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Mutations in the BRCA1-associated RING domain (BARD1) gene in primary breast, ovarian and uterine cancers

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Germline alterations of *BRCA1* result in susceptibility to breast and ovarian cancer. The protein encoded by *BRCA1* interacts *in vivo* with the BRCA1-associated RING domain (BARD1) protein. Accordingly, BARD1 is likely to be a critical factor in BRCA1-mediated tumor suppression and may also serve as a target for tumorigenic lesions in some human cancers. We have now determined the genomic structure of *BARD1* and performed a mutational analysis of 58 ovarian tumors, 50 breast tumors and 60 uterine tumors. Seven polymorphisms were detected within the 2.34 kb coding sequence of *BARD1*. Somatically acquired missense mutations were observed in one breast carcinoma and one endometrial tumor; in at least one of these cases, tumor formation was accompanied by loss of the wild-type *BARD1* allele, following the paradigm for known tumor suppressor genes. In addition, a germline alteration of BARD1 was identified in a clear cell ovarian tumor (Gln564His); again, loss of the wild-type *BARD1* allele was observed in the malignant cells of this patient. The Gln564His patient was also diagnosed with two other primary cancers: a synchronous lobular breast carcinoma and a stage IA clear cell endometrioid cancer confined to an endometrial polyp 6 years earlier. These findings suggest an occasional role for BARD1 mutations in the development of sporadic and hereditary tumors.

INTRODUCTION

Alterations in the *BRCA1* gene (1,2) account for 40–50% of early onset familial breast cancer cases and >75% of cases of familial breast/ovarian cancer (3,4). In families with germline *BRCA1* alterations, tumor formation requires loss or alteration of the wild-type allele (5), in keeping with the hypothesis that BRCA1 has tumor suppressor function.

BRCA1 encodes a large polypeptide of 1863 amino acids that contains an amino-terminal RING domain and two carboxy-terminal BRCT domains (2,6). Although the functions of these amino acid motifs are not precisely determined, the RING and BRCT domains of BRCA1 are well conserved phylogenetically and serve as common sites for missense mutations that predispose women to early-onset breast cancer. Therefore, these domains appear to be critical for tumor suppression by BRCA1.

We recently described an interaction between BRCA1 and a protein termed BARD1 (the BRCA1-associated RING-domain protein) (7). BARD1 is structurally related to BRCA1 in that it harbors an amino-terminal RING domain and two carboxy-terminal BRCT motifs. Studies of their subcellular distributions have shown that both proteins co-localize to discrete nuclear structures at defined stages of cell cycle progression (8,9). The *in vivo* association between BRCA1 and BARD1 is mediated by sequences within their respective RING domains. The fact that disease-related missense mutations within the RING motif of BRCA1 ablate the BRCA1–BARD1 interaction suggests that this protein complex mediates the tumor suppression function ascribed to BRCA1 (7).

If the BRCA1–BARD1 complex is indeed essential for tumor suppressor activity, then it is reasonable to ask if alterations of BARD1 are also involved in oncogenesis. Therefore, we determined the genomic structure of *BARD1* and confirmed its localization by fluorescence *in situ* hybridization (FISH) to 2q34–q35, a region that is not frequently altered in breast or ovarian tumors. We also screened a panel of human tumors for genetic alterations of *BARD1*. Of these, 1/58 ovarian tumors, 1/50 breast tumors and 1/60 uterine tumors harbored different missense alterations, each of which lies within or close to the BRCT domains of BARD1. The wild-type allele of *BARD1* was clearly absent in

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both the ovarian and uterine tumors, implying that loss of BARD1 function may contribute to oncogenesis. Although the BARD1 mutations in the breast and uterine tumors (Val695Leu and Ser761Asn) were acquired somatically, the ovarian tumor mutation (Gln564His) was of germline derivation. This patient had also developed independent primary cancers of the breast and endometrium. Thus, although further studies are needed, these data imply that loss of BARD1 function may be a contributing factor in the formation of some sporadic and hereditary tumors.

RESULTS

Genomic structure of BARD1

To obtain the genomic DNA encoding BARD1, bacteriophage λ and BAC (bacterial artificial chromosome) libraries of human genomic DNA were screened by hybridization with fragments of *BARD1* cDNA (7). Eleven hybridizing λ clones and two hybridizing BAC clones were subjected to nucleotide sequence analysis with oligonucleotide primers derived from the *BARD1* cDNA sequence. This analysis revealed that the BARD1-coding sequences are derived from 11 exons distributed over at least 65 kb of genomic DNA [GenBank accession nos AF038034 (exon I), AF038035 (exons II and III), AF038036 (exon IV), AF038037 (exon V), AF038038 (exon VI), AF038039 (exon VII), AF038040 (exon VIII), AF038041 (exon IX) and AF038042 (exon X)]. The chromosomal origin of *BARD1* was then established by FISH of normal human metaphase chromosomes with plasmid subclones containing *BARD1* genomic sequences. FISH analysis localized *BARD1* to bands 2q34–35, consistent with the *BARD1* mapping data obtained previously with the Genebridge panel of whole genome radiation hybrid DNAs (7).

BARD1 mutation screening

The primer sets that were used to amplify the 11 coding exons of *BARD1* from cDNA or genomic DNA templates are presented in Table 1.

Table 1. PCR primers for the amplification of BARD1 sequences from cDNA or genomic DNA template

Exon	Forward primer (5′→3′)	Reverse primer (5′→3′)	PCR product size (bp)	Template	Annealing temp (°C)
I	R135S: CCGAGGAGCCTTTCATCCGA	R135AS: CGAGCGCGGCGCGACTGT	154	cDNA/genomic	59
I–III	B202-Z1S: ATGGAACCGGATGGTCGCGGT	B202-ZAS: TCTTCAAGTCTTGTATCCAGGC	213	cDNA	59
III–IV	B202-A: GCCTGGATACAAGACTTGAAG	B202-N:AACATCTGCAGGAGGACTTGG	306	cDNA	57
IV	B202-B: AAAGCTTCAGTGCAAACCCA	B202-BAS: TCCAGATCTTGCAGAAGCC	132	cDNA	53
IV	B202-X: CAGATGTTTCTGAGAGGGCT	B202-XAS: ATTCCTCTTTGGAGTCAAATTC	138	cDNA/genomic	55
IV	B230-A: GAGGCAGAAAAAGAAGATGGT	B230-AS: AGGAGCCACTTGCTAGTAAG	136	cDNA/genomic	55
IV	B202-Y: ATGGTGAAATAGACTTACTAGC	B202-YAS: GCAGACCTTCTCAGGAGTC	149	cDNA/genomic	55
IV	B230-B: AAGAGCAGGAATGAAGTAGTG	B230-BAS: CTCCACTGGTGCTCAGAATG	163	cDNA/genomic	55
IV	B230-C: AGTGGAGATTTTGTTAAGCAA	B230-CAS: AGGTGGTGTACCTGGTGAA	159	cDNA/genomic	51
IV	B230-D: GGTACACCACCTTCTACATT	B230-DAS: GTCTCTCCTCTATGATTTCTT	113	cDNA	53
IV–V	B230-PS: CAATGAAGCTGTTGCCCAA	B230-P: GTCTTTAACATTTGGATCACT	137	cDNA	51
V–VI	B230-E: AGTGATCCAAATGTTAAAGAC	B230-EAS: CCCATTCTTGGCTGCATC	162	cDNA	51
VI–VII	B230-F: CAAAATGACTCACCACTTCAC	B230-FAS: ATCGACAGGCCGCAGACC	120	cDNA	55
VII–IX	B230-FF: CCTGTCGATTATACAGATGAT	B230-FFAS: AACATGAGTTACTGTACTGTC	234	cDNA	57
VIII–X	B230-WS: TATACTGAGTTTGACAGTACAG	B230-WAS: CATACTTTTCTTCGTAGACATG	146	cDNA	55
X–XI	B230-G: TGGGTAAAAGCATGTCTACGA	B230-R: GTAGCATCCATCAAACAGCTT	126	cDNA	55
XI	B230-H: GGATGCTACTTCTATTTGTG	B230-HAS: GAGTCACGTCACTGTCTG	124	cDNA	51
XI	B230-TS: CCTCAGTAGAAAGCCCAAGC	B230-TAS: GCCCTGCCGAACCCTCTC	154	cDNA	57
XI	B230-US: GAGAGGGTTCGGCAGGGC	B230-UAS: TTCAATTTGAAATGTTCATCTGGT	124	cDNA/genomic	57
I	R1352S: ACAGTCGCGCCGCGCTCGA	R13AAS: CAGAAACTGTGCGACCCGTG	107	genomic	59
II	R12AS: AGATGTTTATCTAACAATGACTC	R12BAS: AGTTGTACTATATACATCAAACC	146	genomic	55
III	R13BS: ATTCTGCTGAATGGGTTGCTT	R13CAS: TAACTAAGAGAGATAGGGATAG	226	genomic	55
IV	R5C5: GGAGCTCCATGTGGGAGCAA	B202-N: AACATCTGCAGGAGGACTTGG	270	genomic	59
IV	B230-D: GGTACACCACCTTCTACATT	R5DAS: TCTGAGATGGTATTTCAGAGT	170	genomic	53
V	R34DS: TGCTTTTTAATTTCCATTTTGTTC	R34FAS: AAGAACTGTAAAACACAGAAAGA	163	genomic	55
VI	R34FS: TGCTCTTTCTTATCACTTCTTTC	R34GAS: CTTGACTCAAGAATATAGGTCC	278	genomic	57
VII	FFGS2: TTGAGTCGAGTCACACATTTGA	B2305FGAS: CTATTATGTTCCTTTCATAACCA	233	genomic	55
VIII	3FGR: TAATGTCTTTGTCTAGTCGTCTAA	WSGAS: GGTAGTTCTCCAAAAGGATCA	264	genomic	55
VIII	3FGR: TAATGTCTTTGTCTAGTCGTCTAA	3F: TTCTGAAGACAGCCCACTGC	123	genomic	55
IX	B230IXS: GAGTTATAAGAAGCAGGCCAA	B230IXAS: ATTTCTTAATTCTCTCAAATCCAA	199	genomic	55
X	R36H5: TAGTGCTCACTTGATACTTAGT	R36EAS: CATAATAAGAACAATGAAAGTTGT	187	genomic	55
XI	R36E5: TTGATCTGCCTTTAACAAATG	B230-TAS: GCCCTGCCGAACCCTCTC	296	genomic	57

Table 2. *BARD1* polymorphisms

Altered *BARD1* nucleotide	Amino acid change	Population	Allele	Frequency	No. chromosomes screened
C143T	Pro24Ser	Caucasian	Pro	0.59	22
			Ser	0.41	
		African	Pro	0.82	22
			Ser	0.18	
A530G	Lys153Glu		Lys	n.d.	n.d.
			Glu	n.d.	
C1121G	Silent (Thr351)		C	n.d.	n.d.
			G	n.d.	
1140–1160 (Deletion of CCATTGCCTG-AATGTTCTTCA)	1139del21 (del aa 358–364: PLPECSS)	Caucasian	WT	0.99	216
			1139del21	0.01	
		African	WT	0.88	32
			1139del21	0.12	
A1592G	Val507Met		Met	n.d.	n.d.
			Val	n.d.	
G1765C	Cys557Ser	Caucasian	Cys	0.98	80
			Ser	0.02	
C2045T	Arg658Cys	Caucasian	Arg	0.98	138
			Cys	0.02	
		African	Arg	1.00	42
			Cys	0	

The *BARD1* nucleotide positions are based on the GenBank accession no. U76638.
n.d. not determined.

Table 3. BARD1 alterations in breast/ovarian/uterine tumors

Sample (age)	Tumor type	Codon change	Nucleotide change	Effect	Loss of wild-type allele	Germline or somatic
ov61 (73 years)	Ovarian clear cell adenocarcinoma (Stage 3A)	CAG→CAC	G1765C	Gln564His	Yes	Germline
ut8 (50 years)	Malignant mixed mullerian tumor (Stage 4B)	AGC→AAC	G2354A	Ser761Asn	Yes	Somatic
BC64	Intraductal adenocarcinoma of the breast	GTC→CTC	G2156C	Val695Leu	None detectable	Somatic

The mutation positions are based on the GenBank entry of BARD1 (accession no. U76638).

We used single-stranded conformational polymorphism (SSCP) analysis (10) to screen genomic DNAs or cDNAs from 50 breast tumors, 58 ovarian tumors, 60 uterine cancers (primarily endometrial), six breast cancer lines and six ovarian cancer lines for genetic alterations in *BARD1*. Variant bands were excised from the SSCP gel, subjected to a second round of amplification and DNA sequenced.

BARD1 polymorphisms

The BARD1 polypeptide is comprised of 777 amino acids and includes an amino-terminal RING domain (residues 46–90), three tandem ankyrin repeats (residues 427–525) and two carboxy-terminal BRCT motifs (residues 605–777) (see Fig. 1). Seven polymorphisms were detected within the 2.34 kb of BARD1-coding sequence (Table 2 and Fig. 1). One of these is an in-frame deletion of seven amino acids between its RING domain and the ankyrin repeats (residues 358–364). This deletion was seen in 1% of Caucasian and 7% of unrelated African chromosomes. Given the low frequency of this allele in Caucasians, it was surprising to find that both the MCF7 breast cancer cell line and the PEO4 ovarian cancer cell line harbored this deletion since they were both derived from Caucasian patients (11,12). However, gene deletions do not necessarily account for disease or cancer susceptibility. For example, a polymorphic stop codon within the 3′ end of the coding sequence of *BRCA2* results in loss

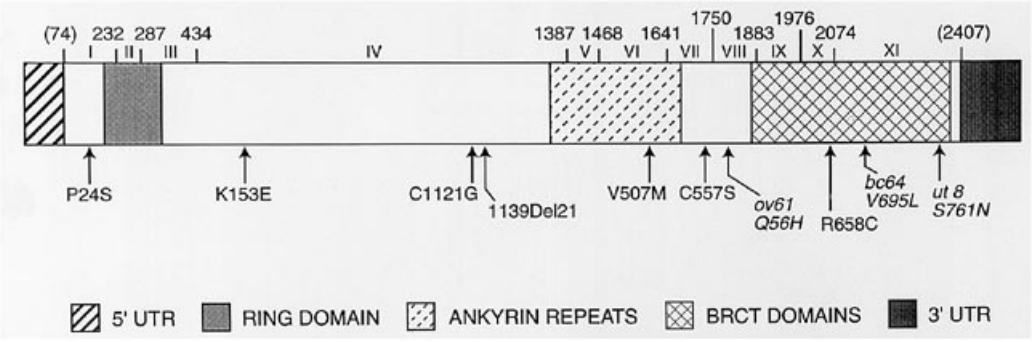

Figure 1. Schematic diagram of the *BARD1* cDNA. The RING domain, ankyrin repeats, BRCT sequences and 5′ and 3′ untranslated regions are shaded as indicated. The locations of splice sites according to the nucleotide sequence of the gene (GenBank accession no. U76638) are indicated above the diagram. Mutations (italics) and polymorphisms (plain text) are indicated below the cDNA diagram. Designations of amino acid changes are according to the nomenclature proposed by Beaudet and Tsui (1993) (35).

of the 93 most terminal amino acids (Lys3326ter) with as yet no described deleterious effect (13).

A germline BARD1 alteration

When 58 ovarian tumors were analyzed with SSCP, one (ov61) was found to harbor a missense mutation within *BARD1* that resulted in a glutamine to histidine (CAG→CAC; Gln564His) change. This alteration falls between the ankyrin repeats and the BRCT domains of BARD1 (Figs 1 and 2, Table 3). The patient with this tumor was a woman of African–American origin who was diagnosed at age 73 with a clear cell adenocarcinoma of the ovary (stage 3A) and a synchronous infiltrating lobular carcinoma of the breast. SSCP analysis of RT-PCR products indicated that only the mutant transcript was present in the ovarian tumor mRNA from this individual, indicating that the wild-type transcript was either expressed at undetectable levels or was completely absent (Fig. 2).

To examine the BARD1 status in ov61 further, RT-PCR products from the tumor were cloned and sequenced and the fraction of clones with wild-type versus mutant sequence was determined. When 10 subclones were sequenced, all were found to harbor the mutant allele, indicating that the mRNA derived from the wild-type allele was truly absent in the ovarian tumor. When corresponding germline DNA from benign uterine tissue was cloned and sequenced, 4/9 clones harbored the mutant allele and 5/9 clones harbored the wild-type allele, indicating that the alteration in the tumor was of germline origin.

At the time of hysterectomy 6 years earlier, this patient had been diagnosed with an incidental stage IA endometrial clear cell tumor. It is likely that the tumors of the endometrium and ovary were independent primaries since the initial endometrial tumor was a small focus of carcinoma confined to an endometrial polyp.

We used SSCP analysis to examine genomic DNA extracted from paraffin-embedded tissue obtained from the three primary tumors from this patient as well as from benign uterine tissue (Fig. 3). This revealed the variant allele in all samples, including normal uterine tissue, again confirming that this alteration was of germline origin. Moreover, the wild-type allele of *BARD1* was reduced in the genomic DNA of the ovarian tumor, explaining the loss of wild-type *BARD1* transcripts. We detected both the wild-type and mutant alleles in genomic DNA of both the endometrial and breast cancers; however, a large proportion of the paraffin sections was normal tissue, so that it was not possible to determine if the wild-type allele had been lost in these tumors. Further histological examination indicated that a significant proportion of normal tissue had infiltrated these tumor specimens rendering microdissection of tumor tissue from these samples impossible.

The Gln564His missense alteration was not seen in >300 individuals examined (>600 chromosomes), suggesting that this alteration is not a polymorphism. Since the patient was African–American we also screened an additional 30 African individuals (60 chromosomes) for this variant and did not see it, indicating that this change is unlikely to be a polymorphism exclusive to the African population. In light of the known interaction of BARD1 with BRCA1 and the observed loss of the wild-type *BARD1* allele in the ovarian tumor, it is tempting to speculate that the germline Gln564His alteration resulted in predisposition to endometrial, breast and ovarian cancer in this patient.

Somatic BARD1 alterations

The diagnosis of endometrial cancer in the patient with the Gln564His mutation prompted us to examine an additional 60 uterine cancers for genetic alterations in BARD1. One additional endometrial tumor (ut8) was identified with a novel BARD1 alteration in a patient of European origin who had been diagnosed at age 50 with a stage 4B malignant mixed mullerian tumor (carcinosarcoma) (Table 3). This alteration, a Ser761Asn change in the second BRCT domain, was not seen in >600 other Caucasian chromosomes by allele-specific oligonucleotide (ASO)/dot-blot analysis. Direct sequencing of genomic DNA derived from this tumor only revealed the mutant allele, indicating that the wild-type allele had again been lost. Microdissection of normal DNA from archival sections of this tumor followed by direct sequencing revealed only a wild-type allele indicating that the alteration was somatic in origin.

Finally, a breast tumor (BC64) obtained from a 60-year-old woman, and diagnosed as an invasive ductal carcinoma, was found to harbor a BARD1 alteration (Val695Leu) in one of the BRCT domains. The Val695Leu substitution was not seen in the germline DNA of the patient (Table 3) or in >600 chromosomes, indicating that it arose somatically upon malignant transformation. It was not

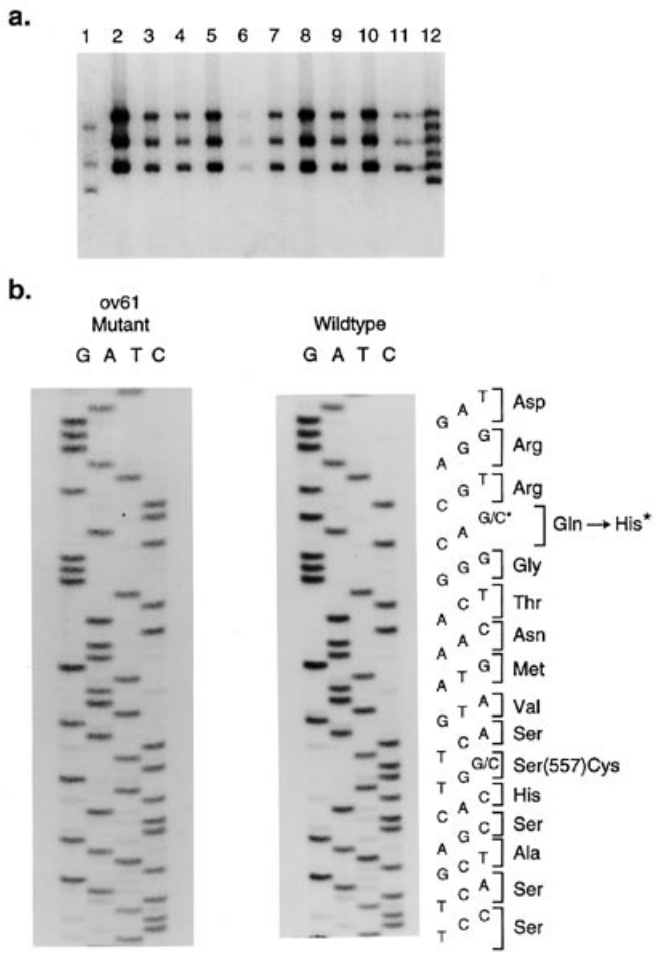

Figure 2. (**a**) SSCP analysis of cDNA from 12 ovarian tumors after amplification of template cDNA with primers B230-FF and B230-FFAS. The tumor in lane 1 was derived from patient ov61. In this sample, no wild-type allele can be seen. The tumor in lane 12 is heterozygous for the Ser(557)Cys polymorphism. Tumors in lanes 2–11 did not exhibit any alteration in this region. The lower two bands represent non-denatured strands. (**b**) Sequence analysis of PCR products of SSCP fragments from a1 (patient ov61) (left sequence) and corresponding normal cDNA sequence obtained from variant SSCP fragments in lane a12 (right sequence). Primer B230-FF was used for sequencing. The amino acid sequence is indicated to the right of the DNA sequence. A one-base substitution (C→G) (Gln564His) was seen in tumor DNA from patient ov61 (the altered base and amino acid are both indicated with an asterisk. The location of the polymorphic change from Ser to Cys (G→C) is indicated.

possible to determine whether the wild-type allele had been lost in the malignant cells of this patient since genomic DNA from microdissected tumor cells was not available.

DISCUSSION

The genomic structure of BARD1 and amplimers described here will enable mutational analysis of this gene in a variety of human tumors and cancer patients. FISH analysis confirms the localization of *BARD1* to chromosome 2q, and sub-localizes it to within 2q34–35, a region not shown to be altered frequently in neoplasia.

Screening a panel of primary breast (n = 50), ovarian (n = 58) and uterine (n = 60) tumors revealed three distinct missense alterations in the BARD1-coding sequence. One of these was a clear cell ovarian tumor that harbored a Gln564His mutation of germline

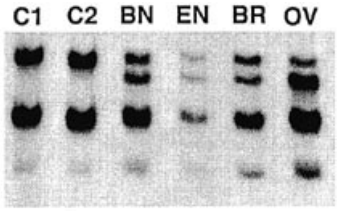

Figure 3. SSCP analysis of genomic DNA obtained from paraffin sections of tumors from patient ov61. PCR products for SSCP were obtained with primers 3F/3FGR (Table 1) that flanked the Gln564His change in exon VIII that is seen in this individual. C1, C2: control samples; BN: benign uterus; EN: endometrial tumor; BR: breast tumor; OV: ovarian tumor. A novel SSCP band is seen in all samples from the patient that is not seen in the control samples. The upper wild-type allele is decreased in intensity in the ovarian tumor. This decrease is not seen in the DNA from the endometrial and breast tumor sections that have a substantial amount of contamination of normal tissue.

origin. Notably, the wild-type allele of *BARD1* had been lost from the malignant ovarian cells, but not normal cells, of this patient. The patient was 73 years of age and had developed three independent primary tumors: the aforementioned clear cell ovarian carcinoma, a synchronous lobular breast carcinoma and, 6 years earlier, a clear cell endometrial cancer. Unfortunately, it was not possible to obtain a family history on this patient to determine if other carriers of Gln564His had also developed cancer. Insufficient material was available to rule out conclusively the presence of a germline *BRCA1* alteration in this patient. The Gln564Arg alteration is of unknown significance, and it is conceivable that the loss of the wild-type allele in the ovarian tumor was driven by a mutation in a neighboring gene. However, it is of interest that this residue, along with flanking amino acids, is conserved in the mouse ortholog of BARD1 (NTGQR in both species; unpublished data).

The diagnosis of endometrial cancer in this patient 6 years earlier was intriguing and prompted us to investigate the frequency of BARD1 alterations in an additional 60 uterine tumors. This resulted in the identification of a somatic BARD1 alteration (Ser761Asn) in a malignant mixed mullerian tumor (Ut8). Again, the wild-type allele of *BARD1* was clearly missing from this tumor. Moreover, the altered residue (Ser761) lies within the 30 amino acid core of the second BRCT domain, adjacent to an invariant tryptophan that is characteristic of the BRCT motif (6,14,15). Finally, a somatic alteration (Val695Leu) was detected in a breast tumor. This change also lies within the region containing the BARD1 BRCT domains.

The three alterations that we describe lie within, or close to, the two tandem BRCT domains of BARD1. While the functions of the BRCT motif are not known, the corresponding region of BRCA1 has been shown to have transactivational potential (16,17). BRCT domains are often found in proteins that function in the cell cycle checkpoints induced by DNA damage, including RAD9, XRCC1 and RAD4 (6,14,15). This, together with the findings that BRCA1 associates with RAD51 (18) and that BRCA1, BARD1 and RAD51 co-localize to nuclear dots in S phase but not in G1 (8,9), suggest that these proteins may be involved in cell cycle checkpoint control in response to DNA damage.

BARD1 is altered in only a small percentage of sporadic breast and ovarian tumors and, in this respect, is similar to BRCA1 and BRCA2. In the case of BRCA1, no genetic alterations have been detected in sporadic breast tumors; however, 10% of ovarian tumors

harbor somatic *BRCA1* mutations that result in protein truncations. In these tumors there is also loss of the wild type allele (19,20). In the case of BRCA2, four independent studies collectively identified two sporadic missense alterations and one truncating mutation in 281 primary breast cancers and two somatic alterations in 185 ovarian carcinomas (21–26).

The role of BARD1 in the development of uterine cancer is intriguing. BRCA1 alterations have not been described in this type of tumor, although some BRCA1 linked families occasionally have members with a linked *BRCA1* mutant haplotype and endometrial cancer [e.g. family 4, (27)]. An analysis of patient ov61 suggests that germline mutations of *BARD1* may confer a predisposition to certain neoplastic conditions and that studies of *BARD1* mutations in familial cases of breast, ovarian, or uterine carcinoma may be warranted, particularly in those patients that develop synchronous or metachronous tumors of endometrial, breast or ovarian tissues.

MATERIALS AND METHODS

Clinical specimens

Tumor tissue, matched normal tissue and blood specimens were obtained as part of protocols approved by the University of Texas Southwestern Medical Center Human Subjects Review Board, St Paul's Medical Center of Dallas, Medical City of Dallas and The Southern division of the Co-operative Human Tissue network. The breast cancers were primarily infiltrating ductal carcinomas. The ovarian carcinomas were of mixed histology although the majority were papillary serous carcinomas. Of the 60 uterine cancers, 44 were endometrial, 11 were mixed mullerian tumors and five were sarcomas. The following breast and ovarian cancer cell lines were obtained from the American Type Culture Collection: MCF-7, ZR75-1, BT-483, BT-20, T-47D, BT-474, OVCAR3, CAOV-3. BG-1 was kindly provided by Dr E.J. Modest, Boston University, and 2008 and 2774 have been described elsewhere (28,29). The ovarian cancer line PE04 was obtained from Dr Simon Langdon (Medical Oncology Unit, Western General Hospital, Edinburgh, Scotland). Tumors were frozen immediately in liquid nitrogen and stored at –70°C prior to RNA extraction. Buffy coat was prepared from blood. In some cases, DNA was prepared from paraffin-embedded tissue. RNA and cDNA were prepared by standard procedures.

Genomic structure of *BARD1*

A human genomic library cloned into bacteriophage λ was first screened by hybridization with fragments of *BARD1* cDNA (7). Eleven hybridizing λ clones were identified and subjected to nucleotide sequence analysis with oligonucleotide primers derived from the *BARD1* cDNA sequence.

This identified exon–intron boundaries for all exons except for VII, VIII and IX. To obtain genomic sequence of this region, three BACs were identified by hybridization with B230EX-1.3, a probe comprised of *BARD1* cDNA residues 1229–2530 (accession no. U76638) (Research Genetics). DNA was prepared by standard methods. The same primers were used to sequence two of these BAC DNAs generating exon–intron boundary sequences for this region.

Mutational screening for *BARD1* alterations

SSCP was performed as described elsewhere (10) with oligonucleotide primers for *BARD1* with cDNA or genomic DNA as shown in Table 1. cDNA was derived from tumor, matched normal tissue or cell lines. Genomic DNA was obtained from tumor tissue, matched normal tissue, cell lines, blood and paraffin-embedded tissue.

SSCP analysis

PCR of tumor or blood DNA/cDNA was performed in 20 µl volumes containing 100 ng of cDNA or genomic DNA template; 1× PCR buffer (Perkin Elmer); 200 µM each dATP, dGTP, dCTP and dTTP; 10 pmol each primer (Gibco BRL); 0.3 µCi of [^{32}P]dCTP (Amersham); and 0.5 U of *Taq* DNA polymerase (Perkin Elmer). PCR conditions were 30 cycles of 94°C, 30s; 55°C (or as specified for annealing temperatures in Table 1), 30s; 72°C, 30s. A final extension reaction at 72°C was performed for 1 min. Amplified samples were diluted 1:10 in formamide buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 5 min then cooled rapidly to 4°C. For each sample, 4 µl was loaded onto an SSCP gel and run at 8 W (constant power) for 8–16 h in 0.6× TBE at room temperature. Gels contained 0.5× MDE (AT Biochem), 0.6× TBE, 240 µl of 10% ammonium persulfate and 24 µl of TEMED. Duplicate gels were prepared with a supplement of 10% glycerol. Gels were subjected to autoradiography with or without being dried. Film was exposed for 12–24 h with an intensifying screen.

DNA sequencing of SSCP variants

Variant bands were excised from the gel after alignment with the autoradiograph and purified with Qiaquick Gel Extraction kit (Cat #28706). DNA was resuspended in 20 µl of H_2O and 5 µl was treated with 10 U of exonuclease I (manufacturer) and 2 U of shrimp alkaline phosphatase (manufacturer) at 37°C for 15 min. Following inactivation of this reaction with heat (80°C for 15 min), the DNA template was subjected to cycle sequencing with Thermosequenase (Amersham Life Science) and [α-^{33}P]ddNTPs. Sequencing reactions were electrophoresed in 8% acrylamide/bis gels with 1× glycerol tolerant gel buffer at 70 W constant power for 2 h. Gels were dried and subjected to autoradiography.

Cloning of *BARD1* PCR products for sequence analysis

Products obtained following PCR amplification were cloned directly into the TA-cloning vector pCR 2.1 (Invitrogen Cat #K2000-01). Cloning and transformation were performed according to the manufacturer's instructions. Colonies with inserts of the correct size were identified by direct amplification of the colony DNA in a PCR reaction with vector-specific primers (M13 forward/M13 reverse). In general, 70% of the colonies had inserts of the correct size; 30% of the colonies had no detectable insert. DNA was prepared from colonies with correct insert sizes according to standard procedures (30) and subjected to DNA sequencing with the M13 forward oligonucleotide primer and ABI DNA sequencing kit (Cat #402112). Sequencing reactions were analyzed with an ABI377.

PCR/ASO analysis of mutant alleles

PCR products were applied to replica Hybond filters as described elsewhere (31), except that instead of UV cross-linking, filters were baked for 2 h at 80°C.

Prehybridization and hybridization was performed as described elsewhere (32). The filters were subjected to a final wash in the Me4NCL solution for 2×20 min at 60°C.

To discriminate between the Ser761 and Asn761 alleles, PCR products were obtained with the US and UAS primers, and hybridized with the following ASOs: Asn761, GCTCCTTCG-AACTGGTTTATA; Ser761, GCTCCTTCGAGCTGGTTTATA.

To obtain allele frequencies for the Arg658Cys polymorphism, PCR products obtained with R36H5/R36EAS primers were hybridized with: Cys658, TGAAGGTCCATGCAGAAGCAG; and Arg658, TGAAGGTCCACGCAGAAGCAG.

FISH mapping of *BARD1*

The cytogenetic location of *BARD1* was obtained through FISH of normal human metaphase chromosome spreads with pooled DNA from three plasmid subclones in puc12 of λ clones with inserts of 14, 16 and 13 kbp, representing 5′, middle and 3′ parts of the gene (R13S-14, R16S-16 and R36S-13) and containing a total of 43 kb of human sequence. Plasmid DNA was labeled with biotin by nick translation and subjected to FISH analysis as described elsewhere (33,34).

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