Exclusion of Breast Cancer as an Integral Tumor of Hereditary Nonpolyposis Colorectal Cancer

Annegret Müller, Tina Bocker Edmonston, Diana A. Corao, Deborah G. Rose, Juan P. Palazzo, Heinz Becker, Robert D. Fry, Josef Rueschoff, and Richard Fishel

Genetics and Molecular Biology Program, Department of Microbiology and Immunology, Kimmel Cancer Center [A. M., T. B. E., R. F.], and Departments of Pathology [T. B. E., D. A. C., J. P. P.] and Surgery [D. G. R., R. D. F.], Thomas Jefferson University, Philadelphia, Pennsylvania 19107-5541; Department of General Surgery, University of Göttingen, Göttingen, Germany [A. M., H. B.]; and Department of Pathology, Klinikum Kassel, Kassel, Germany [J. R.]

Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant genetic predisposition syndrome that accounts for 2–7% of all colorectal cancers. Diagnosis of HNPCC is based on family history (defined by Amsterdam or Bethesda Criteria), which often includes a history of multiple synchronous or metachronous cancers. The majority of HNPCC results from germ-line mutations in the DNA mismatch repair (MMR) genes hMSH2 and hMLH1 with rare alterations in hMSH6 and hPMS2 in atypical families. Both HNPCC and sporadic MMR-deficient tumors invariably display high microsatellite instability (MSI-H). Two types of HNPCC families can be distinguished: type I (Lynch I) with tumors exclusively located in the colon; and type II (Lynch II) with tumors found in the endometrium, stomach, ovary, and upper urinary tract in addition to the colon. A proposed association of breast cancer with type II HNPCC is controversial. To address this important clinical question, we examined MSI in a series of 27 female patients who presented with synchronous or metachronous breast plus colorectal cancer. Although MSI-H was found in 5 of 27 (18.5%) of the colon cancers, in all cases the matched breast cancer was microsatellite stable. We also examined the breast tumors from three women who were carriers of MMR gene mutations from HNPCC families. None of these three breast tumors displayed MSI nor was the expression of MMR proteins altered in these tumors. We conclude that breast cancer largely arises sporadically in HNPCC patients and is rarely associated with the HNPCC syndrome.

Introduction

HNPCC is the most common human cancer predisposition syndrome, accounting for approximately 2–7% of the total colorectal cancer burden (1–4). HNPCC is clinically characterized by colorectal cancers, a proclivity to the proximal colon, and high frequency of metachronous and synchronous cancers (5). The inclusion of extra-colonic cancers (endometrial, gastric, ovarian, and upper urinary tract) in addition to colorectal cancer, as well as a less stringent family history, distinguishes Amsterdam Criteria families from Bethesda Criteria families (6–8). The addition of breast cancer in Bethesda Criteria and/or type II (Lynch II) HNPCC is controversial (9–17).

Alterations of the human MMR genes hMSH2 and hMLH1 account for the vast majority of HNPCCs (18–22). Mutations of other MMR genes are either absent (hMLH3, hPMS1, and hMLH3) or rare and largely associated with atypical families (hMSH6 and hPMS2; Refs. 23 and 24). The detailed mechanism of tumorigenesis in HNPCC remains enigmatic (25, 26), although it clearly begins with a “second hit” on the remaining MMR allele (27). Loss of MMR function results in elevated spontaneous mutation rates (mutator phenotype) that accelerate the adenoma-carcinoma transition associated with HNPCC tumors (3, 28). The mutator phenotype associated with MMR defects is an example of induced genomic instability that leads to tumorigenesis (26, 29, 30). Malignant transformation ensues when genes relevant for growth control and differentiation are affected as secondary mutation events.

Simple repeat sequences (microsatellites) are particularly prone to replication errors associated with the type of genomic instability that results from the loss of MMR function (31–33). Tumor DNA containing such replication errors can be distinguished by MSI-H (when >30% of the microsatellite markers are altered) or MSI-L (when <30% of the microsatellite markers are altered; Ref. 34). Tumor tissues from >80% of patients with an HNPCC syndrome (Amsterdam or Bethesda Criteria) and 100% of patients with verified germ-line alterations of the hMSH2 or hMLH1 gene display MSI-H (8, 34, 35). Mononucleotide repeat sequences appear to be the most unstable in bona fide HNPCC tumors (34). These observations have led to the widespread use of microsatellite analysis as a diagnostic screening test for HNPCCs (36). In addition to HNPCC, MSI-H is also found in 9–17% of sporadic colorectal carcinomas (34, 37).

Breast cancer affects one in nine women in the United States population (38, 39). Such a high incidence makes it difficult to determine whether breast cancer is coincidental in HNPCC families or caused by the germ-line mutation in one of the MMR genes linked to HNPCC. The inclusion of breast cancer as an integral tumor in the HNPCC (Lynch II) spectrum rests largely on an analysis of breast and colon cancer tissues from a single female from a large HNPCC kindred (10). The breast tumor from this individual displayed MSI-H (8 of 20 or 40% of microsatellites analyzed) and a distinct pattern of MSI-H in the colon tumor. Moreover, loss of the wild-type MMR allele, in this case hMLH1, was observed in the breast tumor tissue. Supporting evidence for the inclusion of breast cancer in the type II (Lynch II) syndrome comes from MSI analysis of single breast tumors from five additional HNPCC kindreds. Using the NCI and the ICG-HNPCC criteria (8, 34), three of these breast tumors displayed MSI-H. Only one of these three breast tumors displayed instability at mononucleotide repeats. Although these studies were performed prior to a clear definition of diagnostic MSI (34, 40), it appeared to provide compelling evidence for a correlation between breast cancer, HNPCC, and the litmus of MMR defects, MSI-H. The frequency of MSI in sporadic breast tumors is also controversial, with some reports suggesting the occurrence of MSI in breast tumors (11, 41–43) and others suggesting a lack of MSI in breast tumors (14, 44, 45).

Recent interest in the relationship of breast cancer to the HNPCC
 type II (Lynch II) spectrum has been intensified by the observations of Scott et al. (16). A survey of 95 HNPCC families suggested a significant elevation in breast cancer susceptibility when hMSH2 or hMLH1 mutation carriers are compared with hMLH1-carrier families. These observations would appear to have important implications for patient management and cancer surveillance, just as the exclusion of endometrial carcinoma as an integral HNPCC tumor resulted in a significant delay in the diagnosis of HNPCC (46). In a response to these studies, Vasen et al. (17) reported that a similar analysis of a 200 HNPCC family cohort found that only 7 of 328 hMSH2 or hMLH1 mutation carriers had developed breast cancer. There was no difference in the breast cancer risk for hMSH2 or hMLH1 carriers. Both the Scott et al. (16) and Vasen et al. (17) studies cited the need for an analysis of colon and breast tumors from HNPCC individuals for clear signatures of MMR defects (16, 17).

Synchronous or metachronous cancers are frequently associated with HNPCC and are among the criteria for identifying HNPCC patients and families (7). Here we have examined MSI in patients identified with synchronous or metachronous breast plus colon cancer. We found that 5 of 27 (18%) of the colon tumors displayed MSI-H. None of the corresponding breast tumors showed MSI. In addition, independent breast cancer tissues from female members of three MMR mutation-positive HNPCC families were analyzed. Although all of the colon tumors of those families displayed MSI-H, all of the breast tumors were MSS. Taken together, these results suggest that breast cancer caused by mutation of the MMR genes is rare and should not be included in the HNPCC type II (Lynch II) spectrum.

Materials and Methods

Patients. This retrospective study is based on women with breast plus colon cancer who were treated at Thomas Jefferson University in Philadelphia between 1959 and 2000. A total of 3,538 female patients with colorectal cancer were seen as well as 12,361 with breast cancer. A total of 58 women had both breast and colon cancer. Paraffin blocks of both breast and colon tumors were acquired for 27 of these patients. Three additional women with breast cancer from confirmed mutation-positive HNPCC families where recruited from the German HNPCC Registry. Tumor blocks and blood were studied after obtaining informed consent. Data on MSI status of synchronous or metachronous extracolonic cancers (e.g., endometrial, stomach, ovarian, and upper urinary tract) were obtained from the Thomas Jefferson University Familial Cancer Registry. Of the 27 patients analyzed with breast plus colon tumors, only 1 was a member of the Thomas Jefferson University Familial Cancer Registry.

DNA Extraction. Paraffin-embedded tumor and corresponding normal tissue sections were mounted on glass slides and stained with H&E. Microdissection was performed on paraffin sections stained briefly with methylene blue. Areas of interest were selected by a surgical pathologist (T. B. E. and J. P. P.), scraped off the slide, and subjected to proteinase K digestion at a final concentration of 2 mg/ml (Qiagen, Valencia, CA). DNA was extracted with the QIAamp DNA Mini kit (Qiagen), according to the manufacturer’s recommendations. For sequence analysis, DNA from blood was extracted with QIAamp DNA Maxi Blood kit (Qiagen) following the manufacturer’s recommendations.

Microsatellite Analysis. In accordance with the recommendations by the NCI and the ICG-HNPCC (8, 34), six microsatellite loci were used to detect MSI-H. Three loci with mononucleotide runs (BAT25, BAT26, and BAT40) and three loci with dinucleotide repeats (D2S123, DSS346, and D17S250). These microsatellite loci had been recommended by Dietmaier et al. (34) as having the highest diagnostic sensitivity and specificity. Primers were 5’-end labeled with HEX, TAM, or TET (Perkin-Elmer, Foster City, CA). DNA was amplified in a standard reaction mix using AmpliTaq Gold (Perkin-Elmer). The PCR products were run on an ABI 377 automated sequencer using fragment analysis software (GeneScan; Perkin-Elmer).

Additional peaks in tumor tissue DNA, when compared with normal tissue DNA, indicated MSI. Instability in >30% of the markers was reported as MSI-H, <30% was classified as MSI-L, and no instability was reported as MSS. Losses of heterozygosity were excluded as MSI.

Immunostaining for hMSH2 and hMLH1. To determine the presence or absence of MMR proteins, the breast tumors of the three members of the HNPCC families were subjected to immunohistochemical analysis. Briefly, slides were deparaffinized and hydrated through graded alcohols. Antigen retrieval was achieved by microwave treatment (800 W for 20 min) and incubation with 3% H2O2. Non-specific binding was blocked by incubation with goat serum for 30 min. The slides were incubated with primary antibody against hMSH2 (0.5 μg/ml; Oncogene Sciences, Cambridge, MA) or against hMLH1 (1 μg/ml; Clon G168-728; Pharmingen, San Diego, CA) overnight at 4°C. The sections were then washed with PBS and incubated with the secondary biotinylated antibody. After rinsing with PBS, the sections were incubated with streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA). For detection, the chromogen 3-amo-no-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) was used according to the manufacturer’s recommendations, and counterstaining was done with hematoxylin.

Mutations Analysis. Germ-line mutation analysis in the three HNPCC families was done by automated DNA sequencing using Big Dye terminator chemistry (Perkin-Elmer) for sequence analysis on the Applied Biosystems Model 377 DNA sequencing systems (PE Applied Biosystems, Foster City, CA). PCR was performed on 75 ng of genomic DNA for each exon. The PCR products were purified using the QiAquick PCR purification kit (Qiagen) and submitted for sequence analysis. The sequence was analyzed with the DNAStar Sequencer (Gene Codes Cooperation, Ann Arbor, MI).

Results

Synchronous and metachronous cancers have been established by the ICG-HNPCC as one of the criteria (Bethesda Criteria) for identifying HNPCC individuals and families (7). For HNPCC type II (Lynch II), synchronous and metachronous cancers include both colon as well as well-defined extracolonic tumors such as endometrial, ovarian, stomach, and upper urinary tract cancer (7).

To examine the role of breast cancer in type II (Lynch II) HNPCC, we identified 58 patients that presented with synchronous or metachronous colon plus breast tumors at Thomas Jefferson Hospital. The tumor blocks for both the colon and breast tumor were acquired for 27 of these patients, and microsatellite analysis of six different NCI/ICG-HNPCC recommended markers was performed on DNA isolated from microdissected normal and tumor tissue regions (Fig. 1). The pathology and MSI status of the breast and colon tumors from these 27 patients are shown in Table 1. MSI-H was observed in 5 colon tumors (18%; Table 1). None of the breast tumors examined displayed MSI-H, which includes the corresponding breast tumors associated with the MSI-H colon tumors (Table 1). Consistent with previous observations, 100% of the MSI-H colon tumors displayed instability in at least one of the mononucleotide microsatellites (BAT25, BAT26, or BAT40). Immunohistochemical analysis of a subset of the tumor tissue pairs confirmed the loss of expression of MMR proteins in the colon tumor tissue but not the breast tumor tissue (Fig. 2).

We have previously characterized low microsatellite instability (MSI-L; defined as instability in <30% of examined microsatellite sequences). The genetic causes of the MSI-L phenotype are unknown, although rare alterations of the hMSH6 MMR gene have been found within this cohort (data not shown; Ref. 25). Approximately two-thirds of MSI-L tumors display methylation inactivation of the MGMT promoter (47). Loss of MGMT exacerbates mutation rates by leaving unrepaired methylguanine damage in DNA (47). The majority of MSI in MGMT-deficient tumors appears to be confined to dinucleotide repeats. These data suggest that the MSI-L phenotype is unlikely to be significantly associated with MMR defects (47). We found that 7 of 27 colon tumors (26%) and 4 of 27 breast tumors (15%) displayed MSI-L (Table 1 and Table 2). In 60% of the MSI-L tumors, instability was confined to the dinucleotide microsatellite markers. MSI-L was found in two pairs of the synchronous and/or
metachronous breast plus colon tumors. These data appear similar to sporadic colorectal tumors, which appear unrelated to HNPCC (47).

To further investigate the role of breast cancer in HNPCC, we identified three kindreds fulfilling the Amsterdam criteria in which at least one female family member was diagnosed with breast cancer. Molecular diagnostics of a proband in two families revealed deletion mutations within the hMSH2 gene (del C at codon 335; del TC at codon 232; Fig. 3, A and B). A proband in the third family was found to contain an insertion in the hMLH1 gene (ins C at codon 496; Fig. 3C). All three of these mutations cosegregate with HNPCC and are pathogenic as a result of a premature truncation of the protein. Moreover, the colon tumor tissues from affected family members of each of these kindreds display MSI-H (data not shown). Immunohistochemical analysis of the respective colon cancers suggested the loss of expression of hMSH2 in tumor tissues from family members that carried the hMSH2 deletion mutations and loss of expression of hMLH1 in the colon cancer from family members that carried the hMLH1 insertion mutation (see Fig. 2 for illustrative example; data not shown). In contrast, analysis of multiple colorectal or endometrial tumors from affected family members of all three families invariably displayed MSI-H (data not shown). These data strongly suggest that the breast tumors of the MMR mutation-positive female family members arose independently of the HNPCC predisposition syndrome.

Discussion

The lifetime risk of developing breast cancer in United States women is approximately 11% (one of nine). The lifetime risk of developing colon cancer in the general population is ~5% (1 of 20). The risk of colon cancer increases 2–3-fold in persons who have a first-degree relative with colon cancer (48). In the case of the development of two different cancer types, synchronous or metachronous, a hereditary predisposition must be suspected (48).

The recognition of HNPCC-associated cancers led to the Bethesda Criteria or the type II (Lynch II) cancer predisposition syndrome. In 1995, Aarnio et al. (49) performed a detailed pedigree analysis of 40 HNPCC families to evaluate the cumulative risk of cancers other than colon cancer. At that time, endometrial cancer generated a cumulative risk high enough to suggest a specific surveillance program. These results underline the importance of identifying the correct HNPCC-associated tumors toward the recognition of affected families as well as appropriate clinical surveillance. Endometrial, gastric, ovarian, and upper urinary tract tumors are generally accepted as integral tumors of the type II (Lynch II) HNPCC syndrome. The inclusion of breast cancer in type II (Lynch II) HNPCC is controversial (11, 14, 41–45).

The use of well-defined microsatellite markers has established diagnostic MSI as a valuable tool in the diagnosis of HNPCC (8, 34). Tumors from HNPCC patients that have been identified as carriers of pathogenic MMR mutations invariably display MSI-H (34, 37, 50). Moreover, MSI-H has been observed in 80% of the early adenomas from HNPCC MMR mutation carriers (51). These studies have confirmed the importance of MSI-H as a fundamental indicator of MMR defects.

**Table 1 Pathology and MSI status of breast and colon tumors**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Breast cancer pathology</th>
<th>MSI status (breast)</th>
<th>Colon cancer pathology</th>
<th>MSI status (colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Muc. Adeno</td>
<td>MSS</td>
</tr>
<tr>
<td>2</td>
<td>LCIS</td>
<td>MSI-L</td>
<td>Carcinoid</td>
<td>MSS</td>
</tr>
<tr>
<td>3</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
<tr>
<td>4</td>
<td>Inv. Duct. CA</td>
<td>MSI-L</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
<tr>
<td>5</td>
<td>DCIS</td>
<td>MSI-L</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
<tr>
<td>6</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
<tr>
<td>7</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
<tr>
<td>8</td>
<td>Inv. Lob. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>9</td>
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<td>MSS</td>
<td>Adenoca</td>
<td>MSI-H</td>
</tr>
<tr>
<td>10</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>11</td>
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<td>MSS</td>
<td>Adenoca</td>
<td>MSI-H</td>
</tr>
<tr>
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<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>13</td>
<td>DCIS</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
<tr>
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<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>15</td>
<td>DCIS</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSI-H</td>
</tr>
<tr>
<td>16</td>
<td>LN-met&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MSI-L</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>17</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>18</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
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<tr>
<td>19</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
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</tr>
<tr>
<td>20</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>21</td>
<td>DCIS</td>
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<td>MSI-H</td>
</tr>
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<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
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<tr>
<td>23</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
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<td>DCIS</td>
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<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
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<td>MSS</td>
<td>Adenoca</td>
<td>MSS</td>
</tr>
<tr>
<td>26</td>
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<td>MSS</td>
<td>Adenoca</td>
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<tr>
<td>27</td>
<td>Inv. Duct. CA</td>
<td>MSS</td>
<td>Adenoca</td>
<td>MSI-L</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inv. Duct. CA, invasive ductal carcinoma; LCIS, lobular carcinoma in situ; DCIS, ductal carcinoma in situ; Inv. Lob. CA, invasive lobular carcinoma; Muc. Adeno, mucinous adenocarcinoma; Adenoca, adenocarcinoma.

<sup>b</sup> Metastasis in axillary lymph node consistent with breast primary; primary tumor not found in mastectomy specimen.
To clarify the involvement of breast cancer in HNPCC, we examined the tumors from patients with synchronous and metachronous breast plus colon cancer as well as the breast or colon tumors from affected members of HNPCC families for MSI-H. In our experience, ~78% of patients with metachronous or synchronous HNPCC-related cancers display MSI-H. Of these, 86% were found to harbor a mutation in either hMSH2 or hMLH1. We found that none of the synchronous or metachronous breast tumors examined in the present study displayed MSI-H. Moreover, in this cohort the frequency of MSI-H in the colon tumors (18%) was similar to that found in sporadic colon tumors (34). These data suggest that, unlike bona fide HNPCC-associated tumors, identifying synchronous or metachronous breast plus colon tumors does not enrich for MMR-defective type II (Lynch II) HNPCC tumors.

None of the breast tumors from confirmed mutation-positive HNPCC family members displayed MSI. Combined with the observation of a normal MMR protein expression pattern in the breast tumor tissue, these data suggest that the breast tumors from these MMR mutation carriers developed independently of HNPCC and their MMR mutation-carrier status.

Previous reports have described the identification of MMR mutations in two unrelated families fulfilling the Amsterdam criteria, where both male and female gene carriers were affected with breast cancer (12, 52). In one family, the affected female patient diagnosed at the age of 35 with breast cancer revealed a mutation in both hMLH1 and BRCA1 (52). The second family was also diagnosed with an hMLH1 mutation (12). Patients from this family presented with both colon and endometrial cancer. A male member was affected with colorectal cancer in addition to breast cancer. Importantly, microsatellite analysis of DNA isolated from the breast cancers from these two families revealed an MSI-L status.

Several studies have concluded that a defective MMR system is uncommon in human breast cancer and that breast cancer should not

### Table 2. MSI analysis of synchronous or metachronous breast plus colon tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>MSI-H (% MN MSI)</th>
<th>MSI-L (% MN MSI)</th>
<th>MSS (% MN MSI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MSI-L</td>
<td>0</td>
<td>2 (0%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>MSS</td>
<td>5 (100%)</td>
<td>5 (60%)</td>
<td>13</td>
</tr>
</tbody>
</table>

* The percentage of tumors with instability in at least one mononucleotide repeat microsatellite sequence.
EXCLUSION OF BREAST CANCER AS TUMOR OF HNPCC

We gratefully acknowledge speculative discussions with Henry Lynch as well as comments and criticism by Jeremy Jass and Kristine Yoder.

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