Chromosome Microdissection Identifies Cryptic Sites of DNA Sequence Amplification in Human Ovarian Carcinoma

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ABSTRACT

DNA sequence amplification contributes to the multistep process of carcinogenesis, and overexpression of amplified genes has been shown to contribute to the malignant phenotype. Cytogenetic analyses of human tumor cells, including ovarian malignancies, frequently show cytological evidence of DNA amplification in the form of double minutes and homogeneously staining regions. In this report, we have combined the techniques of chromosome microdissection and fluorescence in situ hybridization (P. S. Meltzer et al., Nat. Genet., 1: 24-28, 1992) to identify the composition and chromosomal origin of seven homogeneously staining regions from seven cases of ovarian cancer. Twelve specific chromosome band regions were identified as amplified including 11q, 12p, 16p, 19p, and 19q. These results provide important insights into the organization of amplified sequences within ovarian malignancies and add further to our recognition of regions likely to harbor genes important to the development or progression of ovarian cancer.

INTRODUCTION

Ovarian cancer continues to be the leading cause of mortality due to gynecological malignancy in the United States. The lifetime risk of developing this disease is about 1.4%, and the risk increases with age (1). Relatively little information is available about recurring cytogenetic alterations in direct biopsy samples from primary ovarian cancers, although recent studies are beginning to provide insight into recurring sites of chromosomal changes (2-6). Obtaining cytogenetic data on ovarian tumors is particularly challenging because of the complexity of the tumor karyotypes and the frequency of markers that are unrecognizable by routine chromosome-banding techniques. One frequent finding in ovarian cancers is the presence of amplified DNA sequences recognized in the form of extrachromosomal double minutes or integrated hsrs. Genes reported previously to be amplified in ovarian cancer (including their chromosomal loci) are: MYC (8q24; Ref. 7); FGF3/INT2 (11q13; Refs. 8 and 9); KRAS (12p12.1; Ref. 10); ERBB2 (17q12; Ref. 11); and AKT2 (19q13.1-q13.2; Ref. 12).

We have recently developed a strategy based upon chromosome microdissection (13) to directly detect the chromosomal origin of cytogenetically visible chromosome aberrations, including double minutes and hsrs (14, 15). In this report, we present the results of microdissecting hsrs from seven ovarian carcinomas, identifying the chromosomal origin of DNA sequence amplification, and thereby identifying chromosomal regions likely to harbor genes amplified and of potential biological significance in ovarian cancer.

MATERIALS AND METHODS

Ovarian Cancer Cell Culture. Microdissection of chromosomal DNA was performed on a total of seven cases of ovarian carcinoma. Clonal karyotypic alterations for cases not published previously are provided in Table 1 (16, 17). Five of the seven cases of ovarian carcinoma (T91-007, T91-175, T92-023, T92-208, and T92-281) were derived from short-term cultures of primary tumors, while two cases (UACC-326 and 2727) represent early passage (P-11 and P-10, respectively) cell lines. The aforementioned cases used for microdissection were generated by the Tissue Culture Core Laboratory of the Arizona Cancer Center (Tucson, Arizona). Metaphase chromosomes from an additional four cases of ovarian cancer (OVA145, OVA142, OV34A, and OA39B) were provided by the Cancer Therapy and Research Center (San Antonio, TX) and were used for cross-hybridization experiments. CaOV-4 and OVCAR3 obtained from the American Type Culture Collection (Rockville, MD) were also used for cross-hybridization. All cells were grown to confluence, arrested with Colcemid, harvested for metaphase chromosomes, and G-banded as described previously (18). The clinicopathological information on ovarian carcinomas used in this study (except CaOV-4 and OVCAR3) is presented in Table 2.

Microdissection and Topoisomerase I Treatment. The procedure for chromosome microdissection was performed essentially as described previously (19). Briefly, one to five copies of the targeted hsrs were dissected from GTG-banded tumor metaphase chromosomes. The microdissection was performed with a glass microneedle controlled by a micromanipulator attached to an inverted microscope. The dissected chromosome fragment(s) was transferred into a 5-µl collection drop [containing 40 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 50 mM NaCl, 200 µM of each dNTP, 1 unit topoisomerase I, and 5 pmol of a universal primer (UNI); 5'-CCGACTCGAGNNNNNNATGTTG-3'; Ref. 20]. After the desired number of dissected DNA fragments were collected, the collection drop was covered with a drop of mineral oil, incubated at 37°C for 1 h, and denatured at 90°C for 10 min.

Amplification of Dissected DNA. An initial eight cycles of PCR (denaturation at 94°C for 1 min, annealing at 30°C for 2 min, and extension at 37°C for 2 min) was conducted by adding approximately 0.3 units of T7 DNA polymerase (Sequenase, version 2.0; USB) at each cycle [Sequenase (13 units/µl) was diluted 1:8 in enzyme dilution buffer (USB), and 0.2 µl was added to 5 µl reaction mixture]. Following this preamplification step, a conventional PCR reaction catalyzed by Taq DNA polymerase was performed in the same tube. The components of the PCR reaction were then added to a final volume of 50 µl [10 mM Tris-HCI (pH 8.4), 2 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 200 µM of each dNTP, 1 µM UN1 primer, and 2 units Taq DNA polymerase (Perkin-Elmer/Cetus)]. The reaction was heated to 95°C for 3 min, followed by 35 cycles at 94°C for 1 min, 1 min at 56°C, and 2 min at 72°C, with a 5-min final extension at 72°C.

FISH. Amplified, microdissected DNA (2 µl) was labeled with biotin-16-dUTP (Boehringer Mannheim GmbH) or spectrum orange-dUTP (Imageneics) in a secondary PCR reaction identical to that described above except for the addition of 20 µM biotin-16-dUTP or 50 µM spectrum orange-dUTP. The reaction was continued for 15-20 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, with a 5-min final extension at 72°C. The PCR products were then purified with a Centricron 30 (Amicon) filter and used for FISH. Hybridization of the FISH probes is based upon the procedure of Pinkel et al. (21). Briefly, for each hybridization, about 100 ng of probe was used in 10 µl hybridization mixture (containing 50% formamide, 10% dextran sulfate, 2X SSC, and 1 µg human Cot I DNA; BRL) that was denatured at 70°C for 5 min. The slide with metaphase spreads was denatured in 70% formamide-2X SSC (pH 5.3) at 70°C for 3 min and then hybridized with probes at 37°C in a moist
chamber overnight. The slide was then washed two times in 50% formamide, 2X SSC (pH 5.3) at 45°C for 15 min each, once in 2X SSC (pH 7.0) at 45°C for 10 min, and once in PN buffer [0.1 M sodium phosphate-0.1% (v/v) NP40, pH 8.0] at 45°C for 10 min. The hybridization signal of the probe was detected by three layers of FITC-conjugated avidin (Vector) and amplified with two layers of anti-avidin antibody (Vector). The slide was counterstained with 0.5 µg/ml propidium iodide (or 0.5 µg/ml DAPI for two-color FISH) in an antifade solution and was examined with a Zeiss Axiophot microscope equipped with appropriate epifluorescence filters.

Pre-Banded FISH. To unequivocally identify the chromosomal constitution of the hsr regions, the labeled, dissected DNA was hybridized to normal mitoses that had been banded previously. After GTG-banding, metaphase spreads from normal peripheral blood lymphocytes with good banding were selected and photographed. The slide was then destained by washing in absolute methanol for 10 min and then rinsed in 70% and 100% ethanol for 1 min each, followed by washing in 3:1 methanol:glacial acetic acid for 10 min and air-dried. The slides were then washed with 3.7% formaldehyde in IX PBS for 10 min and washed twice in 1X PBS for 5 min each. FISH using the selected hsr painting probe was then carried out as described above. The metaphase spreads were photographed and directly compared to the identical G-banded chromosomes to identify band regions encompassing the amplified DNA.

Southern Blot Analysis. Genomic DNA from human placenta and ovarian carcinoma cell line UACC-326 was isolated by standard techniques (22) and quantitated by spectrophotometry. DNAs were digested with EcoRI, fractionated on a 0.8% agarose gel, transferred to a nylon membrane (Zeta-probe; Cetus) with 100 ng of cDNA. Twenty-five amplification cycles were performed in a total of 5 of 12 cases of ovarian tumors studied (Table 4). As an example of the use of Micro-FISH probes in identifying the chromosomal constitution of the hsr regions, the labeled, dissected DNA was hybridized to normal mitoses that had been banded previously.

RESULTS

Chromosome microdissection was performed on seven cases of ovarian cancer with cytologically documented evidence of gene amplification. The karyotype of case T91–007 has been published previously (2). A description of the clonal karyotypic alterations of the remaining six cases is provided in Table 1. In all seven cases, one or more unidentified marker chromosomes containing an hsr were observed. An example of an hsr-bearing marker chromosome chosen for microdissection is illustrated in Fig. 1.

Chromosome microdissection was used to establish the chromosomal constitution of putative hsr regions using a recently developed strategy (15). Briefly, 1–5 copies of an hsr were microdissected and amplification have not yet been recognized, including 4qpl6, 15q22, 16pl1-13.2, 16q21, and 16q24 (Table 3).

As documented in Table 4, Micro-FISH probes generated from the hsr regions of six cases were cross-hybridized to a panel of ovarian cancer cell lines to study the frequency of amplification of selected chromosomal regions. Cross-hybridization experiments demonstrated that the most frequently amplified region, 19q13.3-13.2, was amplified in a total of 5 of 12 cases of ovarian tumors studied (Table 4). As an example of the use of Micro-FISH probes in identifying the composition of DNA sequences within an hsr, probes from the hsr of T92–023 and UACC-326 were hybridized to the same hsr-bearing marker chromosome in UACC-326. Of interest, the hybridization
patterns look different for each probe (Fig. 2), and the probe for UACC-326 is unable to hybridize to the hsr in case T92–023 (Table 4). This suggests that the two hsrs have limited shared homology of amplified sequences, despite the fact that both probes map, in normal cells, to a common region on 19q13.1 (Table 3). Dual-color FISH was then used to distinguish the specific region hybridized within the hsr. Specifically, a Micro-FISH probe for UACC-326 was labeled red/orange (using spectrum orange-dUTP), and a second probe for T92–023 was labeled green (with a biotin-16-dUTP), and both were simultaneously hybridized to metaphase spreads from the UACC-326 cell line. Fig. 2 shows a CCD image of the hybridization pattern of each probe individually as well as both probes superimposed on the DAPI-stained chromosome that contains the hsr. The Micro-FISH probe for T92–023 demonstrates a continuous hybridization pattern throughout the hsr with enhanced signal at the ends of the amplified region (Fig. 2). In contrast, the hybridization signal for the Micro-FISH probe for UACC-326 is more evenly distributed over a smaller portion of the hsr (Fig. 2).

Table 3 Origin and composition of hsrs from ovarian tumor biopsies and cell lines

<table>
<thead>
<tr>
<th>Biopsy specimen</th>
<th>Chromosomal loci</th>
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<tbody>
<tr>
<td>T91-007</td>
<td>12p12.1</td>
</tr>
<tr>
<td>T91-175</td>
<td>12p11.2-p12.1 / 4p16</td>
</tr>
<tr>
<td>T92-023</td>
<td>19p13.2-q13.1 / 16q24</td>
</tr>
<tr>
<td>T92-208</td>
<td>11q13.2-q14</td>
</tr>
<tr>
<td>T92-281</td>
<td>16p11-p13.2 / 16q21 / 15q15 / 15q22</td>
</tr>
<tr>
<td>Cell line</td>
<td></td>
</tr>
<tr>
<td>UACC-326</td>
<td>19q13.1–q13.2</td>
</tr>
<tr>
<td>UACC-2727</td>
<td>19q13.2</td>
</tr>
</tbody>
</table>

* Boldface, chromosomal regions that are involved in more than one hsr.

Table 4 Summary of cross-hybridization of Micro-FISH hsr probes

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Micro-FISH probes</th>
<th>007</th>
<th>175</th>
<th>208</th>
<th>281</th>
<th>023</th>
<th>326</th>
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* +, hybridization identifies hsr; –, hybridization does not identify hsr.
Fig. 2. Cross-hybridization analysis of a hsr-containing marker chromosome in ovarian carcinoma cell line UACC-326. A. DAPI-stained UACC-326 metaphase chromosomes hybridized with the Micro-FISH probes T92-023 (FITC signal) and UACC-326 (spectrum orange signal). B. marker chromosome from UACC-326-containing hsr: column 1. DAPI stain; column 2. T92-023-FITC signal; column 3. UACC-326-spectrum orange signal; column 4, superimposed outline of DAPI, FITC, and spectrum orange signals detected by CCD camera; column 5. superimposed DAPI, T92-023-FITC, and UACC-326-spectrum orange signals. C. intensity profile of each signal as seen by CCD camera, expressed as signal intensity/pixel position along the marker chromosome: blue line, DAPI; green line, FITC; red line, spectrum orange.

In addition to revealing the composition of an hsr, cross-hybridization of the Micro-FISH probes identified previously unknown amplified DNA in 3 of 7 cases (Table 4). For example, in case T91-007, the microdissected hsr was shown to encompass 12p12.1 (Table 2). However, following FISH with the hsr probe for UACC-326, a cryptic amplification of a19q13.1–q13.2 sequences was
from data recently reported for human breast cancer (15). Specifically, in breast cancer, the overwhelming majority of hsrs (13 of 16 cases) were shown to contain two or more chromosomal regions coamplified in the same hsr (15). This difference may suggest a different mechanism for hsr formation in ovarian and breast carcinogenesis.

*AKT2* is a serine-threonine kinase gene that has been mapped to 19q13.1–q13.2 and found to be amplified in several cases of ovarian carcinoma (12). Our data demonstrated that 3 of 7 ovarian cancer hsrs studied exhibited DNA sequence amplification for 19q13.1–q13.2. Southern analysis of the *AKT2* gene in one ovarian carcinoma case, UACC-326 (shown by Micro-FISH to contain 19q13.1–q13.2 amplification), demonstrated increased copy number when compared to the genomic DNA from normal cells (Fig. 3). This data provides genespecific evidence for amplification that corroborates the cytological evidence of DNA sequence amplification of chromosomal region 19q13.1–q13.2.

In this series of ovarian cancers, chromosome 19q is the most frequently amplified region. Microdissection recognized 3 of 7 hsrs with 19q amplification, with an additional two cases recognized by cross-hybridization of 19q hsr probe against our panel of ovarian tumors (Table 3). Aberrations of chromosome 19 material have been recognized in other cytogenetic studies of ovarian cancer. Pejovic et al. (4, 24) reported that approximately one-half of the ovarian carcinomas cytogenetically characterized demonstrated a 19p+ marker in which the additional chromosomal material was of unknown origin, a finding that was recently confirmed.5

In this study, microdissection of amplified DNA sequences in human ovarian carcinoma has enabled the identification of chromosomal regions involved in hsrs that would otherwise be unidentifiable by routine G-banding. Likewise, the probes generated from microdissected hsrs identified cryptic areas of DNA sequence amplification in ovarian tumors not detected by cytogenetic analysis. In addition to identifying amplified chromosomal regions containing previously recognized amplified genes, this study has identified novel chromosomal regions, the selected amplification of which may contain genes biologically relevant to ovarian carcinogenesis.

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REFERENCES


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