Prognostic Value and Clinicopathological Profile of Microsatellite Instability in Gastric Cancer

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ABSTRACT

In this study, microsatellite instability (MI) was investigated in 126 gastric carcinomas and correlated with clinicopathological features and prognosis; at least 5–10 microsatellite loci were analyzed. MI was identified in 56 (44.5%) of all investigated carcinomas, one locus being affected in 40 (31.7%) carcinomas, two loci being affected in 6 (4.8%) carcinomas, and more than two loci being affected in 10 (8.0%) carcinomas. MI was correlated with neither age and sex of the patients nor with depth of invasion, lymph node metastasis, tumor differentiation, or histological type according to WHO and Laurén classification. The frequency of MI was the same in early gastric carcinomas as it was in advanced gastric carcinomas, suggesting that MI arises early during the tumorigenesis of gastric cancer. No significant differences in survival could be demonstrated between patients with MI-negative and MI-positive gastric carcinomas.

INTRODUCTION

MI is a well-known phenomenon of hereditary non-polyposis colorectal cancer due to mutated MMR genes (1–7). Meanwhile, a number of MMR genes (hMSH3 (8), hMSH6 (9), hFMS1, and hPMS2 (10]) that are correlated with MI have been detected, and some of these (hMSH3 and hMSH6) are also mutated in sporadic cancer (8, 9). Likewise, MI has also been reported in several types of sporadic cancer, such as colorectal cancer (11, 12), endometrial cancer (13), ovarian cancer (14), breast cancer (15), prostatic cancer (16–18), and pancreatic cancer (19). Interestingly, MI-positive tumors associated with the hereditary non-polyposis colorectal cancer syndrome are characterized by a particular clinicopathological profile (20) and are supposed to have a better prognosis than CRC (21). Concerning the prognostic value of MI in sporadic tumors, contradictory results have been reported. Whereas MI could be correlated with a better prognosis in CRC (22, 23), it was correlated with a decreased survival in sporadic breast (15) and endometrial (13) cancer.

In gastric cancer, the frequency of MI varies between 15% (24) and 38% (25), depending upon the number of loci investigated. Concerning the prognostic value of MI in gastric cancer, so far, only one study on 61 patients has been published, reporting a correlation of MI with a better prognosis (26). To investigate the clinicopathological profile of MI and to substantiate its putative prognostic role in an adequate number of patients, 126 gastric carcinomas were investigated, and between 5 and 10 microsatellite markers were analyzed.

MATERIALS AND METHODS

Patients. Specimens from 126 curatively resected patients (R0, resection according to the tumor-node-metastasis classification; Union International Contre Cancer (27]) were analyzed. Follow-up letters from all patients were sent to the surgeons or local tumor registers to obtain up-to-date information on survival or death. Median follow-up time was 1.9 years, with a range of 2 months to 9.1 years. The mean age of the patients was 57.4 years, with a range from 23 to 89 years. Seventy-nine patients were male (62.7%) and 47 patients were female (37.3%). None of the patients received adjuvant chemotherapy. To eliminate bias due to deaths directly resulting from operation, patients who died within 4 weeks after surgery were excluded from the statistical analysis of survival.

Tumors. In this study, 35 early carcinomas (pT1) and 91 advanced carcinomas (52 pT2 and 39 pT3-4) were included. Lymph node metastasis could be recognized in 64 cases, and 62 cases were lymph node negative. Lymphatic vessel invasion was observed in 54 patients, whereas blood vessel invasion could be verified in only 24 of the 126 cases. According to WHO classification (28), 49 carcinomas were classified as signet ring cell carcinomas, 48 were classified as carcinomas of the tubular type, 18 were classified as undifferentiated carcinomas, 7 were classified as carcinomas of the mucinous type, and 4 were classified as carcinomas of the papillary type. According to the Laurén classification (29), 60 tumors were the intestinal type, 54 carcinomas were the diffuse type, and 12 carcinomas were the mixed type. Two tumors were well differentiated (G1), 20 tumors were moderately differentiated, 82 were poorly differentiated, and 22 were undifferentiated.

DNA Extraction. DNA from 126 tumors and corresponding nontumorous tissues was isolated from formalin-fixed, paraffin-embedded tissue sections by microdissection. In all
microdissected samples, the tumor tissue comprised at least 70% of the cells in the microdissected area. Microdissected tissues were dewaxed with xylene, washed with ethanol, air-dried, and resuspended in 200 μl of 25 mM EDTA (pH 8), 75 mM NaCl, 0.5% Tween 20, and 25 mg/ml proteinase K and incubated at 55°C for 3 days. DNA was extracted by using the QIAGen tissue kit (Qiagen, Hilden, Germany).

**Microsatellite Analysis.** For microsatellite analysis, two (A)n mononucleotide markers and eight (CA)n polymorphic markers covering seven different chromosomes were investigated (Table 1). Microsatellite loci on chromosomes 2, 5, and 18 are localized in the neighborhood of the known tumor suppressor genes: hMSH2, APC, and DCC. The mononucleotide marker BAT 25 and BAT 26 are part of intronic sequences of the hMSH2 gene and the c-kit oncogene, which are known to be frequently mutated in carcinomas of the mutator phenotype (30).

PCR was performed in a total volume of 50 μl containing 200 ng of DNA, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 50 mM KCl, 5% DMSO, 0.1% Triton X-100, 200 μM dNTP, 400 μM each primer, and 2 units of Prime Zyme Polymerase (Biometra, Göttingen, Germany). After an initial denaturation step at 94°C for 4 min, 35 cycles were performed, consisting of 1 min at 94°C, 2 min at 52–60°C (depending on the locus under investigation), and 2 min at 72°C, followed by a final extension for 4 min at 72°C. Five μl of the PCR product were diluted 1:1 with a denaturing loading buffer [98% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 10 mM EDTA acetic acid (pH 8.0)] and were electrophoresed on a denaturing polyacrylamide gel (SequaGel-8, Hessisch-Oldendorf, Germany) consisting of 8% acrylamide/bisacrylamide (19:1) and 8.3% urea in 0.1 M Tris-borate-2 mM EDTA buffer (pH 8.3) for 2 h at 100 W. After electrophoresis, gels were fixed in 10% ethanol and 1% acetic acid for 20 min, followed by an incubation in 0.017 M silver nitrate solution for 20 min. Gels were developed in a solution consisting of 0.38 M NaOH and 0.19% formalin. The reaction was neutralized with a 0.07 M sodium carbonate solution. Subsequently gels were vacuum-dried and stored for permanent record.

Each tumor specimen was investigated with at least five different microsatellites. The median number of loci analyzed per case was seven. A case was considered MI positive when at least two loci showed a different mobility of bands in gel electrophoresis. All PCR and electrophoretic runs were performed twice.

**Als.** Al was defined as a visible reduction of 50% or more in the band intensity of the tumor sample when compared to the corresponding normal tissue.

**Statistical Analysis.** Tests for differences between MI-positive and MI-negative groups were performed using Fisher’s exact test for dichotomous variables, χ² test for other categoric variables, and Wilcoxon-Mann-Whitney test for ordinal variables. Analyses of survival were performed using the Kaplan-Meier method (31), and differences between patients’ groups were tested by the log-rank test. Differences with P <0.05 were considered significant.

### RESULTS

**Frequency of MI in Gastric Cancer Patients.** MI could be demonstrated in 56 (44.5%) of the 126 gastric carcinomas investigated, one locus being affected in 40 (31.7%), two loci being affected in 6 (4.8%), three loci being affected in 4 (3.2%), and four and more loci being affected in 6 (4.8%) carcinomas. In cases with MI in multiple loci (Fig. 1), both gains and losses of alleles could be observed with an allelic size variation up to 14 bp, whereas in most cases with MI in one locus, allelic size varied between 2 and 4 bp only.

**AI of Polymorphic Markers in Gastric Cancer.** Als were found at a frequency of 6.6% on locus D2S123, which is part of the MMR gene hMSH2 (Fig. 2). The highest frequency of AI (6.9%) was observed at the locus D5S107 on the long arm of chromosome 5. The marker D5S346 is strongly related to the

**Table 1** Microsatellite loci analyzed

<table>
<thead>
<tr>
<th>Marker</th>
<th>Localization</th>
<th>Primer</th>
<th>Annealing temperature (°C)</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2S119</td>
<td>2p21/hMSH2</td>
<td>5'cttggggaaacagaggtca</td>
<td>58</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D2S123</td>
<td>2-21-16/hMSH2</td>
<td>5'gaagctttctcaatattct</td>
<td>60</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D5S107</td>
<td>5q11.2-13.3</td>
<td>5'ctacacaaatctgatatattgtc</td>
<td>52</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D5S346</td>
<td>5q21-22/APC</td>
<td>5'gatcagctcaagatctctttcc</td>
<td>58</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D10S197</td>
<td>10q11-12</td>
<td>5'acctgacttgctcttacctgtc</td>
<td>60</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D11S904</td>
<td>11p13</td>
<td>5'atgggaagatagctctttcc</td>
<td>58</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D17S261</td>
<td>17p12-11.1</td>
<td>5'gcagggctctgtctgatgacta</td>
<td>52</td>
<td>(CA)n</td>
</tr>
<tr>
<td>D18S34</td>
<td>18q12.2/DCC</td>
<td>5'tctgaaaccctactctcga</td>
<td>52</td>
<td>(CA)n</td>
</tr>
<tr>
<td>BAT 25</td>
<td>Intron c-kit</td>
<td>5'tccacacctctgctgaaagat</td>
<td>58</td>
<td>(A)n</td>
</tr>
<tr>
<td>BAT 26</td>
<td>Intron hMSH2</td>
<td>5'tcatactttggactctgcccc</td>
<td>58</td>
<td>(A)n</td>
</tr>
</tbody>
</table>

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APC gene and showed Al at a frequency of 4.7% of the informative cases (Fig. 2). Furthermore, Al was found in 5.9% of the D10S197 and D11S904 loci, whereas no Al was observed at D2S119, D17S261, and D18S34. BAT 25 and BAT 26 proved to be homozygous in all cases under investigation.

Correlation of MI with Clinicopathological Findings. MI in two or more loci occurred more frequently in female (17.3%) than in male (10.2%) patients, but this difference was not significant (Table 2). According to patients' age (Fig. 3), no MI-positive tumor was observed in patients 40 years old or younger (n = 7). Concerning depth of invasion widespread MI (two or more loci affected) occurred in almost the same percentage in early gastric cancer (pT1) as in advanced pT3-4 carcinomas (11.5% and 13.5%, respectively). No significant correlation could be observed regarding lymph node involvement (pN category) or blood and lymphatic vessel invasion. Finally, no significant correlation could be detected between MI and the different histological types according to the WHO and Lauren classification or the grade of differentiation (Table 2). Interestingly, however, all four papillary type gastric cancers according to the WHO classification showed MI in more than one locus, whereas no gastric cancer of the mucinous type (n = 7) showed a widespread MI.

Survival Analysis. Using the internationally established definition of MI (instability in at least two loci), no statistically significant differences (P = 0.64) between the survival of patients with MI-positive tumors and those with MI-negative tumors (Fig. 4) could be found. Furthermore, using other definitions of MI (MI at three or more loci or MI at four or more loci, respectively), no significant difference was observed. No prognostic influence could be found when different subgroups of the patients according to the pT category or pN category were analyzed separately.

DISCUSSION

An ongoing problem are the criteria to classify a malignant tumor as a tumor of the mutator phenotype. In accordance to the definition used for MI in colorectal cancer (1, 3, 14), in this study, up to 10 loci were analyzed, and a tumor was classified as a carcinoma of the mutator phenotype when MI could be demonstrated in at least two loci. Following this definition, in this study, MI could be found in 16 of 126 (12.8%) gastric carcinomas. This frequency is in line with some other reports on MI in gastric cancer (4, 32, 33), but there are also several studies that have found a distinctly higher frequency of MI-positive gastric carcinomas (26, 34–36). This discrepancy may be due to
Concerning the patients' outcome, contradicting results had been presented in different types of human malignancies. In CRCs, MI was correlated with a better prognosis (22, 23), whereas in sporadic breast as well as in sporadic endometrial cancer, MI was correlated with a poorer outcome (13, 15). In this study, a significant difference between MI-positive and MI-negative gastric carcinomas could not be verified. This is in contrast to the study of dos Santos and coworkers (26), who were able to show a better outcome for widespread MI (MI at three or more loci) in a series of 61 gastric cancer patients. Even if we applied the definition of MI (MI at three or more loci), which dos Santos and coworkers used (26), we could not find a difference concerning the survival. This was also true when different subgroups of patients were analyzed separately according to different pT categories and pN categories. Besides some differences in the methodological approach, the discrepancy between the results of dos Santos and coworkers (26), and our results may be also due to inherent differences between high-risk populations like Japan, Taiwan, Korea, or Portugal and low-risk populations from central Europe, as in this study (37).

In this study, no differences in the frequency of MI could be observed between early gastric cancer (pT1) and more advanced carcinomas (pT2-pT4), suggesting that a mutator phenotype caused by alterations of MMR genes and revealed by MI arises early during tumor progression. Correspondingly, Semba and coworkers (38) could demonstrate MI already in intestinal metaplasias and gastric adenomas supporting the hypothesis of a multistep tumorigenesis in gastric carcinomas of the mutator phenotype.

Comparing patients' age, no MI could be found in patients younger than 40 years. Similarly, Hayden and coworkers (39) have reported ~10 gastric carcinoma patients younger than 40 years of age without MI. In this study on 126 gastric carcinomas, 7 patients younger than 40 years were included, and none of them had a mutator phenotype. Larger studies including sequence analysis of the MMR genes are necessary to elucidate this phenomenon.

Considering tumor histology, the frequencies of MI-positive and MI-negative tumors did not differ between the different histological types of the WHO and Lauren classification. This is in accordance with data from Keller and coworkers (32) and Lin and coworkers (36). On the other hand, a more frequent occurrence of MI in intestinal type carcinomas (26, 35) was observed in high-risk populations, suggesting again that there might be different mechanisms of gastric carcinogenesis in high-risk populations compared to populations being on a lower risk.

Summarizing, we show that MI manifests early during tumorigenesis of gastric cancer. However, MI is not associated
REFERENCES


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