Microsatellite Instability Correlates with Reduced Survival and Poor Disease Prognosis in Breast Cancer

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ABSTRACT
Size changes in microsatellite sequences have been detected in many types of cancer, but the influence of this form of genetic instability on disease progression remains unclear. We determined the incidence of microsatellite instability in breast cancer by comparing PCR-amplified sequences from paraffin-embedded samples of normal and tumor tissue from affected individuals. This analysis showed that at least 30% of breast cancers exhibit microsatellite instability (MI). Of importance, MI correlated with indicators commonly associated with poor disease prognosis, including lymph node status, tumor size, and advanced tumor stage. Individuals with MI+ tumors also showed significantly reduced disease-free and overall survival. These data contrast with studies showing that MI correlates with improved prognosis in colon and gastric cancers. We propose that defects resulting in MI promote disease progression and result in a poor prognosis in breast cancer.

INTRODUCTION
The development of breast and other cancers is a multistep process (1, 2). At least six genetic changes may be required to convert normal breast epithelium to malignant breast cancer (1, 3), with each alteration presumably increasing proliferative or survival capacity. In contrast, many of the types of changes commonly observed in cancer cell genomes develop at immeasurably low frequencies in normal cells. This is consistent with previous proposals that one or more changes occurring during cancer evolution increase the endogenous mutation rate beyond the normal repair capacity or decrease the ability to detect and/or repair mutations, resulting in a mutator phenotype in affected cells (4, 5).

Mutations in cell cycle control and MMR genes enable cells to undergo genetic alterations at greatly increased rates. Tumor suppressor genes that affect the G1-S transition, such as p53 and Rb, maintain genomic fidelity by inducing cell cycle arrest or apoptosis in response to DNA damage or to specific metabolic limitations (6–10). Mutations in tumor suppressor genes, or the pathways they control, eliminate the normal arrest/apoptosis responses to DNA damage or growth-limiting conditions, resulting in dramatically higher rates of chromosome breakage, rearrangement, and aneuploidy.

Mutations in genes involved in maintaining the fidelity of DNA replication result in increased mutation rates via small scale sequence alterations such as base changes, additions, and/or deletions. For example, in normal cells, MMR proteins recognize and correct errors made during DNA replication (for reviews, see Modrich (11) and Fishel and Kolodner (12)), minimizing the rate at which sequence changes accumulate in daughter cell genomes. Mutations in MMR proteins allow errors made during replication to be passed on to daughter cell genomes, resulting in 100-1000-fold increases in mutation rate (13–16). In addition, inactivation of the murine MMR gene MSH2 results in a hyperrecombination phenotype, indicating that members of this repair pathway are also involved in preventing promiscuous chromosomal rearrangement (17). Thus, defective MMR provides a mechanism for increasing the rates of both localized and large scale chromosomal alterations.

Defective MMR is often manifested as alterations in simple sequence repeats, a phenomenon alternately referred to as MI (MI+ or MIN), replication error phenotype, or the mutator phenotype. First detected in patients with HNPCC (18–21), MI has been described in a wide variety of malignancies (22–26) including breast cancers (20, 27–31). MI has been observed in both hereditary and sporadic cancers, although some differences in overall incidence and in the percentage of microsatellites affected have been observed (32–34). Although it is not known whether the MI observed in diverse cancer types derives from the same basic mechanisms, it is clear that MI+ tumors undergo mutations in repeat sequences at higher rates than their MI− counterparts, and that this type of genetic instability is found in a significant fraction of human tumors.

Mechanisms resulting in genetic instability (e.g., germline p53 mutations in patients with Li-Fraumeni syndrome) should lead to accelerated tumorigenesis and poor disease outcome in affected individuals. It is surprising, therefore, that MI correlates with a better prognosis and increased survival of patients with HNPCC (28, 35, 36). The prognostic implications of MI-generating mechanisms in sporadic cancers, or cancers in other tissues, have not been determined. If different mechanisms (or mutations in different MMR genes) generate the MI observed in sporadic cancers or if different target genes are mutated in specific tissues, the ultimate effects on disease progression and patient survival may be different than that observed in colon cancer. Furthermore, since HNPCC represents a small percentage of human cancers (5% of all colon cancer patients), it is clearly important to determine the consequences of MI-generating mechanisms in sporadic tumors, which constitute the majority of human cancers.

The goal of this study was to determine the incidence of MI in breast cancer and to analyze its relationship with known prognostic factors and patient survival. Our results reveal that MI in breast cancer is associated with more advanced, aggressive disease and poor patient prognosis. These findings contrast with those made in Lynch syndrome and advance our understanding of the impact of MI on disease outcome outside of HNPCC and colorectal cancer. The implications for diagnosis and treatment of breast cancer are discussed.

MATERIALS AND METHODS

Samples and DNA Isolation. Archival paraffin-embedded material from the surgical pathology files of the University of California, San Diego Medical Center was used for this study in accordance with a protocol approved by the Institutional Review Board for investigations involving human subjects. Samples consisted of matched normal breast, primary breast tumor, and, where available, LN material from 48 patients diagnosed with invasive breast carci-
noma. Samples were selected in a consecutive fashion from those for which ploidy status and sufficient material was available for analysis. Determination of a family history of breast cancer was not made for this study, and it would be expected that only 5% of these samples would be from patients with familial disease. All primary tumors had been analyzed for hormone receptor status (ER) and DNA ploidy at the time of diagnosis. All patient charts were reviewed by one investigator (B. A. P.), and tumor registry data were accessed to confirm clinical and pathological staging, disease-free survival, and overall survival. Ten-μm sections were cut from each paraffin-embedded tissue block, taking precautions to avoid cross-contamination of specimens. The first and last section from each block were stained with H&E and examined with light microscopy to confirm that normal sections were free of atypia or carcinoma in situ, and that tumor and LN sections were composed of ≥90% tumor cells. Suitable DNA for PCR analysis was obtained after extraction of paraffin with xylene and treatment with proteinase K as described previously (37). Extracted DNA was stored at -20°C prior to PCR amplification.

**PCR Primers.** Tumor and normal DNA were amplified using 11 different primer pairs specific for known human microsatellites. TP53, EDH17B, DI75855, DI75250, and DI75579 have been described previously (for TP53, see Ref. 38; for others, see Ref. 39). The following primers were a gift from Dr. Val Sheffield: ATA6G09 (5’-ACTTGAGTCCAGGTTGAG-3’ and 5’-AACACAAGAGGCTGCTGTAAGC-3’; 295-321 bp), ATA6E01 (5’-CTGCCTGCTTCTCCTGATG-3’ and 5’-ATGTTGACAGGAAAGCA-3’; 198-223 bp), GGAAD07 (5’-GAGAGGCAAGTCGTAAGC-3’ and 5’-ATGGAAGAGCTGTCCTAAACA-3’; 185-256 bp), GGA2E02 (5’-AGGAAAGAGGAAACGAGGA-3’ and 5’-TATATAGGAAATAATGTTGAGG-3’; 172-219 bp), and GATA1BI2 (5’-AACAAAAACAAAACAAAC-3’ and 5’-GGTTGAATGATCTGATG-3’; 365-403 bp).

**PCR Conditions.** PCR reactions were performed in a volume of 30 μl, using 1× PCR buffer (Boehringer Mannheim), 200 μM dNTPs, 20 μM each primer, 1-3 μl of extracted DNA, and 0.75 units Taq polymerase (Boehringer Mannheim). PCR amplification was done in an Eppendorf Thermal Cycler using the following conditions: 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and completed with a 7-min extension at 72°C. PCR reactions were kept on ice until placed into the preheated thermal cycler to avoid production of unwanted, low stringency PCR by-products. Two μl of this reaction were reamplified in fresh reaction mix using the conditions given above, with the addition of 0.2 μl [32P]dCTP (Amersham) reaction. Controls containing no DNA were run with each PCR reaction. After amplification, this second reaction was mixed with two volumes of loading buffer (95% formamide, 20 mM NaOH, and 0.1% bromophenol blue) and heated at 95°C for 3 min. Three μl of the denatured product were run on 6% denaturing polyacrylamide sequencing gels. Gels were dried, exposed, and analyzed on a Molecular Dynamics PhosphorImager according to the protocol supplied by the manufacturer.

**Statistical Analysis.** Statistical analyses were performed using SAS version 6.09 (SAS Institute, Cary, NC). All analyses were performed at a significance level of 0.05 using two-sided hypothesis tests. Data transformations (e.g., log) were examined and applied where appropriate. Comparisons of continuous responses by MI status were performed using two-sample t tests. Wilcoxon’s rank sum test with adjustment for ties was used to calculate MI incidence. Statistical Analysis. Statistical analyses were performed using SAS version 6.09 (SAS Institute, Cary, NC). All analyses were performed at a significance level of 0.05 using two-sided hypothesis tests. Data transformations (e.g., log) were examined and applied where appropriate. Comparisons of continuous responses by MI status were performed using two-sample t tests. Wilcoxon’s rank sum test with adjustment for ties was used to calculate MI incidence.

**RESULTS**

**MI Incidence in Breast Cancer.** The lack of a standard definition for the presence of MI in a tumor has led to widely divergent estimates of its incidence in sporadic cancers. We utilized the following protocol to control for the quality and quantity of input DNA isolated from the paraffin sections. All samples showing changes in microsatellite sequences were verified by isolating DNA from a new paraffin section and subjecting it to a second, independent PCR analysis. This as well as control PCR reactions lacking DNA were done to eliminate the chance of false positives due to PCR artifacts or sample contamination. DNA from a subset of verified positives was sequenced to show that alterations in band size were due to changes in the number of microsatellite repeats and not to random sequence alterations or amplification of nonmicrosatellite sequences (data not shown). Finally, we used the stringent criteria established by Aaltonen et al. (18) and utilized in several studies of MI (32, 33) that require alterations in microsatellite size at two independent genomic sites to classify a tumor MI+.

Fig. 1 summarizes the results of analyzing microsatellite sizes at 11 different loci in primary tumors from 37 breast cancer patients. Differences in microsatellite repeats between tumor, LN, and normal DNA were scored in two ways. D2 changes indicate a clear difference in size of one or more microsatellite bands between normal tissue and either the primary tumor and/or LN sample from the same patient (Fig. 2). Changes in allele intensity between tumor and normal tissue were also observed. These changes, designated D1 in Fig. 1, are most likely due to loss of heterozygosity within the tumor and were not used to calculate MI incidence.

Using the stringent criteria of two or more D2 changes between the primary tumor and normal adjacent tissue to indicate MI, 11 of 37 (30%) patients in this study had tumors with MI. One patient, case 37, was found to have one D2 change in both the primary and LN samples and another D2 change in the LN alone. This patient was scored as MI+ since the primary and the LN together showed evidence of two D2 changes. This MI incidence is within the range of previously reported values for sporadic breast cancer (20, 27-31).

**Correlations between MI and Tumor Characteristics.** The potential impact of MI on tumor growth and progression was determined by examining the association with a variety of clinical and pathological characteristics of the primary breast cancers. Unintentional bias was prevented from entering the analyses by coding patient tissue samples so that MI determination was done without knowledge of the patient and tumor characteristics. The results are summarized in Table 1.

The presence of MI+ tumors correlated significantly with the presence of involved LNs at diagnosis. Patients with MI+ tumors had involved LNs in 80% of the cases tested compared to 37% of those with MI− tumors (P = 0.05).

Tumor size also correlated strongly with MI (Table 1). MI+ tumors were found to be significantly larger than MI− tumors (median size, 3.5 cm versus 1.5 cm, respectively) using a t test for populations with unequal variances (t13 = -2.45, P = 0.027). Furthermore, the size differences between diploid and aneuploid tumors were greater in MI+ patients than in MI− patients, an observation supported by a general linear model for log (tumor size) with a significant interaction term MI status X ploidy (P = 0.031). Stated another way, aneuploid MI+ tumors were more than four times as large as diploid MI+ tumors (5.8 cm versus 1.4 cm, respectively). In contrast, aneuploid MI− tumors were less than twice the size of their diploid counterparts (2.6 cm versus 1.6 cm).
from the exact null distribution for the Wilcoxon statistic yielded a similar value of $P = 0.012$.

Other parameters commonly analyzed in breast cancer include patient age, tumor cell ploidy, S-phase fraction (the percentage of cells in S-phase), and hormone receptor status. As shown in Table 1, our findings indicate that these parameters do not correlate with the MI status in the 37 patients analyzed.

**Correlation between MI and Patient Performance.** A significant relationship was observed between patients with MI+ tumors and disease-free survival (defined as the time from diagnosis to the first recurrence of cancer outside the breast). As shown in Fig. 4a, patients with MI+ tumors developed metastatic disease at a significantly higher rate than did patients with MI− tumors ($P = 0.050$). Overall, 64% (7/11) of patients with MI+ tumors developed metastatic disease compared to 16% (4/25) of the MI− group. A univariate analysis of the data gives a risk ratio of 5.62 for the MI status, with a 95% confidence interval of 1.03 and 30.5. We also examined whether the MI status correlated with overall survival (defined as the time from initial diagnosis to death). We again found that patients with MI+ tumors showed significantly reduced survival compared to patients

### Table 1 Correlation of MI with pathological parameters

<table>
<thead>
<tr>
<th>Category</th>
<th>MI+</th>
<th>MI−</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of involved LN</td>
<td>8/10 patients</td>
<td>7/19 patients</td>
<td>$P = 0.050^a$</td>
</tr>
<tr>
<td>Tumor size (median)</td>
<td>3.5 cm</td>
<td>1.5 cm</td>
<td>$P = 0.027^b$</td>
</tr>
<tr>
<td>Median tumor stage at diagnosis</td>
<td>IIB</td>
<td>HA</td>
<td>$P = 0.017^c$</td>
</tr>
<tr>
<td>Age (median)</td>
<td>58 yr</td>
<td>62 yr</td>
<td>None</td>
</tr>
<tr>
<td>Tumor ploidy by flow cytometry</td>
<td>3/11 diploid</td>
<td>12/25 diploid</td>
<td>None</td>
</tr>
<tr>
<td>S-phase fraction</td>
<td>5/9 high</td>
<td>12/24 high</td>
<td>None</td>
</tr>
<tr>
<td>Presence of ER</td>
<td>7/11 positive</td>
<td>18/25 positive</td>
<td>None</td>
</tr>
<tr>
<td>Development of metastatic disease</td>
<td>7/11</td>
<td>4/25</td>
<td>$P = 0.007^d$</td>
</tr>
<tr>
<td>Estimated disease-free survival proportion (3 yr)</td>
<td>0.35</td>
<td>0.78</td>
<td>$P = 0.050^d$</td>
</tr>
<tr>
<td>Estimated overall survival</td>
<td>0.47</td>
<td>0.87</td>
<td>$P = 0.020^d$</td>
</tr>
</tbody>
</table>

The presence of involved LN, tumor size, tumor stage, patient age, tumor ploidy, S-phase fraction, and the presence of ERs were determined at the time of initial diagnosis. Disease-free and overall survival were determined by examination of patient charts and tumor registry data. Analysis of involved LN excluded patients for whom involved LN existed, but were unavailable for PCR analysis (cases 6, 16, 20, and 51) or for those patients who had undergone chemotherapy prior to surgery (cases 5, 19, and 38). All statistical tests were performed using two-sided alternatives. Significance was determined using various tests.

- $^a$ Fisher’s exact test.
- $^b$ Fisher’s test for populations with unequal variances.
- $^c$ Wilcoxon rank sum test.
- $^d$ Log rank test for differences in the two survival distributions.

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**Fig. 2.** Examples of alterations of microsatellite sequences in breast cancer. Six percent polyacrylamide/urea sequencing gels were used to resolve changes in microsatellite size. $a$, differences between tumor (T) and normal (N) DNA from patient 21 using TP53 primers. The presence of multiple bands at each allele is due to polymerase stuttering, a reproducible phenomenon that can occur when amplifying DNA sequence repeats (51). $b$ and $c$, changes in the number of microsatellite repeats between tumor and normal DNA from patients 31 and 33, respectively, using GGAA2E02 primers.
with MI− tumors ($P = 0.020$; Fig. 4b). This is the first demonstration of MI correlating with disease outcome outside of Lynch syndrome cancers and raises the possibility of the use of MI as marker of disease prognosis.

**DISCUSSION**

The stringent criteria we used to determine MI incidence in breast cancer revealed MI in 30% of the patients examined. The characteristic microsatellite size changes observed and the results from sequencing a subset of the alterations indicate that the changes we detected are the same as the MI reported in colon, breast, and other cancers (18–31). However, it is possible that the incidence we determined underestimates the actual frequency, since we analyzed only 11 of the many thousands of microsatellite sequences in the genome. In addition, we detected MI in two cases of recurrent tumors which were not included in our statistical analyses. Such cases also emphasize the association of MI with poor prognosis and advanced disease. Thus, the incidence of MI in breast cancer and its impact on prognosis may be greater than our conservative estimates indicate.

Our findings that MI+ breast cancers are larger, more frequently found in patients with involved LNs, and correlate with poor disease outcome are consistent with the presence of a mutator phenotype. It has been well established that mutations in MMR genes produce genome-wide MI and contribute to tumor formation in mammals (17, 41). For example, the inability to detect DNA mismatches generated during replication have been reported to result in structural gene inactivation (13, 14). Such a process could also activate quiescent oncogenes and/or inactivate tumor suppressor or cell cycle control genes. Indeed, mutations in the transforming growth factor β type II receptor have been found in MI+ colon cancer cell lines, resulting in enhanced tumorigenicity (42). We have found many cell lines with MMR defects that also have inactivated cell cycle control pathways, implying that the failure to detect and repair replication errors can produce variants unable to respond to other forms of DNA damage. Loss of cell cycle control could then potentiate further genetic alterations, resulting in rapid tumor progression. This idea is supported by data showing that an intact MMR system is required for an appropriate G2-M arrest in response to unrepaired DNA mispairs (43). The finding in this study that MI+/aneuploid tumors were significantly larger than MI+/diploid tumors may indicate that the presence of a mutator phenotype, coupled with loss of ploidy control, can result in accelerated growth. Similarly, the absence of adenomas in patients with HNPCC may mean that the MMR defects result in the evolution of fast-growing tumor cells that rapidly progress to carcinoma (44).

In this context, it is somewhat surprising that germline mutation of either of two MMR genes selectively increases the probability of developing colorectal and other related cancers, yet patients developing these cancers have a better than average prognosis (28, 35, 36). These contrasts between the different prognoses associated with MI in colon and breast cancer suggest that there is either a gap in our understanding of how defective MMR ultimately affects genomic stability, or that the actions of a specific mutational mechanism may have variable outcomes in cells of different tissues. Our finding that MI correlated with indicators of poor disease prognosis and reduced survival in breast cancer indicates that there may be a tissue-specific response to the effects of MI. It is possible that the unique architecture of each tumor type could affect disease outcome differently, although they may share a common underlying mutational mechanism. Thus, the growth of a MI+ tumor in breast tissue, with its close proximity to the lymphatic system, may allow invasion and metastasis to occur before detection of the malignancy. In contrast, MI+ tumors in the proximal colon, limited in their invasiveness on the basal side by the muscularis, grow into the lumen, where the potential routes of me-
tochastic are more limited. An alternative that is not exclusive of the above possibility is that the different underlying mutations in colorectal and breast cancer may act either to moderate or intensify the effects of MI on tumor progression. Thus, the same mutational mechanism could have different effects depending on the genetic background of a particular neoplasm. For example, the majority of MI+ colon cancers have been found to be pseudodiploid (45), whereas our data indicate that MI+ sporadic breast cancers are somewhat more likely to be aneuploid. It may be that the effects of MI-generating mechanisms in a pseudodiploid genetic background are different from those in aneuploid backgrounds, perhaps because of a greater opportunity to lose the wild-type alleles of negative growth regulators from aneuploid cells. Regardless of the reasons for the difference in outcome between MI+ colon and breast cancers, our results underscore the need to examine how this type of genetic instability affects the behavior of the diverse cancers in which it occurs.

The statistical correlations between MI and disease outcome reported here are highly significant. We emphasize, however, that analysis of a larger sample set will be required to determine whether MI will be useful as an independent prognosticator. For example, the small sample set limited the utility of multivariate analyses. We performed proportional hazards regression analysis to determine whether other tumor characteristics, such as tumor size or LN status, were responsible for reduced disease-free survival (Fig. 4a). This analysis confirmed that MI status had independent prognostic value in models with multiple predictors (see Fig. 4 legend). However, in the case of overall survival, MI was not found to have independent prognostic value. This discrepancy most likely arises from the fact that the multiple subdivisions of the data set necessary for this type of analysis are only possible in larger sample sizes. Thus, even though the conclusions that can be drawn from the available data are significant, they emphasize the need for expanded studies of the effects of MI on disease outcome in breast and other cancer types.

Some quantitative differences have been noted between the MI observed in sporadic and hereditary cancers. As one example, sporadic tumors display alterations at a lower percentage of microsatellites than do tumors in patients with germline mutations in MMR genes (32, 33). This observation has led some investigators to suggest that different mechanisms may be involved in the generation of MI in sporadic and hereditary cancers. Although it was beyond the scope of this study to determine the molecular defects responsible for generating MI in the samples we examined, the qualitative changes we observed in microsatellite sequences, verified by sequence analysis, were comparable to those found in tumors from HNPCC patients and consistent with a mechanism involving defective MMR. Given that the entire repertoire of MMR genes has not been elucidated, it is possible that the differences in the fraction of microsatellite sequences affected in sporadic and hereditary cancers is due to mutations in different components of the MMR pathway. The fraction of alleles affected in different cell lines is variable (13, 46), perhaps depending on the specific mutations in a given line. For example, it is known that mutation of GTBP, a protein that associates with hMSH2 in the repair of G:T mismatches, causes MI to occur at a lower frequency of alleles than MI caused by mutations in hMLH1 (47, 48). Alternatively, the length of time the mutational mechanism has been active may determine the prevalence of microsatellite alterations in a tumor. Loss of the second copy of the affected MMR gene is thought to be an early event in tumorigenesis in patients with HNPCC, resulting in widespread instability. MI+ sporadic cancers, however, may not experience early disruption of MMR. In these cancers, aberrant MI may not be necessary for initial tumorigenesis. Rather, accumulating the two separate mutations required for the development of MI may occur later in tumor development or as a consequence of other mutational mechanisms, resulting in alterations at fewer microsatellite sequences throughout the genome.

The majority of the allele changes we detected occurred in two tetranucleotide repeats. This raises the possibility that some DNA sequences are more prone to instability than others. Although little is known about the susceptibility of different microsatellite sequences to mutation, instability has been reported to occur more frequently in trinucleotide and tetranucleotide repeats than in dinucleotide repeats in diverse cancers (30). However, hMSH2, the human homologue of bacterial mutS, shows no sequence preference in its binding to mismatched oligonucleotides (49), and the majority of tumors from HNPCC patients have not displayed preferential instability in repeats of a particular size. It remains to be determined whether the apparent differences between alleles altered in HNPCC and the breast tumors analyzed here reflect the involvement of different mutated MMR genes, different molecular mechanisms for generating MI, or simply differences in experimental design.

The correlation between the presence of MI and poor prognosis in breast cancer patients underscores the need for further examination of this phenomenon in this and other types of human cancers. In light of our findings, it may be particularly informative to investigate whether MI is involved in the subset of node-negative breast cancer patients who have poor disease outcomes. In addition to the potential prognostic value, identification of MI+ tumors may allow more effective therapies to be pursued. Since MI+ cells are resistant to some cytotoxic agents (43, 50), reevaluation of chemotherapeutic sensitivity of MI+ malignancies may afford more effective treatment alternatives based on the knowledge of the underlying tumor genotype.

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