**p53 Mutations and Microsatellite Instability in Sporadic Gastric Cancer: When Guardians Fail**

John G. Strickler, Jian Zheng, Qiuping Shu, Lawrence J. Burgart, Steven R. Alberts, and Darryl Shibata

**ABSTRACT**

Genetic instability may underlie the etiology of multistep gastric carcinogenesis. The altered microsatellites observed in tumors with the ubiquitous somatic mutation (USM) phenotype may represent the expression of such instability. Similarly, p53 mutations may allow the accumulation of genetic alterations caused by multiple mechanisms. In 40 sporadic gastric adenocarcinomas, nine tumors (22.5%) with p53 mutations in exons 5–8, and six tumors (15%) with the USM+ phenotype, were detected. None of the tumors had both alterations. The tumors with p53 mutations were predominantly in the proximal stomach whereas the USM+ tumors were predominantly in the distal stomach. The mutant p53 alleles were homogenously distributed throughout the primary tumors, but usually absent from adjacent normal or dysplastic epithelium, indicating that p53 mutations are typically acquired before the bulk of clonal expansion. The loss of mutant p53 alleles during progression was also rarely observed in metastatic foci. Altered microsatellites were homogeneously present in the USM+ primary and metastatic tumors and one synchronous tubular adenoma, but were not detected in adjacent normal and metaplastic epithelium. These findings also demonstrate that the USM+ phenotype is expressed before the bulk of clonal expansion. In most (5 of 6) USM+ tumors, the sizes of the altered microsatellites differed between regions, indicating that the instability usually persists during clonal expansion. These findings indicate that both p53 mutations and the USM+ phenotype are present prior to the bulk of tumor growth and therefore may contribute to, rather than be a late consequence of, malignant transformation.

**INTRODUCTION**

Gastric cancer is one of the most common tumors in the world. Its earliest oncogenic alterations may occur decades before transformation (1). The histological progression associated with the intestinal type of gastric cancer have been well documented, with apparent evolution through a sequence of superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, and dysplasia (2, 3). Similarly, the genetic evolution of cancer involves the accumulation of multiple mutations (4). In gastric cancer, altered loci include p53 (5–10), APC (11, 12), c-K-ras (13–15), and multiple MS in cancers with a replication error (RER) or USM phenotype (16, 17). The specific types and timing of multiple mutations in gastric cancers are poorly characterized as the frequencies of these alterations are typically less than 50% (18).

The presence of a “mutator” phenotype is thought to be critical for multistep carcinogenesis since the normal mutation rate may be insufficient to account for the accumulation of multiple mutations (19). Mutations in the p53 tumor suppressor gene and the loci responsible for the USM+ phenotype may contribute to some aspects of increased genetic instability. The USM+ phenotype is manifest by widespread alterations in the sizes of simple repeat sequences secondary to defects in DNA repair after replication (20–27). USM+ cell lines demonstrate markedly increased mutation rates in MS (26, 27). Although the majority of MS alterations are likely to be neutral or selection independent, the instability may increase the probability that critical oncogenic mutations occur. Recent studies have demonstrated that the USM+ phenotype is present in 18 to 38% of gastric adenocarcinomas, especially in poorly differentiated tumors (16, 17, 28).

p53 is a transcription factor with many diverse roles (29, 30). One apparent role is to ensure genomic integrity by preventing the expansion of cells with genetic alterations caused by diverse mechanisms (31–34). p53 promotes the expression of WAF1/Cip1 which inhibits the cell cycle by binding to cyclin-dependent kinases (35–37). This G1 arrest may allow the repair of DNA damage, or trigger apoptosis (38). Losses of wild type p53 alleles are also associated with gene amplification (39).

Instability would be expected to be most effective at promoting malignant transformation if it were acquired or expressed during early tumor progression (17). Indeed, germ-line mutations of p53 and hMSH2 are present in hereditary cancer families (40) and HNPCC (24, 25). In order to determine possible interactions and when these alterations are acquired or expressed, sporadic gastric cancers were screened for the RER+ phenotype and p53 mutations, and their topographical distributions were analyzed.

**MATERIALS AND METHODS**

**Specimens.** DNA was extracted from 40 formalin-fixed, paraffin-embedded sporadic gastric cancers from the Mayo Clinic (41). Regions of at least 50% tumor cells and normal cells were isolated with microdissection. All tumors were considered primary gastric adenocarcinomas, although the exact origins were uncertain for the 15 proximal tumors arising in the cardia or the gastroesophageal (GE) junction. Only 4 of the 15 proximal tumors had evidence of Barrett’s epithelium. The remaining 25 distal tumors were present in the body or antrum of the stomach. The adenocarcinomas were classified as either of the intestinal or diffuse types (42). Clinical data were collected from review of medical records.

**Detection of p53 Mutations.** The specimens were screened for p53 mutations in exons 5–8 by using PCR and single-strand conformation polymorphism (43). Bands of abnormal mobility were reamplified, cloned (TA Cloning Kit, Invitrogen), and sequenced. The mutations were verified by hybridization of a mutation-specific 17-mer probe back to the DNA amplified from the primary specimen.

**Detection of USM+ Tumors.** Pairs of tumor and adjacent normal tissue were amplified at three MS loci consisting of CA repeat sequences [Mfd27 (44), Mfd41 (45), Mfd47 (46)], and their sizes were compared (27). Tumors with MS sizes different from their normal tissues in at least one-half of the tested loci were designated as USM+ and were analyzed as below. For one case, the Mfd57 locus (47) was also analyzed.

**Topographical Distributions of Altered MS and Mutant p53 Alleles.** The topographical distributions of the mutant p53 alleles or altered MS were determined with selective UV radiation fractionation (41, 48). Multiple blocks of normal, tumor, and metastases were analyzed. From each stained microscopic section, 8–20 different cell groups (100–300 cells) consisting of histologically defined phenotypes were isolated and amplified for 40–50 PCR cycles. Normal tissues were not contaminated with tumor and tumor phenotypes were at least 70% pure. The p53 PCR products were analyzed with mutant and wild type specific oligomer probes (43). The MS from different topographical regions were analyzed as previously described (27).
RESULTS

The 40 sporadic gastric cancers were screened for the USM+ phenotype and p53 mutations in exons 5–8. The USM+ phenotype was detected in six (15%) tumors and p53 mutations in nine (22.5%) tumors (Table 1). p53 mutations were also detected in two other tumors, but could not be confirmed by hybridization of corresponding mutation-specific oligomer probes back to PCR products from the primary specimens or multiple tumor regions obtained by selective UV radiation fractionation. None of the tumors had both a USM+ phenotype and p53 mutation, or more than one p53 mutation. All of the USM+ and p53 mutant tumors were of the intestinal type, while none of three diffuse tumors showed the USM+ phenotype or p53 mutations.

Most p53 mutations (67%) were present in the proximal stomach. All six proximal p53-positive cases were located at the GE junction and did not arise from Barrett’s metaplasia, as determined by routine histological examination. Of the eight proximal adenocarcinomas in which p53 mutations were not identified, four appeared to arise from Barrett’s esophagus and four were at the GE junction. All of the tumors with p53 mutations were of advanced stage with metastasis to lymph nodes.

In contrast to the p53-positive cases, most (83%) USM+ tumors (except Case 15) were located in the distal stomach. Three USM+ cases were metastatic to lymph nodes. The three node-negative USM+ cases included two with invasion limited to the submucosa and one invading through the muscularis propria to the serosa. All six of the USM+ cases are currently alive with no evidence of recurrent neoplasm (16–30 months follow-up).

The nine p53 mutations included one nonsense and seven missense mutations (Table 2). A single tumor had a 4 base pair duplication of the adjacent sequence. All of the point mutations occurred at G or C bases, with one-half consistent with spontaneous deamination at CpG sites. This spectrum of p53 changes is similar to that of other studies.

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p53 mutations were present in the majority of metastatic lesions (5 of 6 possible cases) with predominately homogeneous metastatic topographical distributions. In Case 8, however, the p53 mutation was present in 12 but absent from 2 lymph node metastasis (Fig. 3). There were no morphological differences between the metastases with or without mutant p53 alleles. The small (3 mm) solitary lymph node metastasis in Case 9 also lacked the p53 mutation present in the primary lesion.

The topographic distributions of altered MS were determined for the six USM+ tumors. Altered MS were not detected in adjacent normal, hyperplastic, or metaplastic epithelium, although nonclonal MS changes in individual cells may escape detection. In Case 13, one altered MS locus was detected in a small (0.5 cm) tubular adenoma distinct from the primary tumor. In the primary tumors and their metastases, altered MS from at least one locus could be detected throughout the carcinomas. The patterns were complex as the same or different-sized MS additions or deletions could be detected from adjacent tumor cell groups (Fig. 6). The exception was Case 13 in which intratumor heterogeneity of the altered MS loci was not detected. In Case 10, the primary tumor had a homogeneous distribution of the same altered MS, but its metastases had different-sized MS. These intratumor differences suggest that MS

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### Table 1 Characteristics of gastric cancers

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<tr>
<th></th>
<th>All cases</th>
<th>USM+</th>
<th>p53 mutation detected</th>
<th>USM+ and p53 mutation not detected</th>
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<tr>
<td>No. studied</td>
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<td>6</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>Age</td>
<td>65.6</td>
<td>68.5</td>
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<td>66.6</td>
</tr>
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<td></td>
<td></td>
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<tr>
<td>Proximal</td>
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<td>1</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Distal</td>
<td>25</td>
<td>5</td>
<td>3</td>
<td>17</td>
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<td>Metastases</td>
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<tr>
<td>Absent</td>
<td>8</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Present</td>
<td>32</td>
<td>3</td>
<td>9</td>
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</tr>
<tr>
<td>Type</td>
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<td>Intestinal</td>
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<tr>
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<tr>
<td>Present</td>
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<td>4</td>
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<td>7</td>
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<tr>
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<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>21</td>
<td>2</td>
<td>6</td>
<td>13</td>
</tr>
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*The family history was considered positive if any cancer was noted in parents or siblings.*

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### Table 2 p53 mutation spectrum

<table>
<thead>
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<th>Case</th>
<th>Codon</th>
<th>Mutation</th>
<th>Type</th>
<th>Location</th>
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<tr>
<td>1</td>
<td>282</td>
<td>CGG → TGG Arg to Trp</td>
<td>mC transition</td>
<td>P</td>
</tr>
<tr>
<td>2</td>
<td>276</td>
<td>GCC → GCC Ala to Gly</td>
<td>G or C transition</td>
<td>P</td>
</tr>
<tr>
<td>3</td>
<td>136</td>
<td>CAA → TAA Gin to Stop</td>
<td>G or C transition</td>
<td>P</td>
</tr>
<tr>
<td>4</td>
<td>135</td>
<td>TGC → TGG Cys to Trp</td>
<td>G or C transition</td>
<td>D</td>
</tr>
<tr>
<td>5</td>
<td>282</td>
<td>CGG → TGG Arg to Trp</td>
<td>mC transition</td>
<td>D</td>
</tr>
<tr>
<td>6</td>
<td>261</td>
<td>TCCA*GG → TCCA(TCCA)GG</td>
<td>4-base pair insertion</td>
<td>D</td>
</tr>
<tr>
<td>7</td>
<td>273</td>
<td>CGT → CAT Arg to His</td>
<td>mC transition</td>
<td>P</td>
</tr>
<tr>
<td>8</td>
<td>175</td>
<td>CGG → CAC Arg to His</td>
<td>mC transition</td>
<td>P</td>
</tr>
<tr>
<td>9</td>
<td>179</td>
<td>CAT → AAT His to Asn</td>
<td>G or C transition</td>
<td>P</td>
</tr>
</tbody>
</table>

*P, proximal stomach; D, distal stomach.*

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Fig. 1. Topographical distributions of p53 mutations. The mutant alleles were associated with specific histological phenotypes. The p53 mutations were found in the primary gastric cancers, suggesting that they were acquired prior to the bulk of clonal expansion. p53 mutations were absent from most examples of dysplasia except for Case 1. Intestinal metaplasia was not adjacent to these cancers. In Cases 8 and 9, the p53 mutations present in the primary lesions were absent in some of the metastases.
alterations usually continue to accumulate during macroscopic tumor expansion.

The p53 mutations were not germ line since they were absent from normal tissues. The mutations responsible for the MS instabilities are unknown for the USM+ tumors. However, the family histories were unremarkable for cancer or HNPCC (49) when compared with USM- negative cases (Table 1), except for Case 13 in which both parents had colorectal cancer and a brother had pancreatic cancer. Although this gastric cancer presented at 74 years of age, it may represent a late manifestation of a germ-line mutator mutation. The average age of the USM+ patients was 68.5 years, which was slightly greater than the average for all cases (65.6 years).

DISCUSSION

Genetic instability may underlie the etiology of many tumors as multiple mutations are thought necessary for malignant transformation (19). The altered MS present in USM+ tumors provide evidence of widespread instability or a mutator phenotype (20, 21, 23). This mutator phenotype has been best described for colorectal cancers arising in families with HNPCC. The hMSH2 and hMLH1 genes have been linked to most of these familial tumors (24, 25, 50). A defect in DNA repair after replication is responsible for the multiple MS alterations observed in USM+ tumors (24, 26, 27). Similarly, p53 mutations are associated with defects in G1 arrest after genetic damage, which may allow the genetic changes generated by multiple mechanisms to accumulate (31). In colorectal cancer, there is evidence that both p53 mutations and the USM+ phenotype may frequently (23) or infrequently (20, 51) coexist.

In this study, p53 mutations and the USM+ phenotype were not observed in the same tumors. Marked regional specificity was noted as tumors with p53 mutations were located predominately in the proximal stomach, whereas the USM+ tumors were predominately in the distal stomach. Regional specificity is also present for sporadic USM+ colorectal tumors which predominately arise in the proximal colon (20, 21, 23). This distribution is also consistent with studies which have demonstrated a high incidence of p53 mutations in adenocarcinomas which arise at the GE junction or are associated with Barrett’s metaplasia (10). It is difficult to distinguish between a gastric, or an esophageal origin arising from Barrett’s esophagus, for many adenocarcinomas at the GE junction (52). Interestingly, the epidemiology differs between proximal and distal gastric cancers (53). Most adenocarcinomas of the GE junction exhibit aneuploidy and LOH at multiple loci (54, 55), and p53 mutations are much more common in aneuploid compared to diploid gastric tumors (7). One other study also failed to detect p53 mutations among four USM+ (as defined in this study) gastric cancers (17). Aneuploidy or LOH are relatively rare in USM+ colorectal and endometrial tumors (21, 23, 51, 56).

The strategy used in this study utilizes the topographical distributions of mutations to provide information on when they were acquired relative to the bulk of tumor growth. This strategy requires both the mutation and its clonal expansion, since mutations in individual cells
would be difficult to detect. Therefore definitive conclusions on the p53 mutations or USM+ phenotypes are limited to whether they were acquired prior to, or during the bulk of clonal expansion of the individual cancers.

The homogeneous topographical tumor distributions of the p53 mutations and the lack of the same mutations in adjacent normal tissues suggest that they were acquired somatically before the bulk of clonal expansion, and therefore at or before malignant transformation. Definitive evidence of p53 mutation before malignant transformation was observed in one case in which the identical point mutation was present in adjacent dysplasia, although most examples of dysplasia adjacent to the cancers with p53 mutations lacked the same mutations. Other studies have also provided evidence that p53 mutations can occur in the preneoplastic lesions of gastric adenomas, intestinal metaplasia (57, 58), and Barrett’s esophagus (10, 55).

When the USM+ phenotype is acquired is more difficult to determine as the underlying mutations are unknown. Although the hMSH2 and hMLH1 genes have been linked to most USM+ colorectal cancers in HNPCC, other loci are also implicated (22, 50). As yet, the mutations responsible for the majority of sporadic USM+ tumors have not been characterized. The high average age of presentation of...
the current USM+ gastric cancers suggests that somatic or very weak germ-line mutator mutations were present. The altered MS present in the USM+ tumors represent the phenotype manifestations of the underlying genomic instability. Therefore, the topographical distributions of the MS can approximate both the onset of the phenotype and its persistence during clonal expansion. Similar to the p53 mutations, the altered MS were homogeneously distributed in the tumors, suggesting that instability was expressed before the bulk of clonal expansion. The USM+ phenotype, however, was not detected in histologically normal or metaplastic tissue, although in one case altered MS were present in a synchronous tubular adenoma. Instability likely increases the activation of loci which directly contribute to malignant transformation (25); however, p53 or c-K-ras (data not shown) do not appear to be among these loci. The USM+ phenotype is also expressed early in colorectal carcinogenesis as altered MS are present in adenomas (20, 27).

A mutation acquired early in tumor progression may become unnecessary and therefore lost during later stages. The specific roles of the mutant p53 alleles in the present gastric cancers are unknown, but in two cases they did not persist in the metastases. Similarly, expression of the USM+ phenotype during clonal expansion, or intratumor heterogeneity, was absent in only one case. In this case, the mutator mutation may have been lost or expressed a relatively low mutation rate. These findings demonstrate that loss of p53 mutations or the lack of expression of the USM+ phenotype can rarely occur during gastric tumor progression, consistent with a more critical role during early oncogenesis. Persistence of the mutator phenotype has been observed in most USM+ colorectal tumors and cell lines (26, 27).

In summary, we have demonstrated that p53 mutations and the USM+ phenotype are usually acquired before or at the time of malignant transformation and persist during gastric carcinogenesis. In these roles, p53 mutations and the USM+ phenotype usually appear to act through distinct pathways as both changes were not simultaneously present. Further study of sporadic USM+ tumors may reveal the specific mutator mutations and the critical loci affected by this unique phenotype.

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REFERENCES


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