Isolation and Mapping of a Human Septin Gene to a Region on Chromosome 17q, Commonly Deleted in Sporadic Epithelial Ovarian Tumors 1

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Abstract

Allele losses from chromosome 17 are common in sporadic ovarian tumors. Previously, we reported high rates of LOH (up to 70%) from 17q25 at the marker THH59 in a bank of malignant ovarian tumors. We have extended this study to 70 tumors with 17 markers from the long arm of chromosome 17. In most cases, the data are consistent with whole chromosome loss, but we have identified a minimal region of deletion that is centered around 4 microsatellites with zero recombination at map position 106.9 cM. A P1/BAC contig across the region (~200 kb) was constructed and used to determine the precise position and order of the microsatellites. The contig was shown to hybridize to 17q25 by fluorescence in situ hybridization analysis. The DNA sequence of the entire contig was determined and analyzed by BLAST searches. A 4-kb cDNA was subsequently identified with homology to the yeast, Drosophila and mammalian septin family of genes. We have designated this gene Ovarian/Breast (Ov/Br) septin. Two splice variants were demonstrated within the 200-kb contig, which differ only at exon 1. Within the contig, ~45% of the septin α transcript was identified and 38% of the septin β transcript. The septins are a family of genes involved in cytokinesis and cell cycle control. Their known functions are consistent with the hypothesis that the human 17q25 septin gene is a candidate for the ovarian tumor suppressor gene.

Introduction

Identification of tumor suppressor genes has characteristically been by linkage analysis in families with an inherited predisposition to a particular tumor type or by deletion mapping in a bank of sporadic tumors. In the latter case, polymorphic markers have been used to determine overlapping deleted regions by comparison of tumor DNA with matched control DNA. This LOH 1 analysis has led to the identification of such minimum regions of deletion and the localization of several tumor suppressor genes. In 1990, we and others provided evidence for high rates of LOH from the long arm of chromosome 17 in sporadic epithelial ovarian tumors (1, 2). Whereas LOH could be demonstrated with polymorphic markers from both the long and short arms of this chromosome, the highest rates of loss were detected with the VNTR probe THH59 (D17S154, 17q23–25). Up to 70% of malignant tumors showed loss with this marker, and LOH was detected in a benign tumor, suggesting that such loss may reflect the inactivation of a gene early in malignancy. In a larger follow-up study (3), we showed that this loss was present in all histological subtypes and correlated with tumor stage but could still be detected in early stage disease. The LOH from the short arm occurred in later stage disease, possibly reflecting inactivation of the p53 gene. Numerous other reports have confirmed this high rate of LOH from chromosome 17 and demonstrated that the pattern of LOH in the majority of tumors is consistent with loss of the whole chromosome (4). Moreover, in those tumors in which a partial deletion from 17q can be detected, the partially deleted region is distal of BRCA1, the familial breast/ovarian gene (5, 6). This therefore suggested that BRCA1 was not the target of deletion on 17q in sporadic ovarian disease and that there may be another tumor suppressor on distal chromosome 17q. LOH analysis in a bank of 39 sporadic breast tumors identified a region of interstitial loss of approximately 3 cM around D17S937 on chromosome 17q (7). Subsequently, it was shown that approximately half of a group of 32 ovarian tumors had losses coincident with this region (8). In this study, we report the results of deletion analysis of a large bank of ovarian tumors from patients with no obvious family history of ovarian cancer and provide evidence of a discrete region of loss from distal 17q. We have carried out fine mapping and further localized the common region of deletion to between D17S1790 and afm203wc5, an interval of zero recombination at map position 106.9 cM. By positional cloning efforts, we have identified a gene from this region that is a member of the septin family of proteins that are conserved in organisms as divergent as yeast and humans. Recently, there have been two reports identifying this same septin gene as the fusion partner of the MLL gene in t(11;17)(q23;q25) in two patients with therapy-related AML and de novo AML (9, 10). The septins have been shown to localize to the cleavage site of mitotic cells and are essential for cytokinesis in both yeast and animal cells. However, they are also thought to have a variety of other roles in morphogenesis and the organization of the cell surface (reviewed in Ref. 11). The sequences of the known septins contain a P-loop nucleotide-binding domain consistent with GTPase activity. Many of the septins also contain predicted coiled-coil domains near their COOH termini. However, the sequence similarity is greatest within the central portion of the peptide with the NH2- and COOH-terminal regions being more divergent in both length and sequence.

Materials and Methods

LOH Analysis. Tumor samples were obtained from surgically resected material and snap frozen in liquid nitrogen. Tumors were staged according to the International Federation of Gynaecology and Obstetrics classification. Peripheral blood samples were obtained from each patient. High molecular weight DNA was isolated by standard methods of SDS-proteinase K and phenol chloroform. Standard Southern blot analysis was carried out with the
was carried out in a final volume of 15 μl containing 100 ng of target DNA, 250 nmol primers, 2.5 mm MgCl₂, 200 μM deoxynucleotide triphosphates, 1× Taq buffer, and 0.35 unit of Taq polymerase. The reaction conditions were 250 nM primers, 2.5 mM MgCl₂, 200 μM dNTPs, 1× Taq buffer, and 0.35 unit of Taq polymerase. The reaction conditions were 95°C for 5 min and then 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min with a final extension step of 72°C for 5 min. Two μl of the PCR product were electrophoresed on a denaturing polyacrylamide gel that was end-labeled with [32P]-dCTP by random hexamer priming and hybridized overnight at 65°C. Filters were washed to 2× SSC at 42°C and autoradiographed at 70°C for 8–24 h.

LOH was assessed by direct visual comparison of the relative allelic ratios present in matched normal and tumor DNAs. To ensure that allelic intensities were within the linear range, multiple exposures of each autoradiograph were carried out. Autoradiographs were scored independently by at least two authors. A representative autoradiograph is shown in Fig. 1A. LOH was scored if one allele was absent or exhibited altered signal intensity in tumor DNA relative to the allelic ratio of normal DNA. We have conventionally referred to this alteration of allelic signal within a tumor sample as LOH. However, with this analysis it is not possible to distinguish between allele losses and gains, and such allelic imbalance could also represent amplification of a mutant allele.

PCR reactions with tumors and markers showing partial deletions were repeated twice to confirm the scoring and again read independently by two authors.

Restriction Mapping. P1 and BAC DNAs were restricted with NotI, SalI, and NotI/SalI and NotI/SalI. The digest was fractionated on a 1% agarose gel by pulsed field gel electrophoresis using the CHEF Mapper System (Bio-Rad). The gel was blotted onto Hybond N⁺ (Amersham Pharmacia) and probed with P1 and BAC insert end probes. Briefly, riboprobes were generated from the SP6 and T7 promoters of the P1 and BAC vectors using the riboprobe in vitro Transcription System (Promega).

FISH Analysis. FISH was carried out on normal human lymphocytes metaphase spreads. Biotinylated probes were prepared by nick translation of BAC DNA with biotin-16-dUTP (Boehringer) and DNA polymerase I and DNase I (Life Technologies, Inc.). Slides were hybridized for 72 h and washed to 0.2× SSC (pH 7.0) at 42°C. Detection of bound probe was carried out with avidin/Texas Red, and one round of amplification of signal was performed.

Northern Blot Analysis. A 387-bp PCR product was amplified from HeLa cDNA by primers ABO 01 (5′-CGG AGA TCG TCA GCC AAC-3′) and ABO 02 (5′-ACG TAG CCG AAG TCC ACC GG-3′) corresponding to nucleotides 1207–1594 of Ov/Br septin α. This probe is common to both α and β transcripts because it is derived from exon 2 and the start of exon 3.
DNA was labeled with [α-32P]dCTP (Amersham Pharmacia) by the Mega-prime DNA Labeling System (Amersham Pharmacia). Human multiple tissue Northern blots were obtained from Clontech, and hybridization was carried out according to the manufacturer’s instructions.

**Mutation Analysis.** Total RNA was purified from snap-frozen tumor samples using RNA Stat60 (Biogenesis) according to the manufacturer’s instructions. RNA (5 μg) was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Life Technologies, Inc.) and random hexamer primer. cDNA (1 μl) was amplified by PCR in a volume of 50 μl. The final concentration of reagents in the reaction was 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl2, 50 mM KCl, 200 mM each primer, 200 μM each deoxynucleotide triphosphate, 2 units of Taq DNA polymerase (Life Technologies, Inc.). Cycling conditions of 94°C for 2 min and then 35 cycles of 94°C, 30 s; 58°C, 30 s; and 72°C, 60 s were used. The following primer sets were used to amplify overlapping fragments of the Ov/Br α and β septin open reading frames: primers ABO13F, TGAGAAGGGGAGGCCCGCTCTCG (708–730) and ABO10R, ACCTTTGGGAGGAGGGGCTCT (1236–1217); primers ABO01F, CGGAGATCCACATCGTCAACC (1170–1191) and ABO04R, GGTG- GATTTGGACCTCCTCTG (1916–1985); primers ABO005F, CGAGGAATCTGCGGAGCCCATC (1874–1895) and ABO088R, GAAATGTCCGGGAGGAGATCAG (2525–2500). The numbers in parentheses refer to the positions of each primer with reference to the septin sequence, accession number AF123052. PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega) and sequenced with ABI Prism BigDye Terminator Cycle Sequencing chemistry. Sequence data were compared with the above septin sequence (AF123052).

**Results**

**LOH Studies.** A bank of 70 sporadic ovarian tumors composed of 43 malignant, 5 borderline, and 22 benign lesions was investigated for LOH with 19 markers from chromosome 17. The relative position of these markers along the chromosome and the percentage of LOH observed at this position is shown in Fig. 1B. The highest rates of LOH were detected between the markers D17S785 and D17S785 at distal 17q. The extent of LOH from chromosome 17 was determined for tumors that were informative at a minimum of eight loci (50 of the 70 tumors examined). Tumors were classified as having extensive loss (i.e., loss of the whole chromosome or at least the q arm) or partial deletion. These results are summarized in Table 1. Within the tumor bank, 31 malignant tumors were informative for at least eight loci. No LOH was observed in 3 whereas 20 showed extensive LOH consistent with whole chromosome loss. One malignant tumor had partial LOH from the short arm, whereas 7 tumors demonstrated partial LOH from the long arm. In 2 cases, this LOH was across a large area of proximal 17q and included the region of the BRCA1 gene. In the remaining 5, the LOH was from distal 17q. Within the group of 16 benign tumors examined, 12 had no detectable LOH, 1 had whole chromosome loss, 1 had LOH from the short arm only, and 2 had LOH from distal 17q. Of the three borderline tumors, 1 had no detectable LOH, 1 had LOH from the short arm, and 1 had LOH from distal 17q. Sequencing the original 70 tumors and a further additional 30 tumors with a series of markers that map to 17q, identified a group of 10 tumors with confined deletions at 17q (Fig. 1C). Analysis of these tumors with partial deletions has refined the region of interest to a position within 4 polymorphic microsatellites with zero recombination at map position 106.9 cM. This is also the region to which the VNTR probe, THH59, used in the original LOH study has been mapped (12).

**Physical Mapping.** A P1/BAC contig was constructed to cover this region (13). Three P1s and one BAC were obtained that screened positive for either D17S939 or D17S937. To determine the relationship of these P1s and BAC to each other, a NotI/SalI restriction map was constructed by generating riboprobes from the ends of each P1 and BAC and hybridizing them sequentially to NotI/SalI restrictions on Southern blots. The order and location of the 4 microsatellites was determined in two ways. Primers for PCR with these microsatellites were end labeled and used as probes in Southern blot experiments of NotI/SalI digests. Additionally, individual NotI/SalI fragments were gel purified and used as templates for PCR with individual microsatellites. The complete restriction map and position of the markers is shown in Fig. 2A. The BAC was used as a probe in FISH experiments and hybridized to distal 17q (Fig. 2C).

**Sequencing.** DNA sequencing of the largest P1 (655 g10) and the BAC was performed. Briefly, each was restricted and subcloned to provide approximately a six-deep coverage that was then sequenced automatically. The sequence of the individual contigs was ordered and assembled. The sequence was then subjected to BLAST searches. Further analysis with individual ESTs revealed by the BLAST searches identified 1502 nucleotides of a 4-kb septin cDNA lying within the contig. This cDNA was isolated from brain tissue and lodged recently in GenBank (accession number AB023208). From the sequence of the 4-kb cDNA (which we have named the β transcript), we have been able to identify the genomic structure of the first six exons of this gene. There is an intron of ~27 kb between exons 1 and 2 and the predicted coding sequence begins at position 733 bp within exon 2. The coding sequence is 1266 bp in length and codes for a protein of 422 amino acids and predicted molecular weight of M, 47,500. We have also identified a splice variant of this transcript (which we have named the α transcript) that differs at the 5′ end because of an alternative exon 1. The alternative first exon is located ~57 kb upstream from exon 1β and is 833 bases in length. The coding sequence is 1704 bp in length and begins at position 813 bp of exon 1. It codes for a protein of 568 amino acids and predicted molecular weight of M, 63,600. The available genomic structure is summarized in Fig. 2B. A PCR product generated from exon 2 and the beginning of exon 3 and thus common to both the α and β transcripts was used as a probe in Northern blot experiments against a variety of normal tissue RNAs (Fig. 2D). A 4-kb and 4.3-kb transcript were evident in 10 tissues (spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood lymphocyte, heart, and pancreas), and there was variation in their relative amounts. In the remaining tissues, only the 4.3-kb transcript was detected.

**Mutation Analysis.** A preliminary mutation analysis was carried out by direct sequencing of cDNA from tumor samples with partial deletions in distal chromosome 17q. cDNA was available for 7 of the 10 tumors shown in Fig. 1C (samples 1–3, 5, 6, 8, and 10). Primer sets were used to amplify six overlapping fragments of the Ov/Br α and β septin open reading frames for direct sequencing. Base changes were observed in three of seven samples: in sample 1, a GTG to ATG (Val/Met) at position 2484 bp; in sample 6, a CCC to CCG (Pro/Pro) at position 890 bp, a CGC to TGC (Arg/Cys) at position 984 bp, a CCG to CTCG (Pro/Leu) at position 1192 bp; and in sample 10, a CCG to CCA (Pro/Pro) at position 2510 bp. All base changes were also identified in matched control samples. With the exception of the base change at position 984 bp in sample 6, the other changes result in conservative or semiconservative amino acid changes. However, the C→T change at position 984 bp in sample 6 only alters the coding sequence of the α transcript and represents a dramatic change from a
of the contig. Ov/Br septin, genomic structure of the candidate gene, D17S939, and restriction sites and the position of the microsatellite markers D17S1790, D17S937, D17S939,

Discussion

charged amino acid (Arg) to a polar amino acid with a sulfydryl side chain (Cys).

Despite recent advances in the understanding of the molecular abnormalities associated with sporadic ovarian cancer, the overall 5-year survival still remains very low mainly because of a lack of early diagnosis. There is now clear evidence that genes on chromosome 17 play a crucial role in the development of epithelial ovarian tumors. Indeed, the highest rates of LOH from any chromosomal region in ovarian tumors are from this chromosome.

Since our initial report in 1990 of high rates of LOH from 17q, there have been other reports of LOH from this chromosome in these tumors. Whole chromosome loss was demonstrated in the majority of malignant tumors (4). In our analysis of 70 tumors with 19 markers and considering only tumors that were informative at more than eight loci, a similar pattern of LOH has emerged. In frankly malignant disease, the pattern of loss in 65% of tumors was consistent with loss of one copy of chromosome 17. In 5 of the remaining malignant tumors, a partial loss involving distal 17q was detected. These results would therefore indicate a crucial role in ovarian malignancy for a gene on distal 17q that is often accompanied by allelic imbalance at all chromosome 17 loci.

There has been the suggestion that benign, borderline, and malignant ovarian tumors and also the various histological subtypes may each follow a different molecular pathway (14). Our findings of partial deletions in benign, borderline, and malignant disease and in serous, endometrioid, and mucinous tumors would indicate that this putative tumor suppressor gene is common to all of these pathways and may be an early event.

Previous reports in the literature describing partial deletions from 17q in sporadic ovarian tumors have delineated regions that are proximal to that described in our study. One study described a 16-cM common region of deletion delineated by nm23 and GH (5), whereas another identified a 25-cM region delineated by GH and D17S4 (6). In both studies only a limited number of markers in the 17q23–25 region were used, the most distal being D17S4 and D17S75, respectively. Also, a close examination of the individual cases in both studies reveals that the majority of deletions are in fact quite large, and in only 4 of 14 cases was the distal boundary of the deletion identified. The small region of deletion that we describe falls within the larger deleted regions of 11 of 14 tumors documented in the previous studies.

In addition, this study extends more recent data from an analysis of 39 breast tumors (7) and 32 ovarian tumors (8) in which an overlapping region of interstitial loss of ~3 cM around D17S937 was identified. The common region of deletion that we describe is defined by four microsatellite markers D17S1790, D17S937, D17S939, and afm203wc5, which lie within an area of zero recombination at map position 106.9 cM.

We also demonstrate that this region covers ~200 kb of genomic DNA and contains part of the coding sequence of two splice variants of a human septin gene. It is also now apparent that at least three of the four microsatellite markers used originally to define the minimal region of deletion (D17S937, D17S939, and afm203wc5) fall within the genomic sequence of the septin gene, thus confirming that LOH is actually observed at this locus. In keeping with observations from other septins, this protein has a P-loop nucleotide binding domain. A CLUSTAL alignment of seven human septins is shown in Fig. 3. It demonstrates the conserved central core of the septin proteins and highlights the variability at the NH2 and COOH termini. In addition, the Ov/Br septin α protein has an NH2 terminal extension of 136 amino acids relative to the other human septins. When the clustal alignment is extended with an additional 13 sequences from yeast, Drosophila, rat, and mouse (not shown), it is clear that there is no other septin currently in the databank with such a large NH2 terminus. Databank searches with only this region of Ov/Br septin α has yet to reveal notable or significant similarities with other proteins.

In dividing yeast cells, the septins localize to a ring of 10-nm filaments at the bud neck, where their role appears to be to localize...
membrane proteins such as Bud3p and Bud4p to the site at which budding will occur (15). Other evidence suggests that a major role of the septins is to act as a scaffold or template for recruitment of other proteins that must assemble at specific sites within the cell. In budding yeast, the septins are required for the mitosis-specific regulation of the Nim1-related kinase, Gin4, which functions in a pathway initiated by the Clb2 cyclin (16). A link between the organization of the peripheral cytoskeleton and cell cycle progression has been demonstrated recently, when it was shown that the function of the Nim1-related kinase, Hsl-1, was dependent on proper septin function and that Hsl-1 colocalized and coprecipitated with the septins in yeast (17). Hsl1 induces entry into mitosis by negatively regulating the Wee1-related kinase, Swe1.

The availability of a coiled-coil domain at the COOH terminus would suggest that individual septins can participate in protein-protein interactions and as such can organize into filamentous structures within cells. In Drosophila, a purified septin complex was shown to be composed of three previously identified septin polypeptides Pnut, Sep2, and Sep1. This complex was shown to be a heterotrimer of homodimers and copurified with 1 molecule of bound guanine nucleotide per septin polypeptide. The complex also bound and hydrolyzed exogenously added GTP. It therefore appears that GTP binding and hydrolysis regulate the interaction of septins with each other and/or with other proteins (18).

Recently, a novel mammalian septin, eseptin, has been described that is alternatively spliced and expressed in a variety of tissues (19). Eseptin is the rat orthologue of the septin that we describe in this report. The long and short splice variants of eseptin both had GTP binding sites and were distributed to the plasma membrane; however, the short form also had a more tubular and vesicular, perinuclear distribution. Mutants in the GTP binding site of the short form of the protein were confined principally to the perinuclear region, whereas similar mutants in the long form did not show an altered localization pattern. This may indicate that the two variants have different functions within the cell. A further difference between the two splice variants is the NH2 terminal extension of the long form. As with the septin that we describe, the longer protein includes the entire coding region of the short form, and it may be that the additional sequence at the NH2 terminus specifies different binding properties for this molecule.

The sequences of seven human septins have now been determined and a wide variety of functions attributed to these molecules. It could be hypothesized that the relative amounts of individual septins available to assemble into filaments would dramatically effect the overall substrate specificity of the complex and its cellular function. Studies with eseptin have shown that the relative amounts of splice variants varies between different tissues, and this may therefore represent a level of control of septin function (18). Further studies with antibodies generated to this new human septin on chromosome 17q25 are required to investigate the relative amounts and localization of the protein in normal and malignant ovarian epithelium.

During the preparation of this manuscript, a t(11;17) (q23;q25) was described in a patient with acute lymphocytic leukemia that involved the MLL gene on chromosome 11 (9). The fusion partner gene was cloned, and a cDNA of 2.8 kb was isolated that was found to be a new member of the septin family. The gene, named MSF, had a major transcript of 4 kb that was expressed ubiquitously and a 1.7-kb transcript that was also present in most tissues. A 3-kb transcript was detected in hematopoietic tissues. The 2.8-kb cDNA sequence of MSF is similar to the splice variant α that we have described but is 37 bp
shorter at the 5’ end, *i.e.*, at the start of exon 1 and has a 1639-bp deletion in the 3’ untranslated region. In addition, there has now been an additional report of one patient with de novo AML and one patient with therapy-related AML, both with a t(11;17)(q23;q25), in which the fusion partner of the MLL gene is the chromosome 17q25 septin (10). The 17q25 septin gene therefore becomes one of a number of partner genes for MLL to be cloned from leukemic cells that have reciprocal translocations involving 11q23. However, it is not yet known if it is the MLL gene, the partner gene, or the fusion product that is important in leukemogenesis.

In conclusion, we have identified a gene that is a member of a family with a major role in cytokinesis and control of the cell cycle. We have mapped this gene to a region that is commonly deleted in benign, borderline, and malignant sporadic ovarian tumors of all histological subtypes. In three of seven tumors with confined deletions on distal chromosome 17q25, we have identified a number of germ-line base changes, one of which results in a significant amino acid change of arginine to cysteine. Whether this or any of the other base changes have a major functional effect on the Ov/Br septin still has to be determined.

**Note Added in Proof**

Genomic and expression analyses of this 17q25 septin gene, MSF, and three alternatively spliced transcripts were recently published (Kalikin *et al.*, 2000). The MSF transcripts, including two novel transcripts (MSF-A and MSF-B), were found to share nine coding exons. The intron/exon boundaries of these transcripts were described and six polymorphic variants were identified. Note: MSF-A in that manuscript is called “Ov/Br septin α transcript” and MSF-C in that manuscript is called “OV/Br septin β transcript” in our manuscript. Kalikin, L. M., Sims, H. L., and Petty, E. M. Genomic and expression analysis of alternatively spliced transcripts of the MLL, septin-fusion gene (MSF) that maps to a 17q25 region of loss in breast and ovarian tumors. Genomics, 63: 165–172, 2000.

**References**


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