Microsatellite instability differences between familial and sporadic ovarian cancers

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Introduction

Ovarian cancer (OC*) is the fifth most common cause of cancer-related death in women. Although the majority of women with OC report no prior family history of cancer, OC can recur in families in three clinically distinguishable syndromes: (i) site-specific OC; (ii) breast–OC syndrome; and (iii) Lynch cancer family syndrome (1). Together, these are believed to account for 5—10% of total OC cases. The Lynch cancer family syndrome was originally divided into two subtypes: type I, non-polyposis colorectal cancer (NPCC) in successive generations; and type II, NPCCC and other cancers, including OC, in successive generations, with affecteds often developing multiple, primary cancers during their lifetimes (2). We now know that many extended Lynch, type I pedigrees include persons with cancers seen in Lynch, type II. Therefore, the Lynch cancer family syndrome is best thought of as a single entity with variable expression.

Genes identified as predisposing to cancer in heritable syndromes have often been found to contribute to the development of sporadic examples of the same tumor type. Molecular studies have identified the genes responsible for cancer predisposition in two of the syndromes in which OC occurs. In the breast–OC Syndrome, the vast majority of families demonstrate inheritance of germline mutations in BRCA1 (3,4).

In the Lynch Family Cancer Syndrome, the demonstration of microsatellite instability (MIN) in comparisons between tumor and normal tissue from the same patients, led to the discovery of inherited germline mutations in certain DNA mismatch repair genes as responsible for cancer predisposition (5—10). To date, five DNA mismatch repair genes have been identified in humans. Two, hMSH2 and hMLH1, predominate in the Lynch Syndrome, with mutations in one or the other found in between 80—90% of affecteds in Lynch families identified by HNPCC (11—13). If, in a cell carrying an inherited mutation in a DNA mismatch repair gene, the remaining normal allele at that locus is also lost (through mutation or deletion), the cell becomes deficient in DNA mismatch repair activity. The loss of the DNA repair function makes the cell in which it occurs susceptible to the accumulation of mutations that can occur spontaneously with each cycle of DNA replication (14—16). Included among the new mutations occurring by chance could be those contributing to carcinogenesis: hence, the characterization of DNA mismatch repair gene mutations as predisposing to the development of cancer.

MIN can be demonstrated by differences in the patterns of gel bands corresponding to polymorphic loci, between tumor and non-tumor tissue from the same individual (17). Using the microsatellite markers D2S123, D2S119 and D10S197, we compared DNAs from 90 tumor-white blood cell pairs from patients with OC, with or without a family history of cancer. Our results suggest that the genetic basis of MIN in individual sporadic or familial OC may be different from that reported in Lynch Cancer Families identified through HNPCC.

Materials and methods

Tumor accession and DNA isolation

OC specimens from surgery and venous blood collected within a day of surgery from the same patients were obtained from the Tumor Procurement Service (TPS) at Memorial Sloan-Kettering Cancer Center (MSKCC) and from the Gynecologic Oncology Program at Lenox Hill Hospital (LH). Family history and tumor pathology data were obtained from the TPS/MSKCC and the clinical staff at LH.

The white cells were separated from venous blood on Ficoll-Hypaque, pelleted and digested in Proteinase K. For the tumors, specimens were collected in sterile, isotonic medium and transported to the laboratory, where they were minced and digested in Proteinase K. DNA from both tissue types

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1799
was extracted through phenol/chloroform/isoamyl alcohol, precipitated in ethanol, and resuspended in 1X Tris-EDTA, pH 7.4 and stored at 4°C.

Microsatellite instability testing

Primers for PCR amplification of the variable length CA-repeat segments of locus D2S123 were obtained from Research Genetics, Inc. (Huntsville, AL) and for the loci D2S119 and D10S197 from DNA International (Oswego, OR). These microsatellite markers were chosen because of their high correlation with MIN+ in published HNPCC series.

The primers were end-labeled using 5 μl [32P]ATP (6000 Ci/mm) and 10 units of T4 polynucleotide kinase, and subsequently purified by filtration through a G-25 Sephadex spin column (Boehringer Mannheim (BMB)). The PCR reaction (total volume, 25 μl) contained 2.5 μl 10xincubation buffer, 4 dNTPs (0.11 mM final concentration each); 0.8-1 unit Taq polymerase, all supplied by BMB and 25-200 ng of DNA template. The PCR conditions were: after an initial 5 min of denaturation at 94°C to eliminate mispriming, were: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s, for 35 cycles. Prior to MIN testing, the PCR products were prescreened on an agarose gel to insure amplification of target DNA and the absence of non-specific PCR products. A 2 μl aliquot of PCR products was then treated with buffer (95% formamide, 10 mM EDTA, 0.25% bromphenol blue, 0.25% xylene cyanol) and denatured for 10 min: a 2 μl aliquot was run on a high resolution 10% polyacrylamide gel at 500 V for 10-12 h. Autoradiograms were developed for 24-48 h before analysis.

Microsatellite alterations assessment

OC cases were assessed to be MIN negative when no differences in CA-repeat band patterns were found between tumor and normal DNA from the same patient. Of the three different microsatellite loci tested (17), MIN positives were those in which there were specific differences in the DNAs from tumor and normal tissue from the same person in the CA-repeat band pattern for at least one of the tested markers. All positives were confirmed in at least two independent experiments.

Mutation analysis

Single strand conformational polymorphism analysis (SSCP) was used in an exon by exon screen across two DNA mismatch repair genes, hMSH2 and hMLH1, to distinguish likely sites of mutation. hMSH2 and hMLH1 consist of 16 and 19 exons, respectively, and primer sequences suitable for amplification of each exon have been published (18,19). The primers were end-labeled, as described above. Each PCR reaction mixture contained 1.5 mM MgCl2 or, for hMSH2 exons 6 and 14, and hMLH1 exons 8, 9, 12 and 15, 3.0 mM MgCl2.

Exons were PCR-amplified individually, then combined for multiplex SSCP analysis. The PCR-products chosen for each multiplex run differed by at least 30 bp, to avoid overlaps. For each run, 2 μl of each of two or three PCR amplified exons were combined, diluted to 20 μl (total volume) with loading buffer, denatured and loaded on a non-denaturing high resolution 10% polyacrylamide gel (12 inches). To exclude any false positives arising from differential dilutions of the tested samples, SSCP variants were confirmed in at least two independent experiments. Resolution of the variants by SSCP was significantly enhanced if electrophoresis was at 4–8°C.

cDNA synthesis and PCR amplification (RT/PCR)

Total RNA was extracted using standard protocols: First-strand cDNA was synthesized from 1–5 μg total RNA using oligo-dT primer and Moloney Murine Leukemia Virus (MvMuLV) reverse transcriptase (Pharmacia Biotech). First-strand cDNA was then PCR-amplified (100 μl) using primers—5'-TGT AAA ACG ACG GCC ACT ATC AGG ACC TCA ACC GGT T-3' and (ii) the tumor contains limited or total shifting of CA-repeat bands involving one or both alleles when compared with the normal cell pattern, which can also be associated with changes in the intensity of individual bands and occasionally, with fewer bands in the tumor (compressed MIN pattern) (MIN-2) (Figure 1c: case 3; Figure 1b: case 4); and (iii) the loss of major band(s) corresponding to one of the two alleles in tumor versus normal DNA, a change which in a non-repetitive DNA sequence would constitute loss of heterozygosity (LOH) confined to the tumor (MIN-3) (Figure 1a; case 8).

Of the MIN+ cases, 4/11 can be characterized as MIN-1 (cases 1, 4, 6, 10): of the four, two had a positive family history for cancer and one had only a second degree relative (maternal aunt with breast cancer) and was therefore originally classified as sporadic (see Discussion) (Table II). 7/11 MIN+ cases can be considered MIN-2 (cases 2, 3, 5, 7, 8, 9, 11): all had negative cancer family histories except for case 3, whose mother (first degree relative) had cervical cancer (see Discussion).

In 6/7 tumors demonstrating DNA instability at both D2S123 and D2S119 the same MIN patterns for these chromosome two loci were found (Table II). No correlation could be demonstrated between the MIN classification and particular histopathology (Table II). The average age at diagnosis in

| Table I. Ovarian cancer, age at diagnosis, and family history |
|---------------------------------|-----------------|
| Ovarian cancer/family history   | Min+ / Case     |
| OC with family history of cancer| 3/28 (11%)      |
| OC, family history, age 55 or less | 1/3            |
| OC, family history, over age 55 | 2/3             |
| OC without family history of cancer | 8/62 (13%)   |
| OC, no family history, age 55 or less | 2/8            |
| OC, no family history, over age 55 | 6/8             |

series. Of these, 28 patients have a history of cancers in first and second degree relatives, consistent with one of the familial cancer syndromes that include OC. The 62 cases classified as cancer family history-negative have no first degree relatives with any of the above listed cancers: of these, two patients did have a parent who smoked with lung cancer; two others had only a second degree relative with cancer, a maternal aunt with breast cancer in one instance, a paternal grandfather with colon cancer in the other.

Microsatellite instability screening

Ninety OC and white blood cell DNA pairs were screened for MIN at microsatellite loci D2S123, D2S119 and D10S197. Differences in the gel band patterns between tumor and white cell pairs in at least one locus were found in 11 cases (11/90; 12%) (Table I; Figure 1a, 1b). MIN was found in three of 28 cases (11%) with a family history of cancer in at least two successive generations, and in eight of 62 presumed sporadic cases (13%) (Table I).

The microsatellite alterations found by comparing tumor and normal DNAs from the same patients in this series are of three distinct sub-types: (i) the tumor contains all CA-repeat bands found in DNA from normal cells, plus additional bands (MIN-1 or the type of instability reported in HNPCC) (Figure 1a; cases 1, 4, 6); (ii) the tumor contains limited or total shifting of CA-repeat bands involving one or both alleles when compared with the normal cell pattern, which can also be associated with changes in the intensity of individual bands and occasionally, with fewer bands in the tumor (compressed MIN pattern) (MIN-2) (Figure 1c: case 3; Figure 1b: case 4); and (iii) the loss of major band(s) corresponding to one of the two alleles in tumor versus normal DNA, a change which in a non-repetitive DNA sequence would constitute loss of heterozygosity (LOH) confined to the tumor (MIN-3) (Figure 1a; case 8).

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Results

Family history

Ninety OC cases, consisting of a tumor specimen obtained at surgery and venous blood from the same patient, comprise our

1800
Exon by exon polymerase chain reaction (PCR)-SSCP analysis was performed, looking for heterozygous sequence variation (see below).

MIN+ OC with (three cases) or without (eight cases) a positive family history, was 62 and 67 years, respectively. The latter group excludes a possible outlier, age 40 (case 1). This patient, as noted above, was considered a sporadic case, because there was cancer in only one second degree relative (maternal aunt with breast cancer). However, an extra band was demonstrated in this patient’s tumor when compared with normal cells (MIN-1), and the patient was found to carry a germ-line change in hMSH2 (see below).

To identify possible mutations in hMSH2 or hMLH1, an exon by exon polymerase chain reaction (PCR)-SSCP analysis was performed, looking for heterozygous sequence variation in DNAs from tumors and bloods from the 11 MIN+ cases in our series. The only changes found were in the germline and confined to the tumor in cases 1 and 8, respectively.

In case 1, a 40-year-old with OC, SSCP variation in a segment encompassing hMSH2 exon 5 and surrounding intronic sequences was demonstrated in both blood and tumor DNA (Figure 2). Sequencing of this SSCP variant revealed a 10 bp deletion in an intronic poly-A tract located 2 bp downstream from the splice donor site of hMSH2 exon 5 in both blood and tumor DNA (Figure 3). We did not find any variation in the length of the intron 5 poly-A tract in 27 other patients (including 10 other MIN+ cases, Table II; 11 MIN, OC cases, randomly picked from those in Table I; and six unrelated Lynch cancer family syndrome probands obtained previously, with cancers other than OC [data not shown]).

To determine whether the sequence change found in case 1 is likely to be a pathological mutation or a benign population polymorphism, we studied hMSH2 gene expression in several OC cases, including case 1. Two primers, 4dII and 6d (see Materials and methods), were designed to anneal to the 3' end of hMSH2/exon 4 and the 5'-end of hMSH2/exon 6, respectively. RT/PCR was performed and hMSH2 cDNA encompassing the entire exon 5 and surrounding intronic sequences was amplified from blood and tumor of case 1 and other cases. Data for case 1 was compared with that from three control cases in which the intronic poly-A tract in question was of normal length. Two cDNA products were demonstrated in all samples tested, with one having the size predicted by the normal sequence expressed in several-fold excess over a second, shorter cDNA. Sequencing of these two cDNA species indicates that the longer cDNA corresponds to normally spliced mRNA, whereas the shorter one, present in controls as well as case 1, corresponds to an alternatively spliced mRNA excluding exon 5. Also, analysis of exon 5 sequence included in cDNAs in normal controls and case 1 demonstrates that they are the same (data not shown). Therefore, the presence of the same two hMSH2 cDNA species in both case 1 (blood and tumor) and other OC cases, suggests that the shortened intronic poly-A tract in case 1 is most likely a benign polymorphism or variant, and should not be classified as a pathologic mutation.

In case 8, amplification across the entire hMLH1 gene could be accomplished in both tumor and white cell DNA, with the exception of the 5' end of exon 12 in the tumor. This suggests that a sequence change confined to the tumor, possibly instability in the intronic poly-A and poly-AT tracts adjacent to the splice acceptor site of hMLH1 exon 12, has occurred, thereby preventing PCR-amplification of the indicated segment. Further characterization of this somatic change is underway.

Discussion

The series we present comprises 90 OC cases. DNA instability at one or more microsatellite loci was found in 11 of 90 cases total (12%), including eight of 62 sporadic OC (13%) and three of 28 OC cases with positive family cancer histories (11%). Our findings are largely consistent with previous reports, indicating that MIN occurs infrequently in OC. The MIN percentage in sporadic OC we found is higher than that reported by Wooster et al. (5/27; 18%) (22), but lower than that reported by King et al. (5/27; 18%) (22). However, if cancer staging is taken into account, the discrepancy with King et al. disappears. Our data are based
Table II. Ovarian cancer, microsatellite alterations, age at diagnosis, family history and pathology

<table>
<thead>
<tr>
<th>Case</th>
<th>Microsatellites/instability patterns</th>
<th>Age</th>
<th>Family history</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D2S123</td>
<td>D2S119</td>
<td>D10S197</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>MIN-1</td>
<td>N</td>
<td>N</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>MIN-2</td>
<td>MIN-2</td>
<td>N</td>
<td>55</td>
</tr>
</tbody>
</table>
| 3    | MIN-2  | MIN-2 | N      | 55    | pap.solid serous  
cyst.adeno. |
| 4    | MIN-1  | MIN-1 | MIN-2 | 57    | M:Cx       |
| 5    | MIN-2  | N      | N      | 66    | none      |
| 6    | MIN-1  | N      | N      | 68    | poor diff. adeno. |
| 7    | MIN-2  | MIN-2 | N      | 70    | BET adipose      |
| 8    | MIN-3  | MIN-2 | N      | 71    | none      |
| 9    | N      | N      | MIN-2 | 74    | mesoderm.mixed  |
| 10   | MIN-1  | N      | N      | 75    | none      |
| 11   | MIN-2  | MIN-2 | N      | 77    | S:Br       |

MIN-1, Microsatellite instability with additional CA-repeat bands in tumor.
MIN-2, Microsatellite instability with partial or total CA-repeat band shifting in tumor.
MIN-3, Loss of one allele present in normal DNA from the tumor DNA (loss of heterozygosity confined to tumor).
N, Negative; Age in years.
Family history of cancer: M, mother; B, brother; S, sister.
Cx, cervix; C, colon; P, prostate; Br, breast.
Pathology: pap, papillary; cyst, cystadenocarcinoma; adeno, adenocarcinoma; met, metastatic; mesoderm, mesodermal mixed tumor; carc, carcinoma.

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Pathology: pap., papillary; cyst., cystadenocarcinoma; adeno., adenocarcinoma; met., metastatic; mesoderm., mesodermal mixed tumor; carc., carcinoma.

Fig. 2. hMSH2 exon variation by SSCP. Comparisons of W (white cell) and T (tumor) DNAs from same patients. Variation in exon 5 in both W and T (arrows) from case 1 (Table 2). exclusively on OC, stage II, III or IV, and King et al. reported MIN in 11% (4/37) of OC, stages II, III or IV; they also found MIN in 75% (3/4) OC, stage I. Fujita et al. recently reported an overall MIN frequency in OC 8/47 (17%), but 5/8 MIN+ cases were histologically classified as endometrioid ovarian tumors (23); no endometrioid tumors are found among our 11 MIN+ OC.

Of the 3 MIN+ cases with positive family histories of cancer in our series, the family history in case 4 is consistent with the Lynch cancer family syndrome, and that in case 10, with OC in the proband and breast cancer in a sister, could be Lynch or breast-ovarian cancer syndrome. As with previously reported Lynch families, including HNPCC, both cases 4 and 10 involved extra bands of CA-repeats in the tumor DNAs, when compared with normal cell DNAs from the same individuals (MIN-1 or HNPCC-type of instability). In case 3, compression of the microsatellite banding pattern resulted in fewer CA-repeat bands in tumor versus normal tissue DNA (MIN-2 change). In this case, the only other family member with cancer is the patient's mother, who had cervical cancer. This particular association of cancers (ovary in daughter and cervix in mother) can occur as part of the Lynch cancer family syndrome, or could occur in the same pedigree by chance (two sporadic cancers). We did not identify germline mutations in hMSH2 or hMLH1 in these 3 MIN+ cases.

Our finding of MIN in sporadic OC, also reported in other
series (20–24), suggests that the replication errors causing MIN, resulting from inactivation of both alleles at a single DNA repair locus, can occur exclusively as somatic events in ovarian epithelial cells as they divide. This is supported by the finding that changes in DNA microsatellite patterns have been reported to occur in permanent OC cell lines over time in tissue culture (25).

In HNPCC, MIN is associated with inherited (germline) mutations in DNA mismatch repair genes, predominantly hMSH2 and hMLH1. In our series, we identified a shortened intronic poly-A tract as an hMSH2 germline change in case 1, and an apparent hMLH1 somatic change in exon 12 in tumor DNA from case 8. The functional significance of the latter is unclear; in the former, the fact that the shortened poly-A tract does not affect cDNA length or normal splicing of the adjacent hMSH2/exon 5, is consistent with the indicated change being a benign population variant.

A possible relationship between MIN and tumor subsets defined by specific histopathology has been raised in studies of gastric cancer (26) and OC (23). The latter involved finding MIN more frequently in OC of less common histopathology, such as endometrioid or mixed serous and mucinous type, than among serous adenocarcinomas (23). As noted above, no endometrioid OC is included among the 11 MIN+ OC cases we identified. In our series, MIN in OC is not clearly associated with a specific histopathologic subtype (Table II).

We describe three different classes of banding pattern changes associated with microsatellite instability in our OC series. Four of 11 cases demonstrated gains of extra CA-repeat bands in gels comparing tumor versus normal tissue DNAs from the same patients (MIN-1), the same type of change reported in almost all HNPCC. Wooster et al. had earlier postulated that the additional bands which constitute an MIN-1 change in tumors are not derived by multiplication of existing microsatellite sequence, but are directly derived from division of sequences within one of the original alleles. Using densitometry, they were able to show that the total volume of the allelic and extra bands at each microsatellite locus in the tumor, was essentially equal to that of the original alleles at the tested locus in DNA from the same patient's normal cells (20).

Seven of 11 MIN+ cases demonstrated partial or total shifting of CA-repeat bands in tumor versus normal tissue (MIN-2). It may be that changes in intensity of individual bands and compression of microsatellite bands reflect co-migration of CA-repeat bands, resulting from instability of the CA-repeats at one or both alleles. Such changes would not necessarily lead to any significant loss of DNA from the tumor.

This is the first description of a classification of MIN and the first report of MIN-2 changes in OC. However, published illustrations of MIN in several other cancers, including sporadic stomach cancers (26), non-small cell lung cancers (27), and brain gliomas (28), appear to be consistent with MIN-2 changes.

It is important to differentiate MIN-2 from 'loss of heterozygosity' (LOH) confined to the tumor (MIN-3). LOH refers to the loss from tumor DNA of the major band(s) representing one allele in the DNA from normal tissue of the same patient: the band(s) corresponding to the other allele at that locus will be of equal intensity in both DNAs (see Figure 1a, case 8). The band shifts that characterize MIN-2 need not result in DNA losses from the tumor.

Case 7 demonstrates a totally shifted band pattern for both alleles of a single microsatellite marker in tumor DNA (MIN-2) (Table II). In case 4, a MIN-2 pattern involving both alleles was found for one microsatellite locus and a MIN-1 pattern was noted for two other microsatellite markers (Table II). Dams et al. (28), in discussing MIN in gliomas, have hypothesized that band shifting in MIN that involves both alleles results in qualitatively more extensive sequence changes than those associated with MIN affecting a single allele. This raises the possibility that the genetic changes underlying MIN-2 in cases such as 4 and 7 here, may result in more pronounced DNA instability (and a higher mutation rate?) than is associated with examples of MIN-2 confined to a single allele.

Differences in the patterns of MIN expressed by familial and sporadic OC may also exist. Of the eight sporadic MIN+ OC cases we describe, seven demonstrate MIN-2 changes. One presumed sporadic exception expressing MIN-1, case 1, developed OC at a young age (40 years: the average age of OC in sporadic cases is greater than 55) and has a maternal aunt with breast cancer; she may eventually be placed in the familial category. Of the three MIN+ OC cases with positive family histories for cancer, two demonstrated MIN-1 and one MIN-2 changes. The latter case, number 3, may actually represent a chance association of two sporadic cancers, and not be familial at all. The MIN-1 pattern, therefore, originally described in HNPCC and Lynch syndrome families, most frequently marks familial OC; MIN-2 is most commonly seen in sporadic OC.

As stated above, the development of MIN in sporadic OC implies that MIN can occur exclusively following somatic events. If the genetic bases of MIN-1 and MIN-2 are distinct, the finding of both MIN-1 and MIN-2 changes involving different loci in tumor DNA from the same case, number 4, would require the accumulation of multiple somatic genetic mutations during the course of tumor evolution.

With regard to possible genes that might be responsible for MIN in OC, we focused on the DNA mismatch repair genes implicated in MIN in the majority of HNPCC/Lynch families. We did identify, in one instance, case 1, a germline alteration in hMSH2 (a shortened poly-A tract), but we subsequently inferred that it is likely to be a benign variant. There is also the possibility that variation in length of the poly-A tract is not a primary event, but secondary to instability induced by changes in another DNA replication repair gene, such as that specifying the G/T mismatch-binding protein (GTBP), which can dimerize with hMSH2 (34). Mutations in GTBP have recently been demonstrated to result in alterations in mononucleotide repeat tracts (35). In another case, number 8, we were unable to amplify a single hMLH1 exon for sequencing from tumor but not constitutional DNA from the same patient. Studies are in progress to determine whether this failure resulted from an intra-genic deletion involving the exon, or from changes in intron sequence surrounding the exon.

It is possible that we have missed mutations in parts of the introns of hMSH2/hMLH1 that were excluded from our screen, or in transcriptional control sequences up- or down-stream from the genes themselves. Transcription and translation studies of both hMSH2 and hMLH1 will be required to determine whether we have underestimated the true frequency of inactivation of either gene in OC.

However, the fact that no germline mutations of hMSH2 or hMLH1 were identified in this series of MIN+ OC cases, both familial and sporadic, raises the possible involvement in MIN of OC of genes other than those most frequently implicated in
The identities of other genes that might be responsible for MIN in these cancers is open to speculation. In human and non-human systems, DNA mismatch repair genes other than hMSH2 and hMLH1 have been identified, and a number of DNA repair pathways linked to mismatch repair have also been described (29–32). Studies to determine the range of DNA repair genes contributing to MIN in OC are in progress.

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