

Microsatellite Instability Is Uncommon in Breast Cancer¹

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ABSTRACT

In some tumors, defects in mismatch repair enzymes lead to errors in the replication of simple nucleotide repeat segments. This condition is commonly known as microsatellite instability (MSI) because of the frequent mutations of microsatellite sequences. Although the MSI phenotype is well recognized in some colon, gastric, pancreatic, and endometrial cancers, reports of MSI in breast cancer are inconsistent. We report here our experience with >10,000 amplifications of simple nucleotide repeats in noncoding genomic regions using DNA from 267 cases of breast cancer, including cases that represent all major histological types of breast cancer. We rarely (10 reactions) found unexpected bands in amplifications of tumor DNA that were not present in amplifications of normal DNA. Moreover, repeats of these reactions did not confirm microsatellite instability in a single case. We also evaluated the simple nucleotide repeats in the transforming growth factor type II receptor, insulin-like growth factor type II receptor, *BAX*, and *E2F-4* genes, which are frequently mutated in tumors with microsatellite instability. No mutations of these genes were found in any of the 30 breast cancer cell lines and 61 primary breast cancer samples examined. These results indicate that mismatch repair errors characteristic of the MSI phenotype are uncommon in human breast cancer.

INTRODUCTION

Two distinct forms of genetic instability are presently recognized in neoplastic cells (1), one being chromosomal instability which leads to aneuploidy and the other being MSI,³ which is characterized by frequent errors that occur during the replication of short nucleotide repeats (2). These short nucleotide repeats include those of noncoding microsatellite markers as well as the simple repeats that occur within the coding

regions of some genes such as the *RII* (3), *IGFIIR* (4, 5), *BAX* (6), and *E2F-4* genes (7, 8).

MSI is a prominent feature of a subset of colorectal tumors (9–11), as well as some gastric, pancreatic, and endometrial cancers (12). Cancers with MSI are particularly characteristic of the HNPCC syndromes in which there are germ-line mutations of specific mismatch repair enzyme genes, including *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, or *GTBP* (13–18). Most cancers with MSI, however, are sporadic, and many of these tumors have changes in the promoter region of *hMLH-1* (19–21). Furthermore, there is evidence that some tumors with apparently normal mismatch repair enzymes may also have microsatellite instability, usually affecting smaller numbers of microsatellite loci (2).

Determining whether particular cancers have mismatch repair deficiencies and MSI may have significant biological and clinical importance: (a) recognition of mismatch repair errors could be meaningful for understanding the pathogenesis, and potentially planning the prevention, of the cancer; (b) the ability to detect alterations in microsatellite loci in clinical samples may be a useful diagnostic tool for recognizing rare cancerous cells in these samples (22); and (c) at least in colorectal cancers, MSI may be of prognostic significance and may also be predictive of a tumor's responsiveness to certain chemotherapeutic agents (23–26).

The question regarding whether some breast cancers have mismatch repair errors appears to be unresolved as yet. A high frequency of MSI has been reported in a small number of breast cancers from affected individuals in HNPCC kindred (27), but the overall frequency of breast cancer in HNPCC families does not appear to be significantly increased (28, 29). Previous reports of MSI in presumably sporadic breast cancers are inconsistent, with a few investigators having noted absent or infrequent microsatellite instability in breast tumors (12, 30, 31), whereas a larger number of other investigators have reported remarkably high frequencies of MSI in breast cancers (32–41). To address the issue of mismatch repair errors in breast cancer pathogenesis, we reviewed 10,617 microsatellite amplification reactions performed in our laboratory on breast cancer tissues for purposes of mapping chromosomal deletions. We then carefully repeated each amplification in which we observed preliminary results suggestive of MSI, except for two of our samples that had insufficient DNA for repeat studies. In addition, because mutations of intragenic repeats are also characteristic of tumors with mismatch repair defects, we evaluated primary breast cancers and breast cancer cell lines for mutations of simple nucleotide repeats in the *RII*, *IGFIIR*, *BAX*, and *E2F-4* genes. This constitutes, therefore, the largest published study on MSI in breast cancer.

MATERIALS AND METHODS

Tissues. Paraffin-embedded tissues diagnosed as breast carcinoma were obtained from the surgical pathology files of the Johns Hopkins Hospital and the Johns Hopkins Bayview Med-

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³ The abbreviations used are: MSI, microsatellite instability; RII, transforming growth factor β 1 receptor type II; IGFIIR, insulin-like growth factor type II receptor; HNPCC, hereditary non-polyposis colorectal carcinoma.

Table 1 Breast cancer samples studied for mismatch repair errors
Tissue samples are classified according to histology.

| Breast cancer tissues | No. of samples studied for MSI | No. of samples studied for intragenic single/trinucleotide instability |
|---------------------------------|--------------------------------|--|
| Infiltrating ductal | 154 | 20 |
| Ductal carcinoma <i>in situ</i> | 62 | 0 |
| Infiltrating lobular | 11 | 11 |
| Mucinous (colloid) | 22 | 22 |
| Medullary | 8 | 8 |
| Breast cancer cell lines | | 30 |
| Total | 267 | 91 |

ical Center. For each case, appropriate tissue blocks were selected, and multiple sections 12- μ m in thickness were deparaffinized, stained with H&E, visualized with an inverted microscope, and microdissected using a 26-gauge needle (42). Tissues were digested overnight in buffer containing 0.5% NP40 and 200 μ g/ml proteinase K at an approximate ratio of 1 μ l buffer per 20 cells. This lysate was used directly in PCR reactions.

Breast Cancer Cell Lines. DNA was also isolated from 17 cell lines (BT474, BT483, BT549, DU4475, JACC812, JACC893, MCF-7, MDA-MB-134, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MDA-MB-468, T47D, ZR75-1, and ZR75-30) purchased from American Type Tissue Culture (Gaithersburg, MD) and 13 breast cancer cell lines provided by Dr. M. Sgagias of the National Cancer Institute (43).

Microsatellite Markers. PCR amplification of microsatellite markers was performed using the 104 primers outlined in Table 1. All primers were obtained from Research Genetics (Huntsville, AL), with the exception of primers for *D9S1747*, *D9S1748*, *D9S1749*, *D9S1751*, and *D9S1752*, which were prepared as described previously (44). The set of primers used included the five microsatellite loci proposed to be the initial set used for standardized screening of colorectal cancers for MSI (45).

PCR reactions for microsatellite markers typically contained 1 μ l of lysate and other reagents as described previously (46). Samples were denatured at 95°C before adding Taq polymerase (Life Technologies, Inc., Gaithersburg, MD; *i.e.*, hot start PCR), and most reactions were run for 30–35 cycles with annealing temperatures of 56–58°C. It is notable that these annealing temperatures are higher than temperatures recommended by the manufacturer and would be expected to increase stringency of the reaction. PCR products were then separated by 6% polyacrylamide denaturing gel electrophoresis, and dried gels were exposed to radiographic film. Reactions were scored as noninformative (when only one major band was seen in both normal and tumor DNA), informative with retention of both alleles (when two major bands were seen in both normal and tumor DNA, and molecular weights were identical in both samples), or informative with loss of heterozygosity (when tumor DNA had loss of one of two bands that were present in normal DNA) bands. When amplified tumor DNA was observed to have additional band or bands of sizes that were not present

in the amplified normal sample, we considered the possibility of MSI in the tumor sample. In these situations, we repeated the reactions if additional DNA from the case was available.

Allelic loss data for many of the cases have been presented in previous publications (42, 46–48). Tissues not involved in studies reported previously include 9 lobular carcinomas, 22 mucinous carcinomas, and 6 medullary carcinomas. Microsatellite markers not involved in studies reported previously include *D2S123*, *BAT25*, *BAT26*, *BAT40*, and *D17S250*.

Evaluation of Genes for Mutations of Intragenic Repeats. To test for mutations in the *RII*, *IGFIIR*, *BAX*, and *E2F-4* genes, PCR amplification of tumor DNA was performed using the primers and conditions specified in Table 3. Reactions were radiolabeled using 2.0 μ Ci of [³²P]dCTP in each 10- μ l reaction mixture, and products were electrophoresed on 6% polyacrylamide denaturing gels and visualized by autoradiography. Normally, only a single band is seen for each sample. Frameshift mutation was determined by visualization of bands of altered length in tumor DNA. Positive controls consisted of DNA from HNPCC tumors.

RESULTS

Microsatellite Marker Amplifications. DNA from 267 breast cancer tissues were studied for MSI (Table 1), including cases of infiltrating ductal carcinoma, ductal carcinoma *in situ*, infiltrating lobular carcinoma, mucinous carcinoma, and medullary carcinoma. A total of 10,617 reactions were performed using 104 microsatellite markers distributed on 11 chromosomes (Table 2). In 10 of these reactions, we observed unexpected bands in the amplification of the tumor DNA that were not present in amplification of germ-line DNA. We repeated eight of these reactions using the original PCR conditions and also using an annealing temperature of 58°C if the annealing temperature in the original reaction was <58°C. In each of these repeat reactions, we observed the tumor DNA to have only bands that were also present in the germ-line DNA. Fig. 1 demonstrates two of the original reactions with unexpected bands in the tumor DNA with the repeat reactions that did not show these unexpected bands. Two reactions in which we observed an unexpected band in amplification of DNA were from samples of ductal carcinoma *in situ*, and we had insufficient DNA to repeat these reactions. Thus, we found no repeatable MSI using any of the microsatellite markers, including five markers considered to be highly reliable for detection of MSI in colorectal cancers and nine markers reported previously to have instability in breast cancers.

Amplification of Intragenic Short Nucleotide Repeats. DNA from 61 breast cancer tissues and 30 breast cancer derived cell lines were studied for instability of the intragenic simple nucleotide repeats of the *RII*, *IGFIIR*, *BAX*, and *E2F-4* genes. PCR amplification of tumor DNA was performed using the primers and conditions specified in Table 3. Frameshift mutations, determined by visualization of additional bands in tumor DNA, were seen in control samples from HNPCC colorectal cancer DNA but not in DNA from any of the breast cancer samples. Fig. 1B demonstrates representative amplifications of repeat segments of the *BAX* and *IGFIIR* genes.

Table 2 Microsatellite markers used in amplifications of breast cancer samples

Markers highlighted in bold have been designated to comprise a diagnostic panel to evaluate colorectal cancers for MSI (45), and underlined markers have been reported in other studies to show MSI in breast cancers.

| Chromosome no. | Microsatellite markers | Total number of markers | Total number of reactions |
|----------------|--|-------------------------|---------------------------|
| 1 | <i>D1S500, D1S507, D1S243, D1S305, SPTA1, D1S318, D1S117, D1S158, D1S162, D1S188, <u>D1S228</u>, BAT40</i> | 12 | 1062 |
| 2 | <i>D2S123, BAT26</i> | 2 | 240 |
| 4 | <i>BAT25</i> | 1 | 120 |
| 5 | <i>D5S393, D5S404, D5S415, D5S421, D5S429, D5S656</i> | 6 | 360 |
| 6 | <i>D6S252, D6S268, D6S287, D6S270, D6S292, D6S250, D6S310, D6S314, D6S311, D6S420, D6S473, <u>D6S255</u>, D6S442, D6S305, D6S264, D6S297</i> | 16 | 2613 |
| 8 | <i>D8S133, D8S137, D8S255, D8S259, D8S261, D8S262, <u>D8S264</u>, D8S298, D8S503, D8S504, D8S518, D8S540, D8S549, D8S560, D8S1715, D8S1991</i> | 16 | 934 |
| 9 | <i>D9S104, D9S162, D9S157, D9S156, D9S161, D9S200, D9S169, D9S171, D9S736, D9S816, D9S1747, D9S1748, D9S1749, D9S1751, D9S1752, D9S2230</i> | 16 | 1410 |
| 11 | <i>D11S1324, D11S904, D11S902, <u>D11S988</u>, D11S29, D11S35, D11S528, <u>INT2</u>, D11S911, D11S917</i> | 10 | 1186 |
| 13 | <i>D13S260, D13S263, D13S155, D13S284</i> | 4 | 460 |
| 16 | <i>D16S541, D16S415, D16S265, <u>D16S402</u>, <u>D16S413</u>, D16S420, D16S515, D16S514, D16S511, D16S520</i> | 10 | 596 |
| 17 | <i>D17S513, CHRN1, <u>TP53</u>, D17S786, D17S122, THRA1, <u>D17S579</u>, D17S588, D17S736, D17S926, D17S250</i> | 11 | 1636 |
| Total | | 104 | 10617 |

DISCUSSION

The mismatch repair errors that result in MSI and mutations of intragenic simple nucleotide repeats occur at highest frequency in tumors of individuals with the HNPCC syndrome. Although breast cancers do not occur at significantly increased incidence in HNPCC kindred (29), breast cancers that occur in affected HNPCC kindred do have a high frequency of MSI (27). These data raise the possibility that mismatch repair defects contribute to the pathogenesis of a subset of breast cancers, although it also appears possible that the MSI in the tumors of these individuals was coincidental to the development of the cancers. The present study addresses the issue of whether mismatch repair errors occur in sporadic breast cancers, and our results differ from those of a number of previous studies that reported MSI to occur at a high frequency in some sporadic breast cancers (32–39, 41). Specifically, in our study of 267 presumably sporadic breast cancers, we failed to find reproducible evidence of mismatch repair errors in any of the dinucleotide repeats in 102 microsatellite markers, the mononucleotide repeats in the *BAT25* and *BAT26* markers and the *RII*, *IGFIIR*, and *BAX* genes, or the trinucleotide repeat in the *E2F-4* gene.

It is notable that many of the previous reports of MSI in breast cancer are inconsistent with one another and with the paradigm of MSI described for colorectal carcinoma. For example, it is notable that several laboratories have reported MSI to occur primarily in early stage breast cancers (32–35), whereas other laboratories have reported MSI principally in advanced breast cancers (36–38). Both of these reported situations conflict with the model of MSI in colorectal cancers where mutations appear early in the pathogenesis of the cancer and persist through the development of the tumor (10, 49, 50). Our present study included cases of advanced, metastatic breast cancer as well as samples from *in situ* ductal cancer, and we were unable to find MSI in breast cancers of any stage.

Furthermore, based on the experience of MSI in colorectal

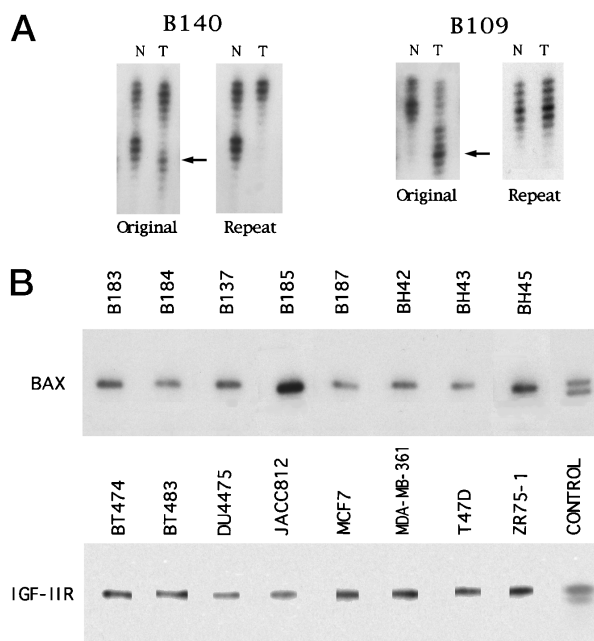


Fig. 1 Absence of mismatch repair errors in breast cancer samples. In A, two examples of microsatellite amplifications are demonstrated in which an unexpected band was seen in the tumor sample of the original reaction (arrows). Repeat reactions failed to show same unexpected bands. Normal (N; germ line) and tumor-derived (T) DNA samples are shown. Sample B140 was amplified with primers for the *D5S419* marker, and sample B109 was amplified with primers for the *D5S404* marker. In B, representative amplifications of *BAX* and *IGFIIR* genes in breast cancer samples are shown. Controls consist of DNA from colorectal cancers with MSI and show frameshift mutations.

carcinoma, it may be expected that breast tumors with MSI would have distinctive clinical and pathological features (9, 40). Although most reports of MSI in breast cancer included ductal

Table 3 Primers used for amplification of intragenic simple nucleotide repeats

| Gene | Repeat | PCR product (bp) | Primers (5'-3') |
|-----------------|-----------------------|------------------|--|
| <i>E2F4</i> | (AGC) _n | 167 | ACAGTGGTGAGCTCAGTTCCTC AAAGGAGGTAGAAGGGTTGGGTC |
| <i>TGF-βRII</i> | poly(A) ₁₀ | 140 | ACAGTTTGCCATGACCCCAAG TCATTGCACTCATCAGAGCTACAGG |
| <i>IGFIIR</i> | poly(G) ₈ | 110 | GCAGGTCTCCTGACTCAGAA GAAGAAGATGGCTGTGGAGC |
| <i>BAX</i> | poly(G) ₈ | 94 | ATCCAGGATCGAGCAGGGCG ACTCGCTCAGCTTCTTGGTG |

cancers or tumors of unspecified histology, two laboratories did report MSI to occur predominantly in a distinctive histological form of breast cancer, lobular carcinoma (37, 39). Again, we were unable to substantiate these findings in our present study, which included cases of lobular carcinoma as well as cases of other major histological types of breast cancer.

In addition to our negative findings reported here, several other investigators have also previously reported low frequencies of MSI in breast cancers. Peltomaki *et al.* (12) found MSI of dinucleotide repeats in some sporadic colonic, gastric, and endometrial cancers but not in breast cancers. Similarly, Hye-Jung *et al.* (30) reported MSI of dinucleotide repeats in some sporadic pancreas and stomach cancers but not in breast cancers. In another report by Wooster *et al.* (31), only 1 of 104 breast cancer cases was found to have instability of dinucleotide repeats. Ten of these cases were found to have instability of individual tri- or tetranucleotide repeats, but alterations of tri- and tetra-nucleotide microsatellite loci have been observed to occur at a modest frequency in other cancers that do not have known mismatch repair defects (22). Although it remains to be determined whether this is related to some form of genetic instability, these infrequent alterations of tri- and tetranucleotide repeats do not appear to be related to the well-described MSI associated with inactivation of mismatch repair enzymes.

To further evaluate the possible involvement of mismatch repair defects in breast cancer, we evaluated 91 samples of breast cancer DNA, including DNA from 30 breast cancer cell lines, for frameshift mutations of four genes with simple nucleotide repeats: the *RII* gene with a poly(A)₁₀ tract (3), the *IGFIIR* gene with a poly(G)₈ tract (4, 5), the *BAX* gene with a poly(G)₈ tract (6), and the *E2F-4* gene with poly(AGC)₁₃ tract (8). These genes are involved in the regulation of cell growth or apoptosis, and mutations of these genes are likely involved in the pathogenesis of cancers with MSI. Mutational analysis of these intragenic repeats in breast cancer has not been reported previously, and our negative findings cast further doubt on the possible importance of mismatch repair errors in breast cancer pathogenesis.

How can we explain the findings of MSI in breast cancers by other laboratories in light of our negative findings? (a) PCR artifact may have in some cases resulted in spurious bands that appear as altered length microsatellites; (b) a second possible explanation for findings of MSI in breast cancer by other laboratories is that some of these studies may have included tumors from women with the HNPCC syndrome (27) or from geographically limited populations of women who have distinctive breast cancers with frequent mismatch repair errors; and (c) it is also

possible that mismatch mutations occur occasionally in some breast cancer cells in the absence of clonal expansion and are thus not detected by our assays. However, if MSI occurs only in a few tumor cells and is not associated with clonal expansion of that tumor, it would seem unlikely that this instability would contribute significantly to the pathogenesis of the cancer.

In summary, a number of findings support our conclusions that replication errors of simple nucleotide repeats are not involved in the pathogenesis of the great majority of breast cancers: (a) breast cancers do not occur at an appreciably increased frequency in HNPCC kindreds, which would be expected if mismatch repair defects contributed to the development of breast cancer; (b) we have found no instability of intronic microsatellite marker repeats in a large number of reactions using a large number of breast cancer samples; and (c) we have not found the intragenic simple nucleotide repeats that are commonly mutated in HNPCC cancers to be mutated in breast cancers. Although it remains to be determined whether all breast cancers have the chromosomal instability described for many colorectal cancers, we conclude that microsatellite instability and mismatch repair deficiencies are uncommon in breast cancer.

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