Loss of Heterozygosity and Microsatellite Instability as Predictive Markers for Neoadjuvant Treatment in Gastric Carcinoma

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INTRODUCTION

Despite its decreasing prevalence, gastric carcinoma is still one of the major causes of cancer death worldwide (1). Because most tumors are diagnosed late in their course, they are generally already locally advanced (UICC3 stages III or IV) and have an overall median survival of only 16 months (2). In these stages the only curative method, complete surgical resection (UICC R0 resection), can be achieved in only 40% of cases (3). Neoadjuvant, cisplatin-based chemotherapy has the goal of reducing tumor volume and systemic tumor cell dissemination in gastric cancer. Although several Phase II trials have shown an increased rate of R0 resection (up to 80%) after neoadjuvant chemotherapy, it is still unclear whether this results in an overall survival benefit (4, 5). Recent published Phase II studies suggest that the individual response to chemotherapy is an independent prognostic factor (5, 6).

Of key importance for the evaluation and further development of neoadjuvant chemotherapy protocols is an accurate means of monitoring and predicting response. A range of interindividual differences are seen in drug response and tolerability that are considered to be due to constitutional genetic differences and specific genetic alterations in the tumor. Markers of response can be considered in terms of clinical, pathological, or molecular parameters, but until now, only limited information concerned with the use of molecular markers for chemotherapy response prediction and monitoring for gastric carcinoma has been available. The role of p53 in determining therapy outcome has been studied in a wide variety of tumor types and cell lines, but the results are conflicting (7). For gastric carcinoma, p53 overexpression, as demonstrated by immunohistochemistry, has been found to be associated with a lack of response to chemotherapy (8–10). The expression of TS, the target enzyme for 5-FU, has been shown to be significantly associated with response to 5-FU-based therapy in gastric carcinoma (11–13). In addition, the expression of ERCC1, an enzyme involved in nucleotide excision repair, has been found to have a significant association with response in a neoadjuvant therapy regimen based on 5-FU and cisplatin (13).

Aside from the expression or mutation of specific genes, analytic methods exist that can detect abnormalities throughout the genome, an example of which is the microsatellite analysis technique, which can detect MSI and LOH. MSI-H is a characteristic feature of tumors of the hereditary nonpolyposis colorectal cancer syndrome (14) and has been linked to defects in the DNA mismatch repair system (15, 16). MSI-H has also been found, although at lower frequency, in sporadic tumors, includ-
ing those of the stomach (17–20). Interestingly, studies of cell lines defective in mismatch repair and exhibiting MSI have demonstrated that mismatch repair deficiency is associated with tumor resistance to cisplatin (21, 22). This led to the suggestion that in vivo resistance to therapy regimens containing cisplatin might be associated with MSI or the development of MSI during therapy.

Chromosomal sites frequently demonstrating LOH can be the location of tumor suppressor genes involved in the carcinogenesis of the tumor. In gastric carcinoma one of the most frequently deleted chromosomal regions is 17p13, the region of the p53 gene (23–26).

To our knowledge, no previous study has used microsatellite analysis to clarify the role of MSI or LOH in determining the response of gastric carcinoma to cisplatin-based, neoadjuvant chemotherapy. Therefore, the goal of our study was to perform this analysis in pretherapeutic tumor specimens at microsatellite loci known to play a role in gastric carcinogenesis and to compare these results with the patients’ clinical responses to therapy.

MATERIALS AND METHODS

Patients and Tumors. In this retrospective study, we analyzed the pretherapeutic biopsies of 45 patients with locally advanced gastric carcinoma who were treated with a cisplatin-based neoadjuvant chemotherapy protocol and were subsequently operated on in the Department of Surgery. Criteria for selection of these patients were the availability of normal and tumor tissue and the suitability of the cases for the isolation of DNA from tumor areas containing at least 50% tumor cells by manual microdissection. From the initially analyzed 45 patients, 8 were later excluded from the study because they received <50% of the planned chemotherapy regimen and were not evaluated clinically with respect to response. The tumors of all of the patients were clinically staged as cT3 or cT4 according to the tumor, node, metastasis classification of the UICC (27). The tumors in the biopsies were graded according to the WHO and were histologically classified according to the Lauren classification (28). The site of the tumor was defined surgically according to the location of the main tumor mass and was divided into tumors located in the proximal, middle, and distal portions of the stomach. The patient data are shown in Table 1.

Neoadjuvant Chemotherapy. To be included in the study, the patient was required to have a locally advanced gastric carcinoma clinically staged as cT3, cT4, Nx. The clinical stage of the tumor was determined by a CT scan and endoscopy with endoluminal ultrasound. To exclude distant metastases, all patients had a diagnostic laparoscopy as well as a bone scan and an abdominal ultrasound. M1 (LYM) and/or a positive preoperative lavage cytology were not exclusion criteria. Patients with previous malignancies or a WHO performance status of higher than 1 were excluded from the study. The patients were treated in one of three Phase II trials with a polychemotherapy regimen based on cisplatin, either according to the EAP scheme (29), the E-PLF scheme (30), or the PLF scheme. The detailed chemotherapy protocols are shown in Table 2. The numbers of patients who received the different protocols are shown in Table 1.

Response Evaluation. After chemotherapy the CT scan and endoscopy with endoluminal ultrasound were repeated and compared with the initial results. A reduction of tumor volume of >50% (according to the WHO classification; Ref. 6) was classified as response, referred to below as “responder” (n = 13), whereas a reduction of ≤50% was classified as nonresponse and referred to below as “nonresponder” (n = 24). Only patients who received at least 50% of the planned total chemotherapy dosage were evaluated for response.

DNA Isolation and Microsatellite Analysis. Paired nontumor and tumor DNA samples from the pretherapeutic biopsy specimens were isolated from formalin-fixed, paraffin-embedded tissues after microdissection, as described previously (31). Tumor DNA was only isolated from tissue areas containing >50% tumor cells.

The microsatellite analysis included 11 microsatellite markers (one mono-, nine di-, and one tetranucleotide repeat). These markers were selected either because of their location at chromosomal sites in, or near, genes known to be involved in gastric carcinogenesis (3p, 5q, 7q, 11q, 16p, 17p, and 18q; Refs. 23–26, 32, and 33) or because of their inclusion in marker panels used for the determination of MSI (BAT 40, D2S123, and DSS346; Refs. 34 and 35). The analyzed markers are listed in Table 3.

The PCR reactions were essentially performed as described previously (20). One of the primers used for amplification was fluorescent labeled. The PCR products were separated and detected with an automated sequencing system according to the protocol of the manufacturer (ABI 377; Perkin-Elmer Corp., Branchburg, NJ).

Scoring of MSI. An additional band in the tumor DNA compared with the nontumor tissue was classified as instability for this marker. Instability in 5 or more of the 11 markers (≥45%) was defined as MSI-H, and instability in 4 or fewer markers as MSI-L. MSI-L was confirmed by a second PCR.

Scoring of LOH. LOH was determined using the calculation method described by Beckmann et al. (36). A tumor was considered to have LOH if the allele 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 the allele peak ratio may have resulted from allelic amplification. BAT 40 was homozygotic or ambiguous with respect to heterozygosity and was not evaluated for LOH. Tumors exhibiting MSI at a given locus were not evaluated for LOH.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient and tumor data</th>
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<tbody>
<tr>
<td>Patients</td>
<td>37</td>
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<tr>
<td>Median age</td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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<td>Intestinal</td>
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<tr>
<td>Nonintestinal</td>
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</tr>
<tr>
<td>G2</td>
<td>6</td>
</tr>
<tr>
<td>G3</td>
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<tr>
<td>Tumor site</td>
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<td>Proximal</td>
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<td>Distal</td>
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<td>EAP</td>
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<td>E-PLF</td>
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The overall extent of LOH for each tumor was calculated as FAL ratio by dividing the number of markers showing LOH by the number of informative markers for each tumor (37). The combined information of the markers D7S644 and D7S1824 for chromosome 7q and of the markers D5S346 and D5S107 for chromosome 5q was used. Only tumors that were informative in at least four of the chromosomal sites analyzed were included in the FAL calculation. This calculation gave a range from 0 – 0.75.

Immunohistochemistry. Immunohistochemistry for p53 was performed using an automated staining device (TeckMate 500; DAKO Corp., Glostrup, Denmark), with the D0 7 monoclonal antibody (DAKO) used at a 1:300 dilution after microwave treatment of formalin-fixed, paraffin-embedded 6-μm tissue sections (once for 20 min, microwave at 750 W, and citrate buffer solution at pH 6.0). The reaction was developed with the reaction indicator. A positive reaction for p53 protein was seen as a nuclear stain, and a case was considered positive for overexpression when at least 10% of the tumor cell nuclei showed definite staining. Positive and negative controls were included with each staining series.

Among the 37 tumors included in the study, sufficient material for immunohistochemical analysis and evaluation was available from 33 cases.

Statistical Analysis. Medians and ranges were reported for continuous variables, and absolute and relative frequencies for categorical variables, respectively. The Fisher’s exact test was used to compare relative frequencies because one or more expected cell counts were frequently lower than 5. A bootstrap procedure was applied to adjust for testing multiple microsatellite markers simultaneously using 100,000 resamples. In this procedure, pseudo-data sets are created by sampling observations with replacement within each microsatellite marker. This resampling was repeated 100,000 times. The adjusted Ps were then calculated from the proportion of resampled data sets with a pseudo-P less than or equal to the actual P (38). Because this was a retrospective investigation, Ps did not have to be interpreted confirmatory. The statistical analyses were performed using SAS software (release 6.12, TS level 0060; SAS Institute Inc., Cary, NC).

RESULTS

MSI. In the study as a whole, MSI was found in at least 1 of the 11 tested markers in the pretherapeutic biopsies in 7 of 37 (19%) tumors, with 2 tumors exhibiting MSI-H (5%) and 5 tumors exhibiting MSI-L (14%). With respect to chemotherapeutic response, in the responder group none of the 13 tumors showed MSI-H and 2 showed MSI-L (15%). In the nonresponder group, 2 of 24 (8%) tumors showed MSI-H and 3 showed MSI-L (13%).

LOH. In the study as a whole, the loci most frequently exhibiting LOH were on chromosome 7q34–35 (D7S1824) with 13 of 27 (48%) tumors, on chromosome 18q (D18S34) with 10 of 29 (34%) tumors, and on chromosome 17p13 (TP53) with 10 of 32 (31%) tumors exhibiting LOH. LOH rates of 20%, 21%, 26%, and 29% were observed for chromosomes 5q21 (D5S346), 3p, 11q, and 5q11 (D5S107), respectively, and at rates ranging from 8–17% for chromosomes 7q21 (D7S644), 2p, and 16q. The overall number of losses per chromosomal site was 63 of 227 informative markers (29%), combining the data for the two loci on chromosomes 7q and 5q, respectively. These results are shown in Table 4.

Chromosome 17p (TP53) revealed a statistically significant difference between the groups, with 7 of 12 (58%) tumors of the responder group versus 3 of 20 (15%) tumors of the nonresponder group exhibiting a LOH (P = 0.018). A borderline statistical difference was observed at chromosome 5q21 with 4 of 9 (44%) responder tumors versus 2 of 21 (10%) nonresponder tumors showing a LOH (P = 0.049). A higher, but not statistically significant, rate of LOH was observed for chromosome 7q34 (D7S1824) in the responder group with 8 of 13 (62%) tumors exhibiting LOH compared with 5 of 14 (36%) tumors in the nonresponder group (P = 0.08). Only slightly higher LOH rates in the responder group were seen at chromosomes 18q, 11q, 5q, and 3p. The total number of losses at all chromosomal sites was with 33 of 81 (41%) significantly higher in the responder group compared with 30 of 146 (21%) in the nonresponder group (P < 0.01), whereas the number of informative markers with respect to all markers tested was nearly equal, with
72% in the responder group and 70% in the nonresponder group. These results are summarized in Table 4 and Fig. 1. Representative examples of LOH are shown in Fig. 2.

With respect to FAL, 19 of the 35 (54%) tumors showed low FAL, 7 (20%) showed medium FAL, and 9 (26%) showed high FAL values. There was a significant difference in FAL rates between the responder and nonresponder groups ($P = 0.02$). In the responder group, 4 of 13 (31%) tumors showed low, 2 (16%) showed medium, and 7 (54%) showed high FAL. In the nonresponder group, 15 of 22 (68%) showed low, 5 (23%) showed medium, and 2 (9%) showed high FAL values. The results are shown in Fig. 3.

**Immunohistochemistry.** Gastric carcinoma biopsies of 33 patients were analyzed for p53 overexpression by immunohistochemistry. Overall, p53 overexpression was found in 14 (42%) of the tumors, whereas 19 (58%) were negative. No correlation was found with respect to p53 overexpression and response, because 5 of 11 (45%) responder and 9 of 22 (41%) nonresponder cases showed p53 overexpression.

**Correlation with Clinicopathological Features.** With respect to the Lauren classification of the tumors, 9 of 13 (69%) tumors in the responder group were of the intestinal type compared with 7 of 24 (29%) tumors in the nonresponder group, a statistically significant difference ($P = 0.036$). The intestinal Lauren type was also more frequent in the tumors with high or medium (>0.25) FAL scores (10 of 16) compared with low ($\leq 0.25$) FAL scores (5 of 19) with a borderline statistical difference ($P = 0.044$).

### DISCUSSION

In this study, we compared the frequencies of LOH and MSI at 11 microsatellite loci in biopsies of primary advanced gastric carcinoma and compared them with the clinical response to neoadjuvant chemotherapy. One of the most statistically significant findings was that responding tumors had an overall higher rate of LOH. Expressing the level of LOH per tumor as FAL, we found high FAL values to be associated with tumors that responded well. A high FAL can be interpreted as an indication of a high level of chromosomal aberrations that may be due to different molecular mechanisms, as, for example, deletions of certain chromosomal regions, aberrant mitotic recombination, or nondisjunctional chromosomal losses (39).

The striking association of tumors with a high rate of LOH and good response, which we saw in our study, is difficult to explain because neither the factors responsible for a high LOH rate nor the precise chromosomal targets of cisplatin and 5-FU are exactly known and also because complex interactions between 5-FU and cisplatin might exist. 5-FU is known to be an inhibitor of TS, a key enzyme in nucleotide metabolism, however, there is also evidence that 5-FU can be misincorporated into DNA and RNA and affect their structure and function (41).
The main effect of cisplatin is assumed to be that of a DNA-damaging agent (42). Our findings seem to fit with the concept that specific types of genetic instability exist that are involved in driving tumor development, but, when exceeding a critical value, instability rises to a level incompatible with viability (43). This could explain why cells with a high level of chromosomal alteration are more sensitive to DNA-damaging agents such as cisplatin or 5-FU.

We found that intestinal tumors had a significantly higher FAL rate than nonintestinal tumors, which may be related to data indicating that diffuse type tumors are more likely to be diploid than intestinal type tumors (44). In our study, good response was associated with both the Lauren intestinal type and a high rate of FAL. A larger study with more cases and multivariate analysis would be needed to clarify which of these is the more important parameter. In addition, one potential source of bias is the fact that the methods used to evaluate response clinically (i.e., CT scan or endoscopy) are more difficult to evaluate with diffusely infiltrating tumors.

Another statistically significant difference between responding and nonresponding tumors was seen with respect to LOH at chromosome 17p13, where the $p53$ gene is located. Fifty-eight percent of the responding tumors and only 15% of the nonresponding tumors showed LOH at this locus. To our knowledge, these are the first reported data for LOH at the $p53$ locus in gastric cancer in comparison with chemotherapy response and indicate that $p53$ mutations might be associated with a better response. Although these results are preliminary and must be confirmed with mutation analysis of the $p53$ gene, they seem to conflict with studies that have found that $p53$ protein overexpression, as seen by immunohistochemistry is associated with a poor response to neoadjuvant chemotherapy containing cisplatin and 5-FU (8–10). In our study, $p53$ overexpression analyzed by immunohistochemistry revealed no correlation with either therapy response or with LOH on chromosome 17p. However, it is well known that $p53$ overexpression does not necessarily correlate with the inactivation status of the gene because certain types of mutations do not produce a stable protein (45), or because protein overexpression may be also due to increased expression of wild-type protein induced by stress factors (46). Furthermore, our findings are contrary to the report of an association between $p53$ mutations determined by allelic loss at 17p and mutation analysis of the $p53$ gene and nonresponse to neoadjuvant chemotherapy in head and neck squamous cell carcinoma (47). These discrepant results may be
partly explained by differences in organ-specific tumorigenesis and tissue-specific response characteristics. Furthermore, a detailed comparison among studies is also complicated by the fact that various clinical parameters and methods are used for pretherapeutic tumor staging and response evaluation that cannot be directly compared (8, 47).

The p53 gene has been implicated in various cellular functions, including the control of the G1-S checkpoint and promotion of correct repair of DNA damage (46). This may be an explanation for our finding that LOH at p53 was associated with response, because defects in p53 would prevent repair of DNA damage resulting from the action of cisplatin or 5-FU. This effect might be even stronger in cells already exhibiting an elevated level of chromosomal alterations. In cell lines, an enhanced sensitivity to cisplatin in cells lacking p53 function has been demonstrated, which supports this hypothesis (48).

With respect to LOH at other chromosomal sites, we found a high rate of LOH at chromosome 7q. Overall, 41% of the tumors showed loss at this chromosomal site, which supports data that show that this chromosomal region is important for gastric carcinogenesis (25, 32). LOH at chromosome 7q was more frequent in the responder than in the nonresponder group (62% versus 29%), although the difference was not statistically significant. The MDR1 gene, which has been reported to be involved in drug resistance (49), is located on chromosome 7q21, but our analyses at 7q21 and 7q34–35 indicate that LOH at 7q34–35 is the more important of the two sites, indicating that the MDR1 gene was not important for determining response in our cases.

At other chromosomal loci we found LOH in the range of 25–35% at 5q, 9q, and 18q, which is in general agreement with previous studies of gastric cancer (23, 31, 37). The frequency of LOH at these chromosomal regions was generally higher in the responder versus the nonresponder group. A difference that was only of borderline statistical significance was observed at 5q21. As a whole, these results suggest that the higher frequencies of LOH in the responder group reflect a generally higher level of CIN in this group rather than specific chromosomal alterations.

MSI has been linked to defects in the DNA mismatch repair system, and cell lines with defects in specific DNA mismatch repair genes have been shown to have a striking resistance to cisplatin (21, 22). The two nonresponder patients with a high level of MSI in our study support this observation. The overall frequency of only 5% for high-level MSI may also partly explain the differences in organ-specific tumorigenesis and tissue-specific response characteristics. However, a significant response or survival advantage is only observed in less than half of treated patients (5).4 Without appropriate predictive markers, patient selection is based on clinical staging parameters and results in the treatment of more than twice as many patients than will eventually profit from neoadjuvant chemotherapy. Treatment of nonresponders may be particularly harmful because it may reduce the chance for a timely surgical tumor resection. The results of our study indicate that microsatellite analysis might be a useful component for the prediction of chemotherapy response in patients with gastric carcinoma. Our finding of an association of the LOH level in the tumor (FAL rate) and therapy response might be particularly helpful in this context and could contribute to a more differentiated, individualized application of neoadjuvant chemotherapy.

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REFERENCES


4 Unpublished results.
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