Detailed Genetic and Physical Mapping of Tumor Suppressor Loci on Chromosome 3p in Ovarian Cancer

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ABSTRACT

Hemizygosity and homozygosity mapping studies show that many common sporadic cancers including lung, breast, kidney, cervical, ovarian, and head and neck cancer display deletions on the short arm of chromosome 3. For ovarian cancer, monochromosomal transfer suppression studies have identified three candidate regions for chromosome 3p ovarian cancer tumor suppressor genes (OCTSGs). To accurately map OCTSG candidate loci, we analyzed 70 ovarian tumors for loss of heterozygosity (LOH) at 20 loci on chromosome 3p that were selected to target those regions proposed to contain tumor suppressor genes for common sporadic cancers. All samples were informative for at least five markers. In 33 (52%) tumors without microsatellite instability, LOH was observed for at least one 3p marker. Analysis of 27 ovarian tumors demonstrating both loss and retention of 3p markers enabled us to define four nonoverlapping minimal deletion regions (OCLOHRs): (a) OCLOHR-1 mapped distal to D3S3591 at 3p25–26; (b) OCLOHR-2 mapped between D3S1317 and D3S1259 at 3p24–25; (c) OCLOHR-3 mapped between D3S1300 and D3S1284, an area that includes the FHIT locus at 3p14.2; and (d) OCLOHR-4 mapped between D3S1284 and D3S1274 at 3p12–13, a region known to contain overlapping homozygous deletions in lung and breast tumor cell lines. However, microsatellite markers from the chromosome 3p21.3 interval homozygously deleted in lung cancer cell lines did not identify a distinct OCLOHR. The frequency and extent of 3p LOH correlated with tumor stage such that LOH at two or more OCLOHRs was present in 53% (16 of 30) of stage III tumors but only 26% (5 of 19) of stage I/II tumors (P = 0.08).

To determine the relationship between the OCLOHRs and the three candidate ovarian cancer suppression regions (OCSRIs) identified previously by monochromosomal transfer studies, we performed detailed genetic and physical mapping studies to define the extent of the three candidate OCSRIs and to establish YAC contigs covering each region. OCSR-A at 3p25–26 and OCSR-B at 3p24 were shown to overlap with OCLOHR-1 and OCLOHR-2, respectively, providing further evidence for OCTSGs in these regions. We also show that OCSR-C overlaps with a locus at 3p21.3 previously implicated in lung and breast cancer.

INTRODUCTION

Ovarian cancer is the fourth most common cause of cancer death in American women. Although ovarian cancer susceptibility genes (e.g., BRCA1, BRCA2, and MSH2) have been identified (reviewed in Refs. 1 and 2), most cases are sporadic, and somatic mutations in familial ovarian cancer genes are not a major feature of sporadic cancers.

Received 3/17/99; accepted 7/20/99.

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1 Supported in part by a Wellbeing grant; the E. B. Jones Bequest, University of Birmingham, United Kingdom; the Nerina and Mario Mattioli Foundation (S. M.); and the Associazione Italiana per la Ricerca sul Cancro (C. M.).
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LOH3 studies have identified frequent chromosome 3p allelic deletions in ovarian tumors (3–8), suggesting the presence of major OCTSGs on 3p. Deletions of chromosome 3p are also frequent in many common sporadic cancers including lung, breast, kidney, cervical, and head and neck cancer (reviewed in Ref. 9). Multiple lines of evidence suggest that 3p contains several TSGs. Thus, in addition to the VHL TSG at 3p25 (10–13), which is implicated in sporadic RCC (reviewed in Ref. 14), a lung cancer TSG locus (LCTSGR1) contained within a 600-kb cosmid contig at 3p21.3 has been defined by overlapping homozygous deletions in breast and lung cancers (15, 16), and a region of 3–4 Mb at 3p12 (LCTSGR2) is implicated by overlapping homozygous deletions in breast and lung tumor cell lines (17–19). Deletions of the FHIT candidate TSG at 3p14.2 have been reported in many sporadic cancers including ovarian cancer (20–25). The importance of chromosome 3p TSGs for ovarian tumorigenesis is demonstrated by the observation that the introduction of a normal chromosome 3b by MMCt suppressed tumorigenicity in an ovarian cancer cell line (26).

In a previous study, we analyzed the VHL TSG in 36 ovarian tumors but found no evidence to suggest that VHL is an OCTSG (7). To delineate candidate regions for chromosome 3p OCTSGs, we have performed detailed LOH studies on 70 ovarian tumors with polymorphic markers selected to include candidate regions (e.g., LCTSGR1 and LCTSGR2) identified in studies on other common cancers. In addition, we have mapped in detail the three candidate suppressor regions identified in the monochromosomal suppression studies reported by Rimessi et al. (26).

MATERIALS AND METHODS

Clinical Material. DNA from fresh frozen tumor tissue samples and from patient-matched normal peripheral blood samples was obtained from collaborative laboratories at Washington University School of Medicine (St. Louis, MO) and Mario Negri Institute (Milan, Italy). In the case of the American samples, genomic DNA was extracted from whole blood and fresh frozen tissue using a proteinase K digestion, phenol/chloroform extraction and subsequent ethanol precipitation. DNA samples provided by the Mario Negri Institute were extracted from tumors and blood samples with QIAamp Tissue and QIAamp Blood kit, respectively, following the manufacturer’s instructions. The tumors were histopathologically classified according to the criteria established by International Federation of Gynecology and Obstetrics (FIGO). Our collection of 70 samples includes 63 epithelial, 5 germ cell (teratomas), and 2 sex cord (granulosa) ovarian tumors. Laser capture microdissection of archival tumor samples was carried out using the National Cancer Institute laser capture microdissection protocol (27).

LOH Analysis. PCR amplification of dinucleotide and tetranucleotide microsatellite sequences was used in this section of the study along with a PCR-based RFLP. Twenty markers spanning the regions of interest on chromosome 3p were selected for use in our array. All are available through the
GBD, with the exception of new primers for the D3S1621 locus. The sequences used for the D3S1621 locus were forward primer 5'-CTCTGATGTTGTA-3' and reverse primer 5'-CCAAAGGAGGTTTTATTCTTTA-3' (size, 140 bp; annealing temperature, 55°C). The forward primers were 5' end-labeled in a 10-μl reaction volume containing 2 pmol of primer per reaction, 1× T4 polynucleotide kinase buffer (MBI Fermentas), 10 units of T4 polynucleotide kinase (MBI Fermentas), and 30 μCi of [γ-32P]ATP (3000 Ci/mmol; Amersham Life Science). This reaction was then incubated at 37°C for 30 min and at 94°C for 5 min. PCR was performed in a 10-μl reaction volume containing 100 ng of genomic DNA; 1× PCR buffer (Life Technologies, Inc.); 3 mM MgCl2; 0.5 unit of DNA Taq polymerase (Life Technologies, Inc.); and 2 pmol of reverse primer. PCR cycling conditions were as follows: (a) 5 min at 94°C; (b) 30 cycles of 30 s at 94°C, 30 s at the appropriate annealing temperature, and 30 s at 72°C; and (c) a final soak at 72°C for 10 min. The reaction products were then diluted 1:1 with a solution of 95% formamide, 10 mM EDTA, 10% bromphenol blue, and 10% xylene cyanol. After heating for 5 min at 94°C, 3.5 μl were loaded onto a 6% polyacrylamide denaturing gel (Sequagel-6; National Diagnostics). Gels were electrophoresed at 90 W constant power to achieve adequate separation of alleles and then dried at 80°C and autoradiographed (Fuji RX film).

LOH was scored based on the absence of alleles in tumor-derived DNA or a difference in the relative intensities of alleles in the tumor-derived DNA upon comparison with the DNA derived from normal tissue.

**Direct Colony PCR of YACs.** YACs obtained from the Human Genome Mapping Project, United Kingdom Resource Center were spread on selective media plates from stock cultures and incubated at 30°C for 48 h. Individual colonies were picked, mixed with 5 ml of water, and treated as a standard PCR template after an initial lysis at 94°C for 5 min. A total of 40 cycles were used, and the end products were separated on a 1.5–2.0% agarose gel (Life Technologies, Inc.); and 2 pmol of reverse primer. PCR cycling conditions were as follows: (a) 5 min at 94°C; (b) 30 cycles of 30 s at 94°C, 30 s at the appropriate annealing temperature, and 30 s at 72°C; and (c) a final soak at 72°C for 10 min. The reaction products were then diluted 1:1 with a solution of 95% formamide, 10 mM EDTA, 10% bromphenol blue, and 10% xylene cyanol. After heating for 5 min at 94°C, 3.5 μl were loaded onto a 6% polyacrylamide denaturing gel (Sequagel-6; National Diagnostics). Gels were electrophoresed at 90 W constant power to achieve adequate separation of alleles and then dried at 80°C and autoradiographed (Fuji RX film).

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**RESULTS**

**Chromosome 3p LOH Analysis.** Seventy matched tumor and normal DNA pairs were analyzed with 20 polymorphic markers (19 microsatellites and 1 RFLP). The locations of these markers and the locations of the VHL, TGF-β R2, and FHIT genes and LCTSGR1 and LCTSGR2 are shown in Fig. 1. Microsatellite instability at ≥40% of informative markers was observed in six (9%) tumors, and these were excluded from the LOH analysis. All tumors were informative for at least five of the markers analyzed. In 33 tumors (52%), LOH was observed at one or more chromosome 3p marker. In six tumors, LOH was observed at all informative markers, consistent with a complete loss of chromosome 3p, and 31 tumors (48%) showed no loss for any informative marker. A subset of tumors (showing both loss and retention of 3p markers) provided the basis for identifying critical nonoverlapping regions of LOH (Table 1). A minimum of four critical LOH regions were identified: (a) OCLORH-1 is distal to D3S3591 at 3p25–p26 (tumor 18 shows retention at D3S3591 and loss at D3S1560; Table 1; Fig. 2; we were not able to obtain a distal limit for this region); (b) OCLORH-2 is contained within the D3S1317 and D3S1259 interval (tumor 17 shows retention of D3S1317 and loss of D3S1038; tumor 15 shows loss of D3S1038 and retention of D3S1259; Table 1; Fig. 2); (c) OCLORH-3 was defined by the D3S1300 to D3S1284 interval (tumor 72 shows retention of D3S1300 and D3S1284 and loss of D3S1481), which contains the FHIT gene and the FRA3B fragile site; and (d) OCLORH-4 was defined by tumor 63 and mapped between D3S1284 and D3S1274. Marker D3S1274 is contained within LCTSGR2 at 3p12, whereas D3S1284 is outside LCTSGR2.

The approximate genetic distances for three of the four minimal deletion regions described above are: (a) OCLORH-2, <3 cM; (b) OCLORH-3, 22.0 cM; and (c) OCLORH-4, 7.0 cM, respectively. Although the LCTSGR1 represented by two microsatellite markers at 3p21.3 and 3p12 (LCTSGR1 and LCTSGR2), regions shown to be functionally important in ovarian tumor development (OC3R A-C), and the four minimal LOH regions (OC3R 1–4) are shown. The positions of the VHL, FHIT, and TGF-β R2 genes are also shown.

**Statistical Analysis.** Comparisons were made using Fisher’s exact test.

![Fig. 1. Summary of polymorphic markers and regions of interest on chromosome 3p.](image)

Genetics markers are listed in descending order from telomere to centromere according to published maps (Marshfield chromosome 3 sex-averaged linkage map), web sites, and approximate cytogenetic (ideogram) positions. Regions implicated in lung cancer development at 3p21.3 and 3p12 (LCTSGR1 and LCTSGR2), regions shown to be functionally important in ovarian tumor development (OC3R A-C), and the four minimal LOH regions (OC3R 1–4) are shown. The positions of the VHL, FHIT, and TGF-β R2 genes are also shown.

**Fig. 1.** Summary of polymorphic markers and regions of interest on chromosome 3p.

**Tumor Histopathology and Grade and Chromosome 3p LOH Analysis.** The relationship between tumor histopathology and the presence of 3p LOH was examined in 56 microsatellite stable tumors. The frequency of 3p LOH was similar in the three major histopathological subgroups: (a) serous tumors, 58% (14 of 24 tumors); (b) endometrioid tumors, 78% (7 of 9 tumors); and (c) other tumors, 53% (10 of 19 tumors). There was a lower frequency of 3p LOH in mucinous tumors (25%; 1 of 4 tumors), but this was not statistically significant.

We then analyzed the relationship between tumor stage and the frequency and extent of 3p LOH. The frequency of LOH was higher with increasing tumor stage. Thus, LOH at one or more loci was detected in 47% (7 of 15) of stage I tumors, 75% (3 of 4) of stage II tumors, and 70% (21 of 30) of stage III tumors (stage I versus stage III tumor, \( P = 0.19 \)). No LOH was observed for all informative markers in any of the four benign tumor samples in our study, whereas all five tumors with loss of every informative marker were stage III.
tumors. We then examined whether later-stage tumors might, in addition to having a greater frequency of 3p LOH, display more extensive LOH (Table 1). Whereas 50% (5 of 10) of stage I/II tumors with 3p LOH had LOH at 1 OCLOHR, most stage III tumors (76%; 16 of 21 tumors) with LOH had LOH at two or more OCLOHRs. Overall, 53% (16 of 30) of stage III tumors had LOH for at least two 3p OCLOHRs, as compared with 26% (5 of 19) of stage I/II tumors (P = 0.08).

**Detailed Genetic and Physical Mapping of Regions Important in Ovarian Tumor Suppression.** Using MMCT of chromosome 3 into the HEY ovarian cancer cell line, Rimessi et al. (26) demonstrated suppression of tumorigenicity. Nude mice were inoculated with the hybrid clones containing a normal copy of chromosome 3. Two clones (clones 3 and 5) were found to revert to the tumor phenotype. Microsatellite analysis of tumors induced by clone 3 revealed it to have lost most of the short arm of chromosome 3, but tumors from clone 5 had undergone loss of only three regions on 3p. To further define these candidate OCTSRs, we have performed genetic and physical mapping studies. Rimessi et al. (26) reported loss at four microsatellite markers (D3S1304 at 3p26–25, D3S1259 at 3p24.1, and D3S1289 and D3S1578 at 3p21.1–21.3) in clone 5. Therefore, we analyzed flanking markers to determine the extent of the deletions. For a microsatellite marker to be informative, the alleles of the recipient HEY cell line had to differ from the allele provided by the donor chromosome 3 (Fig. 3). OCTSR-A was defined by the retention of markers D3S1560 and D3S3591 and the loss of marker D3S1304 in clone 5 (Fig. 3A). OCTSR-B at 3p24.1 was defined by the retention of markers D3S1263 and D3S3701 and the loss of D3S1259 (Fig. 3B), and OCTSR-C mapped in the D3S1568–D3S1613 interval (Fig. 3C). Hence, OCTSR-C overlaps with LCTSGR1 at 3p21.3. The sizes of the candidate OCTSRs were estimated at 6.0 cM (OCTSR-A), 1.0 cM (OCTSR-B), and 1.7 cM (OCTSR-C; Fig. 3C). We then constructed physical maps of the three OCTSRs. The Quikmap Infoclone program6 was used to identify YACS that might cover the three regions. The YACS of interest were obtained from Human Genome Mapping Project, United Kingdom Resource Center,4 Genethon databases, and direct colony PCR, and end labeling of the microsatellite markers was used to confirm the presence or absence of markers on the relevant YACS. OCTSR-A, is covered by two overlapping CEPH YACS, 934H2 and 700B10, (1.7 and 0.8 Mb in size, respectively). OCTSR-B is partially contained in YAC 753F7 (0.77 Mb). OCTSR-C is contained within two overlapping YACS, 745G8 and 819C4 (0.67 and 0.82 Mb in size, respectively; Fig. 3).

**DISCUSSION**

In this large study, we have (a) demonstrated a high incidence of 3p LOH in ovarian cancer of various histopathologies, (b) defined four candidate regions for OCTSGs by LOH analysis, (c) identified a possible correlation between the frequency and extent of 3p LOH and

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6 http://www.cephb.fr/ceph-genethon-map.html.
tumor stage, (d) mapped three OCSRs suggested by tumorigenicity studies, and (e) identified two separate regions of overlap between OCLOHRs and OCSRs and an overlap between an OCSR and LCTSGR1, which provide strong candidate regions for OCTSGs at p21, 3p24, and 3p25–26. The only previous LOH study of similar size was reported by Lounis et al. (8), who also found evidence of multiple regions of 3p LOH in ovarian cancer. Our OCLOHR-3 was not identified as a minimal target region by Lounis et al. (8); however, Hendricks et al. (24) reported alterations of the FHIT gene in 2 of 14 ovarian cancer cell lines studied. OCLOHR-3 contains part of the FHIT gene and is close to the FRA3B fragile site. Frequent LOH at 3p14.2 may indicate a significant role for the FHIT gene in ovarian tumorigenesis or reflect a tendency for chromosomal rearrangements at fragile sites in cancers with chromosomal instability. Although we identified multiple candidate OCTSG regions, we cannot exclude the possibility of further candidate regions that were not represented by the markers used in this study. However the markers studied were chosen because of prior evidence that these regions were likely to be implicated in ovarian and other cancers. Unfortunately, the level of informativeness for each marker was not uniformly high (e.g., D3S1604); however, this is unlikely to have biased our findings.

Fig. 2. Representative examples of 3p LOH. Examples from patients 18, 17, 15, 72, and 63 from each of the proposed minimal regions of allelic loss are shown. Lanes N, T, and L, normal DNA from peripheral blood, tumor DNA, and laser-microdissected tumor DNA, respectively. Alleles, open arrows; allelic losses, filled arrows.
The regions of 3p implicated in our studies can be divided into those that have also been implicated in other tumors (3p12, LCTSGR2; 3p21, LCTSGR1; and 3p14) and those specific to ovarian cancer (3p25–26 and 3p24). OCLOHR-4 at 3p12 is of interest because it overlaps with the homozygous deletion region (LCTSGR2) identified in the U2020 lung cancer cell line (17, 18). Subsequently, overlapping smaller deletions have been found in breast and lung cancer cell lines (19), and our findings would be compatible with a 3p12 TSG implicated in several cancer types. A more recent MMCT study has also shown that the 3p12 region is able to suppress tumorigenicity in a RCC cell line (29). Thus far, preliminary studies of ovarian cancer cell lines have not revealed evidence of homozygous deletions within 3p12. However, mutation analysis of the DUTT1 candidate TSG has not yet been undertaken (19). Chromosome 3p21 LOH is a frequent finding in many common cancer types, and homozygous deletions in lung and breast cancers have identified a 600-kb critical region (LCTSGR1; Refs. 15 and 16). In our study, chromosome 3p21 LOH was frequent, although we could not identify a distinct nonoverlapping deletion; nevertheless, we found an overlap between OCSR-C and LCTSGR1, hence further evidence that the 3p21 region is important in the development of several common sporadic cancers.

A distal 3p25–26 OCLOHR was identified by both Lounis et al. (8) and us. The VHL TSG maps to chromosome 3p25, but we have demonstrated previously that VHL is unlikely to have a role in ovarian tumorigenesis (7). Whereas LOH studies provide mapping data for regions that may contain TSGs, the reliability of this data is greatly enhanced if TSG location can also be inferred from complementary investigations. The three candidate OCSRs identified by Rimessi et al. (26) offered the opportunity to investigate the relationship of these OCSRs to our OCLOHRs. Using an array of microsatellite markers, we carried out fine detail mapping of three OCSRs and demonstrated that OCSR-A and OCSR-B overlapped with OCLOHR-1 and OCLOHR-2, respectively. From the suppression study performed by Rimessi et al. (26), it is not possible to determine which of the candidate OCSRs is required for suppression. However, the coincidence of our candidate OCLOHR-1 and OCLOHR-2 regions with OCSR-A and OCSR-B and the overlap of LCTSGR1 with OCSR-C suggest that all three OCSR regions are likely locations for OCTSGs. The overlaps between OCLOHR-1 and OCSR-A and between OCLOHR-2 and OCSR-B and the overlap between LCTSGR1 and OCSR-C represent three small (6.0, 0.5, and 1.6 cM, respectively) high priority regions for the search for OCTSGs. The availability of cloned DNA for these regions will facilitate the identification of new candidate genes from these regions. The TGF-β R2 gene was recently shown to be mutated in 25% of microsatellite instability and microsatellite stable ovarian tumors (30) but maps outside of OCLOHR-1 and OCLOHR-2. Although genes and ESTs from within the candidate regions have been identified, none have been reported to be mutated in ovarian cancer or other cancers.

It is clear that chromosome 3p contains multiple TSGs, some of which may be implicated in several common cancers. In sporadic RCC, somatic VHL gene mutations and loss are early and frequent events. VHL has been identified as a gatekeeper for RCC; however, inactivation of the VHL gene is not sufficient for tumorigenesis, and we and others have suggested that loss of other (uncloned) 3p TSGs is also necessary (31, 32). For ovarian cancer, we found a possible suggestion that higher-stage tumors had more frequent and extensive 3p LOH. Zheng et al. (4) reported an association between 3p LOH and high-grade malignancy in ovarian tumors. Our suggestion of more extensive 3p LOH in stage III tumors, with many demonstrating LOH at at least two OCLOHRs, is reminiscent of studies in lung cancer by
Chung et al. (33), who found the 3p allele loss in dysplasia to show a much more restricted pattern than in invasive tumors. However, our findings should be interpreted with caution because they are based on small numbers of low-grade and low-stage tumors. There were no obvious indicators that any particular 3p region was implicated solely in low- or high-grade or low- or high-stage tumors. The identification of chromosome 3p TSGs will enable the role of individual TSGs and the importance of serial inactivation in the pathogenesis of ovarian cancer to be determined.

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