

Molecular Characterization of Undifferentiated-Type Gastric Carcinoma

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SUMMARY: As the great majority of gastric cancers develop histologically differentiated, and a significant proportion of differentiated-type carcinomas progress to become undifferentiated, both histological types are likely to share several common genetic abnormalities, such as *p53* mutations at advanced stages. However, a subset of gastric cancers develop as undifferentiated carcinomas, including signet-ring cell carcinoma and poorly differentiated adenocarcinoma, and the molecular pathogenesis of this tumor type remains largely unknown. To characterize the molecular features of undifferentiated-type gastric carcinomas that developed as undifferentiated-type, we examined for *p53*, *APC*, and *epithelial (E)-cadherin* gene mutations, microsatellite alterations including loss of heterozygosity (LOH) and microsatellite instability (MSI), and hypermethylation of the *E-cadherin* gene promoter in 26 early undifferentiated gastric carcinomas, consisting of 14 signet-ring cell carcinomas and 12 poorly differentiated adenocarcinomas. *E-cadherin* expression was evaluated immunohistochemically. *p53* mutations were detected in only one poorly differentiated adenocarcinoma sample (3.8%; 1/26), whereas no *APC* or *E-cadherin* mutations were found. LOH was present only at *D8S261* on the short arm of chromosome 8 in 2 of 14 (14%) informative tumors, both of which were poorly differentiated adenocarcinomas, and MSI was not observed in any of the tumors. No signet-ring cell carcinomas have been found to carry gene mutations or microsatellite alterations. In contrast, hypermethylation of the *E-cadherin* promoter occurred in 69% (18/26) of the tumors; 57% (8/14) of signet-ring cell carcinomas, and 83% (10/12) of poorly differentiated adenocarcinomas, and was significantly associated with loss or reduced expression of *E-cadherin*. Thus, whereas tumor suppressor gene mutation, LOH, and MSI were less common in undifferentiated-type early gastric carcinomas, epigenetic inactivation of *E-cadherin* via promoter hypermethylation may be an early critical event in the development of undifferentiated tumors. (*Lab Invest* 2001, 81:593-598).

Gastric cancers are classified histopathologically as either differentiated carcinomas forming tubular or papillary structures or undifferentiated carcinomas in which such structures are inconspicuous (Nakamura et al, 1968). Different genetic pathways are thought to be involved in the development of these histological types because they exhibit different frequencies of genetic alterations, including mutation of the *K-ras* oncogene and *APC* tumor suppressor gene, amplification of *c-erbB-2* and *c-met* oncogenes, deletion of chromosomal arms, and microsatellite instability (MSI) (Tahara, 1995). However, significant discrepancies in the frequency of genetic alterations among differentiated and undifferentiated carcinomas have been reported (Han et al, 1993; Horii et al, 1992; Kihana et al, 1991; Nakatsuru et al, 1992; Victor et al, 1990; Yamamoto et al, 1999). A significant proportion of the differentiated carcinomas progressed to be-

come undifferentiated, so that the proportion of differentiated carcinomas decreased with increasing tumor size through the increased prevalence of undifferentiated tumors (Endoh et al, 2000b). Thus, advanced-stage undifferentiated gastric carcinomas originate from both differentiated and undifferentiated tumor types, and both histological types at advanced stages share common genetic alterations such as *p53* mutation, which occurs as an early event in differentiated carcinomas of the stomach (Tamura et al, 1995). However, it is clear that a subset of gastric cancers develop primarily as undifferentiated tumors, and early undifferentiated carcinomas rarely exhibit *p53* mutations despite high frequencies in early and advanced differentiated-type and advanced undifferentiated-type carcinomas (Maesawa et al, 1995; Uchino et al, 1993).

Recently it was reported that aberrant DNA methylation of promoter region CpG islands may serve as an alternate mechanism to coding region mutation for the inactivation of tumor suppressor or tumor-related genes (Graff et al, 1997; Herman et al, 1996, 1998; Stirzaker et al, 1997). Also, we have previously reported that transcriptional silencing of the *E-cadherin* via promoter hypermethylation frequently occurred in undifferentiated-type carcinomas of the stomach (Tamura et al, 2000). Because a subset of colorectal

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and gastric cancers exhibited the CpG island methylator phenotype (Toyota et al, 1999a, 1999b), it is possible that undifferentiated-type gastric carcinomas were involved.

To characterize the molecular features of undifferentiated gastric carcinomas that developed as undifferentiated carcinomas, we examined for mutations in *p53*, *APC*, and *E-cadherin* genes, for microsatellite alterations (loss of heterozygosity [LOH] and MSI), and for hypermethylation of the *E-cadherin* gene promoter in 26 early gastric undifferentiated carcinomas.

Results

Mutations of *p53*, *APC*, and *E-cadherin*

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) detected *p53* mutation in only one poorly differentiated adenocarcinoma (3.8%; 1/26) (Fig. 1A). DNA sequencing of the shifted band revealed a silent mutation at codon 249 (CGG to AGG) in exon 7 (Fig. 1B). No *APC* or *E-cadherin* mutations were found.

Microsatellite Alterations

Frequencies of informative cases at each microsatellite locus were 54% (14/26 informative cases) at

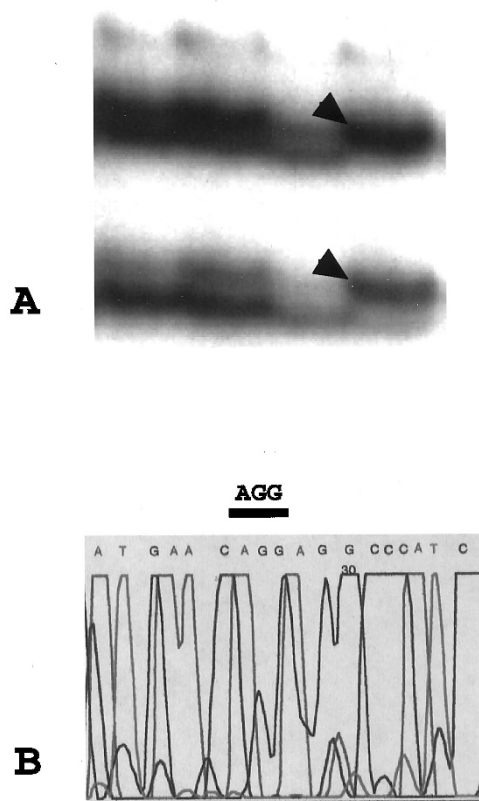


Figure 1.

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) (A) and sequencing (B) analyses of exon 7 of the *p53* gene. Sequencing of the mobility shift band (arrowheads) revealed a silent mutation at codon 249 (CGG to AGG) in exon 7.

D4S404, 92% (24/26) at *D5S409*, 77% (22/26) at *MYH7*, 54% (14/26) at *D8S261*, 23% (6/26) at *D9S171*, 46% (12/26) at *D16S397*, 62% (16/26) at *TP53*, and 69% (18/26) at *D18S46*. LOH was present only at *D8S261* on the short arm of chromosome 8 in 2 of 14 (14%) informative tumors, both of which were poorly differentiated adenocarcinomas, and no MSI was present in any of the tumors (Fig. 2).

Promoter Hypermethylation and Expression of E-cadherin

Hypermethylation of the *E-cadherin* promoter occurred in 69% (18/26) of tumors; 57% (8/14) of signet-ring cell carcinomas, and 83% (10/12) of poorly differentiated adenocarcinomas (Fig. 3). Direct sequencing confirmed the retention of cytosines at CpGs in methylated PCR products and the conversion of all the cytosines to thymines in unmethylated PCR products from several gastric carcinoma samples similarly to our previous findings (Tamura et al, 2000) (data not shown). Loss or reduced E-cadherin expression was observed immunohistochemically in 10 of 12 tumors (83%) with promoter hypermethylation (Fig. 4) and none of 3 tumors without hypermethylation ($p = 0.022$, by Fisher's exact test).

Discussion

Differentiated- and undifferentiated-type gastric carcinomas show different epidemiological characteristics and may be the result of different genetic pathways. Differentiated-type carcinomas are thought to arise from gastric epithelial cells that have undergone intestinal metaplasia and may be associated with *Helicobacter pylori* infection (Correa, 1995), whereas undifferentiated-type carcinomas may arise from native gastric epithelial cells. Because advanced undifferentiated-type carcinomas are composed of cells originating from both differentiated and undifferentiated tumors, it is necessary to study purely undifferentiated-type carcinomas to clarify their molecular pathogenesis. In addition, pure undifferentiated-type carcinomas often show scirrhous

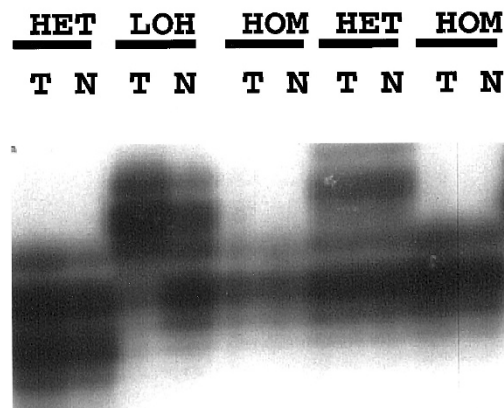


Figure 2.

Examples of microsatellite analysis at *D8S261*. Analysis of tumor (T) and normal (N) DNA from five patients is shown. HET, heterozygosity; LOH, loss of heterozygosity; HOM, homozygosity.

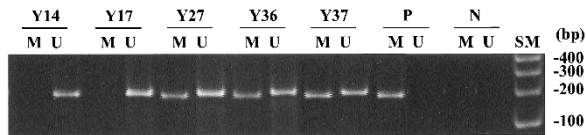


Figure 3.

Methylation-specific polymerase chain reaction (MSP) in five undifferentiated carcinomas. Methylated alleles were present in Y27, Y36, and Y37. M, methylated PCR products; U, unmethylated PCR products; P, positive control (Sss I-treated DNA); N, negative control (unmodified DNA).

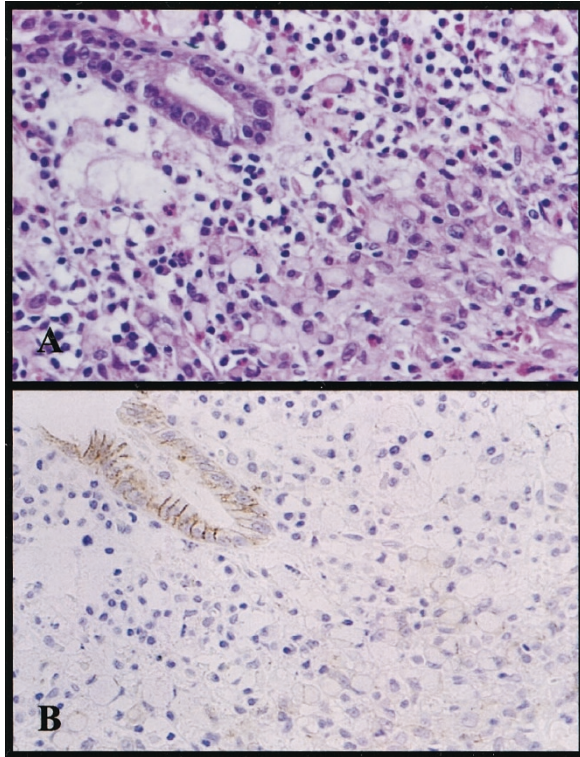


Figure 4.

Histological feature (A) and immunohistochemical reactivity for E-cadherin (B) in a case of intramucosal signet ring cell carcinoma. Whereas foveolar epithelial cells show strong membranous staining, signet ring carcinoma cells show marked reduction in E-cadherin expression.

growth that makes it difficult to investigate genetic alterations because of low tumor cellularity at advanced stages. Therefore, we attempted to investigate genetic and epigenetic alterations in early-stage undifferentiated-type carcinomas with relatively high tumor cellularity.

Mutation of *p53* is one of the most prevalent genetic alterations in human gastric carcinomas, and is considered to be early critical event in the development of differentiated-type gastric carcinomas (Tamura et al, 1995). The frequency of *p53* mutations in early and advanced differentiated-type gastric carcinomas is consistent at around 40% of tumors, similar to that in advanced undifferentiated-type carcinomas (Maesawa et al, 1995; Uchino et al, 1993). Thus, the paucity of *p53* mutations is characteristic of early-stage undifferentiated carcinomas. In the present study, we have confirmed the rarity of *p53* mutations as we found only one in the 12 poorly differentiated adenocarcinomas

tested (8.3%), and that was a silent mutation and therefore probably did not contribute to carcinogenesis, similar to a previous report (Maesawa et al, 1995). Mutation of *APC* is an early critical event in both familial and sporadic forms of colorectal tumorigenesis via the adenoma-carcinoma sequence (Miyoshi et al, 1992a, 1992b). Although *APC* mutations are rare in extracolonic cancers, mutated forms have been found in 3 of 31 undifferentiated-type carcinomas (9.7%), including 10 signet-ring cell carcinomas (Horii et al, 1992), and in 1 of 25 undifferentiated-type carcinomas (4.0%), including 15 signet-ring cell carcinomas (Maesawa et al, 1995). We detected no *APC* mutations in our undifferentiated-type early gastric carcinomas.

Deletions have been frequently reported for several chromosomal loci, including 5q, 7q, 17p, 18q, and 21q, in differentiated-type carcinomas of the stomach (Nishizuka et al, 1998; Sakata et al, 1997; Tamura et al, 1996a). However, few reports have focused on LOH in undifferentiated-type carcinomas, probably because of the difficulty of performing LOH analysis on tissue samples with low tumor cellularity. Nonetheless, frequent LOH on 5q has been reported for this tumor type at advanced stages with or without tumor cell enrichment (McKie et al, 1993; Tamura et al, 1993). In our study, LOH was detected only on 8p in 2 of 16 (12.5%) informative tumors, both of which were poorly differentiated adenocarcinomas, and no other incidences of LOH were found at any other loci, including 5q. The frequency of MSI in gastric cancers varies among reports, so that reports have suggested that undifferentiated-type carcinomas exhibit more frequent MSI than differentiated (Han et al, 1993), or vice versa (Yamamoto et al, 1999). In contrast to our previous report of microsatellite analysis in which LOH was present in 48% (29/61) and high frequency MSI (MSI-H) in 23% (14/61) of early differentiated-type gastric carcinomas (Ohmura et al, 2000), we detected few LOH and no MSI in 26 early undifferentiated-type carcinomas in the present study. We have also reported that the cellular phenotypes of differentiated-type gastric carcinomas were dependent on genetic pathway, so that differentiated foveolar-type carcinomas followed the mutator pathway characterized by MSI (Endoh et al, 2000a; Ohmura et al, 2000). Because differentiated foveolar- and undifferentiated-type carcinomas, both of which may possibly arise from foveolar epithelial cells, rarely exhibit LOH, these carcinomas may constitute the CpG island methylator phenotype (Toyota et al, 1999a, 1999b). Thus, promoter hypermethylation of *hMLH1* in gastric foveolar epithelial cells may lead to differentiated foveolar-type carcinomas, whereas promoter hypermethylation of *E-cadherin* may lead to undifferentiated-type carcinomas.

The *E-cadherin* gene is frequently found to be inactivated via the classic two-hit mechanism in undifferentiated-scattered (diffuse)-type gastric carcinomas at advanced stages, but not in differentiated or undifferentiated-adherent-type gastric carcinomas (Becker et al, 1994; Tamura et al, 1996b). *E-cadherin* mutations were infrequent in undifferentiated gastric

carcinomas including signet-ring cell carcinomas at their early stages (Tamura et al 1996b; Muta et al, 1996), and *E-cadherin* mutations were not detected in our present study. Because *E-cadherin* mutations were detected in the undifferentiated component, but not in the differentiated component, of mixed differentiated/undifferentiated carcinomas, it is possible that *E-cadherin* is mutated when differentiated carcinomas progress to become undifferentiated (Machado et al, 1999). In addition, frequent inactivation of *E-cadherin* via hypermethylation of promoter region CpG islands, which acts as an alternate mechanism to coding region mutations, has recently been reported for undifferentiated-scattered-type gastric carcinomas (Tamura et al, 2000). *E-cadherin* promoter hypermethylation occurs frequently in undifferentiated-type carcinomas, at similar rates in early and advanced stages (Tamura et al, 2000), and can also occur in noncancerous gastric epithelia (Suzuki et al, 1999). In the present study, *E-cadherin* promoter hypermethylation was detected in 69% (18/26) of tumors, 57% (8/14) of signet-ring cell carcinomas, and 83% (10/12) of poorly differentiated adenocarcinomas. We have previously reported that hypermethylation of this region correlated well with loss or reduction of *E-cadherin* protein expression by Western blotting (Tamura et al, 2000), and we confirmed this association with reduced *E-cadherin* expression by immunohistochemistry in the present study. Loss or reduction of *E-cadherin* expression is known to occur frequently in undifferentiated-type gastric carcinomas, even at early stages (Blok et al, 1999). Therefore, epigenetic inactivation of *E-cadherin* via promoter hypermethylation may play a major role in the development of purely undifferentiated-type carcinomas of the stomach, and mutations of this gene may be involved in the progression of differentiated to undifferentiated carcinomas.

In conclusion, mutations in tumor suppressor genes, LOH, and MSI are less common in early undifferentiated-type gastric carcinomas, and epigenetic inactivation of *E-cadherin* via promoter hyper-

methylation may be an early critical event in the development of this tumor type.

Materials and Methods

Samples and DNA Extraction

Twenty-six gastric carcinomas and corresponding noncancerous tissues were obtained surgically from 26 patients. Parts of these tissues were stored at -80°C for DNA extraction, and the remaining tissues were fixed in 10% buffered formalin for histological examination. The carcinomas studied were histologically undifferentiated carcinomas, including 14 signet-ring cell carcinomas and 12 poorly differentiated adenocarcinomas, and consisted of 14 intramucosal and 12 submucosal invasive carcinomas. All these tumor samples were histologically confirmed to contain more than 50% tumor cells, and samples with low tumor cellularity (less than 50%) were excluded from the present study. DNA was isolated by standard proteinase K digestion and phenol/chloroform extraction procedures.

PCR-SSCP Analysis of *p53*, *APC*, and *E-Cadherin*

p53, *APC*, and *E-cadherin* gene mutations were studied by PCR-SSCP analysis according to established protocols (Tamura et al, 1995, 1996a, 1996b). Briefly, extracted DNA (100 ng) was amplified in $10\ \mu\text{l}$ $1\times$ PCR Gold Buffer (Perkin-Elmer Cetus, Norwalk, Connecticut) containing 10 pM each primer, 1 mM MgCl_2 , 0.2 mM each deoxynucleotide triphosphate, 0.5 μl [$\alpha\text{-}^{32}\text{P}$]-dCTP, and 0.5 units AmpliTaq Gold (Perkin-Elmer Cetus). Primer sequences are listed in Table 1. PCR was performed for 35 cycles in a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Cetus) consisting of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds, after initial heating at 95°C for 10 minutes to activate the AmpliTaq Gold. PCR prod-

Table 1. Oligonucleotide Primers (5'–3') for PCR-SSCP

	Upstream	Downstream
<i>p53</i>		
Exon 5	TTCCTCTTCCTGCAGTACTCC	GCCCCAGCTGCTCACCATCG
Exon 6	CACTGATTGCTCTTAGGT	AGTTGCAAACAGACCTCA
Exon 7	TCCTAGGTTGGCTCTGAC	CAAGTGGCTCCTGACCTGGA
Exon 8	CCTATCCTGAGTAGTGATAA	CCTGCTTGCTTACCTCGCT
<i>APC</i>		
Exon 15 ^a	AGACTTATTGTGTAGAAGATAC	ATGGTTCACCTCTGAACGGA
Exon 15 ^b	TCTGTCAGTTCACCTGATAG	CATTGATTCTTTAGGCTGC
<i>E-cadherin</i>		
Exon 6	TCCTCATCGAGCTCAAGTC	GGGTCCAAAGAACCTAAGAG
Exon 7	TGCCAGTCCCAAAGTGACG	TCCACACCCTCTGGATCCTC
Exon 8	AGGTGGCTAGTGTTCTCTGG	CCTTTCTTTGGAAACCTCTAA
Exon 9	GACACATCTCTTTGCTCTGC	GGACAAGGGTATGAACAGCT

PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.

^a Codon 1260–1410.

^b Codon 1389–1547.

ucts were diluted 10-fold with gel-loading buffer, heated to 94° C for 2 minutes, and stored on ice until analysis. Electrophoresis was performed on 6% neutral polyacrylamide gels at 60 W for 2 to 4 hours. Gels were dried and exposed to x-ray film at -80° C overnight. Second-round PCR was performed to confirm the reproducibility.

DNA Sequencing Analysis

Mobility-shift SSCP bands were excised from gels and subjected to a second round of PCR amplification using the same primers as used above, as described (Tamura et al, 1995). The reamplified PCR products were purified and sequenced using a terminator cycle sequencing kit (dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kits, Perkin-Elmer Cetus) and an automated DNA sequencer (310 Genetic Analyzer, Perkin-Elmer Cetus).

Microsatellite Analysis

Eight microsatellite markers, *D4S404*, *D5S409*, *MYH7*, *D8S261*, *D9S171*, *D16S397*, *TP53*, and *D18S46* were investigated. Primers were obtained from MapPairs (Research Genetics, Huntsville, Alabama). PCR was performed by the same procedure as described for PCR-SSCP. Electrophoresis was performed on 6% polyacrylamide gels containing 7 M urea at 60 W for 2 to 2.5 hours. LOH and MSI were defined as visible changes as previously described (Tamura et al, 1995).

Methylation-Specific Polymerase Chain Reaction (MSP)

DNA methylation patterns in the *E-cadherin* promoter region were determined by MSP (Tamura et al, 2000). MSP distinguishes unmethylated from methylated alleles of a given gene based on sequence alterations produced by bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil. Subsequent PCR then uses primers specific to either methylated or unmethylated DNA sequences (Herman et al, 1996). Primer sequences used were 5'-GGTGAATTTTGTAGTTAATTAGCGGTAC-3' and 5'-CATACTAACCAGAAACGCGG-3' for methylated DNA, and 5'-GGTAGGTGAATTTTGTAGTTAATTAGTGGTA-3' and 5'-ACCCATACTAACCAGAAACACCA-3' for unmethylated DNA. The PCR-amplified region for methylated (204 bp) and unmethylated (211 bp) alleles contained 19 CpG dinucleotides, including 5 CpGs within the primer annealing sites. Primer sets spanned the transcriptional start site and were designed to include methylation sites that best corresponded with transcriptional silencing of the *E-cadherin* gene (Baylin et al, 1998). Aliquots of genomic DNA (2 µg) were denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, Wisconsin), again treated with NaOH, precipitated with ethanol, and resuspended in water. Extracted DNA (100 ng) were amplified under the same conditions as described for PCR-SSCP. PCR products were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bro-

me, and visualized under UV illumination. Peripheral blood DNA (100 µg) was treated by Sss I Methylase (New England BioLabs, Beverly, Massachusetts) according to the manufacturer's protocol. Sss I-treated DNA was modified by bisulfite as described above and used as a positive control for methylated DNA. Unmodified DNA was used as a negative control for MSP.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using the standard labeled streptavidin-biotin system (Nichirei, Tokyo, Japan) on 15 of the 26 early undifferentiated gastric carcinomas. Mouse monoclonal antibody to E-cadherin (clone HECD-1; Takara, Kyoto, Japan) was used at 1:250 dilution after antigen retrieval by microwave. Expression was evaluated according to previously published methods (Oka et al, 1992).

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