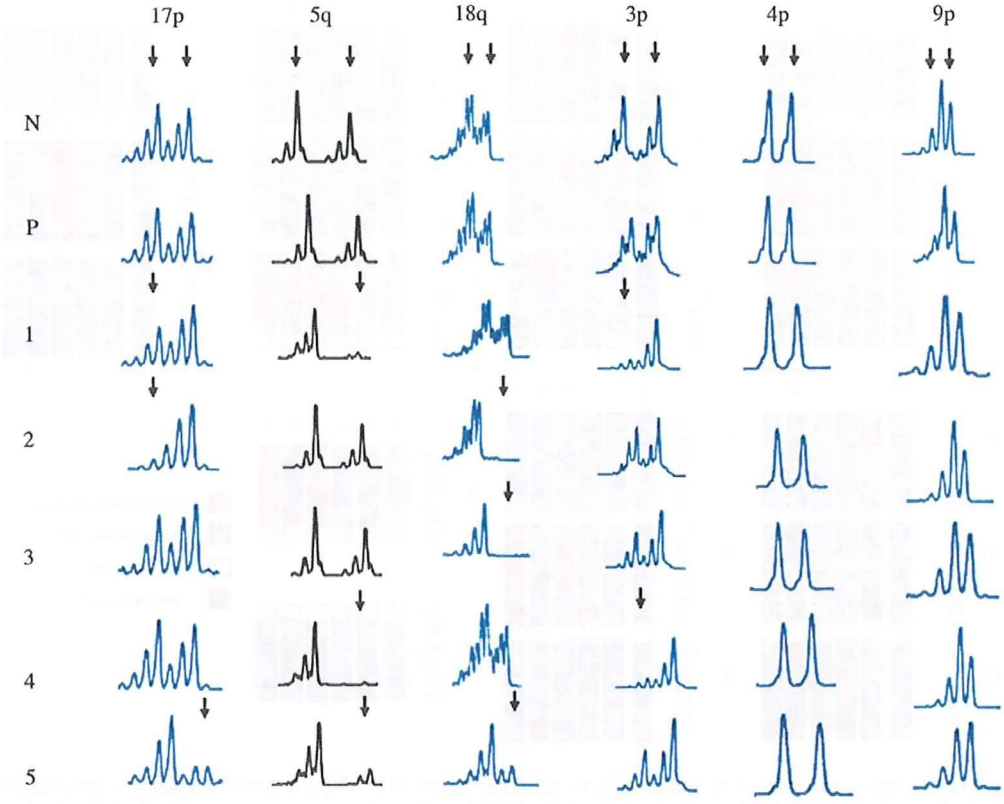

Figure 5. Allelic imbalances on 17p, 5q, 18q, 3p, 4p, and 9p chromosomal loci in an intestinal metaplastic pooled-gland sample and in corresponding intestinal metaplastic single-gland samples (case C). Multiple subclones (genotypes) were seen. Note that although no genetic alterations were detected in the pooled-gland sample, they were frequently found in the paired single intestinal metaplastic gland sample. In intestinal metaplastic single-gland samples, arrows indicate a lost allele at each chromosomal locus. P, pooled gland sample; N, normal.

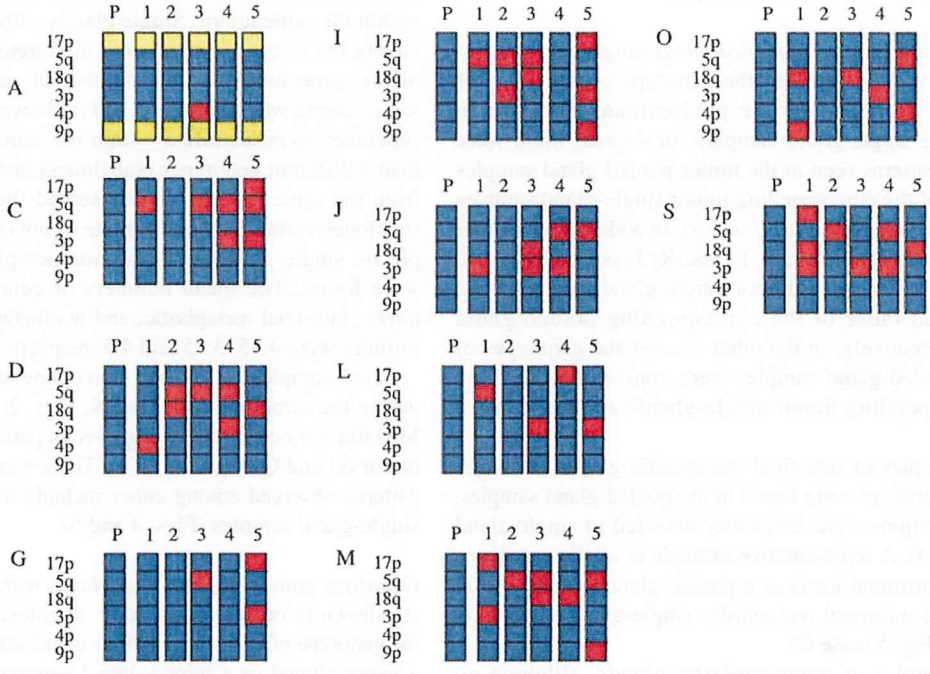

Figure 6. A detailed view of allelic imbalances on 5q, 17p, 18q, 3p, 4p, 9p, and microsatellite instability in nonmetaplastic pooled-gland samples and in corresponding nonmetaplastic single-gland samples (cases: A, C, D, G, I, J, L, M, O, and S). Genetic alterations were seen in the single intestinal metaplastic gland samples. The frequency of allelic imbalances in single nonmetaplastic gland samples is low compared with those of single intestinal metaplastic gland samples. P, pooled gland sample; LOH, loss of heterozygosity; N, negative; NI, not informative; NA, not amplified.

Table III. The number of subclones within the same sample.

Number of subclones within the same tumor	Number of cases		
	Tumor gland	Intestinal metaplastic gland	Nonmetaplastic gland
Two	3	2	1
Three	3	6	1
Four	3	7	3
Five	2	5	5
Six	5	0	0
Seven	2	0	0

with 1 genetic alteration. The major-altered genotype contained 2 types of genetic alterations: multiple AI (AI-type) and MSI. Of 20 carcinomas, 12 contained single-tumor glands with the minor-altered genotype within the same tumor. In contrast, all samples of intestinal metaplastic single glands contained the minor-altered genotype with the exception of one case (sample O). However, surprisingly, at least 1 metaplastic single gland with the major-altered genotype metaplastic glands was detected in 18 of 20 samples. Among the nonmetaplastic single-gland samples, there were major-altered genotypes found in 8 of 10 samples.

Classification of genotype alterations in a single gland within individual tumor. Similar to our previous study (3), we categorized the samples we examined (gastric cancers, intestinal metaplastic glands, nonmetaplastic glands, and including homogeneous tumors) into 5 groups according to criteria based on proportion of genotype alteration: I, all major-altered genotypes; II, major-altered > minor-altered genotypes; III, major-altered = minor-altered genotypes; IV, minor-altered > major-altered genotypes; V, all minor-altered genotypes. According to these criteria, of single glands from tumors, 8 samples were categorized as type I, 8 as type II, and 3 tumors as type IV. The remaining tumor was classified as type III. No tumor was type V. The 2 tumor samples with MSI were grouped as type II and IV, respectively. In contrast, among samples of intestinal metaplastic single glands, 1 was classified as type I, and 9 and 8 samples were categorized as type II and IV, respectively. Two samples were classified as type V. Among nonmetaplastic gland samples, 2 were classified as type II, 2 as types V, and 6 as type IV.

We looked for any association between the genotypes of pooled-gland samples (major-altered and minor-altered genotypes) and the genotypic patterns (I-V) of tumor, intestinal metaplastic, and nonmetaplastic single-gland samples; results are shown in Table IV. In 3 tumor samples (Q, R, and S), the genotype in pooled-gland samples was not consistent with the genotypic patterns of tumor single-gland samples. In contrast, in 10 intestinal metaplastic samples (B, C, D, E, I, J, K, N, O, and Q), the genotype of pooled-gland samples in GIM was different from the genotypic patterns of intestinal metaplastic single-gland samples. In addition, in 7 nonmetaplastic samples (C, D, I, J, M, O, and S), the genotype of pooled-gland samples in nonmetaplastic glands was not consistent with the genotype pattern of nonmetaplastic single-gland samples.

Discussion

In the present study, 12 of 20 tumors showed different predominant genotypes between pooled-gland and single-gland tumor samples. These 12 tumors were primarily classified into 2 groups as an explanation of the discrepancy between pooled-gland and single-tumor gland samples (3). The first explanation is that the AIs in pooled-gland samples at the chromosomal loci we examined may be caused by dilution of minor genotypes within the same tumor (dilution effect). This may represent a mean value of AIs for all collected single-gland tumors. We concluded that the results for 4 tumors (samples: F, I, J, and L) are consistent with this explanation. An alternative explanation may be that the composition of tumor glands in the pooled-gland samples is different from that observed in the single-gland tumor sample (i.e., different composition effect). This suggests that additional subclones, different from those found in the pooled-gland tumor sample, can exist within the same tumor. According to this explanation, it may be likely that a different subclone or different composition of a single-gland sample will not be detected in the pooled-gland samples (B, D, H, O, P, Q, R, and S).

Such a discrepancy between pooled-gland and single-gland samples was prominent in the nonmetaplastic samples. Although no genetic alterations were detected in the pooled-gland samples, multiple genetic alterations were found in corresponding single-gland samples. In particular, this finding was frequently present in GIM samples.

In the present study, it appears that isolation of single intestinal metaplastic glands can be used to increase the sensitivity of tests for allelic imbalance. This finding suggests

Table IV. Frequencies of the genotypic patterns of single-gland samples in gastric tumor, gastric intestinal metaplastic glands, and nonmetaplastic glands according to the genotype of pooled-gland samples.

Genotype of pooled-gland samples	Tumor (n-20)					Intestinal metaplastic gland (n-20)					Nonmetaplastic gland (n-10)				
	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Major	8	6	0	1	0	0	0	0	0	0	0	0	0	0	0
Minor	0	2	1	2	0	1	9	0	8	2	0	2	0	6	2