Identification of Novel Alternatively Spliced *BRCA1-Associated RING Domain (BARD1)* Messenger RNAs in Human Peripheral Blood Lymphocytes and in Sporadic Breast Cancer Tissues

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BARD1 (BRCA1-associated RING domain) is the dominant binding partner of BRCA1 in vivo. The *BARD1* gene has been reported to be mutated in a subset of breast and ovarian cancer patients and *BARD1* germ-line mutations have been identified in breast cancer patients negative for *BRCA1* or *BRCA2* gene alterations. In the present study, we show by RT-PCR and direct sequencing analysis the occurrence of seven novel and one previously identified *BARD1* splicing variants in human lymphocytes and breast cancers. Two of the eight variants (*BARD1*δ and *BARD1* ΔRIN) preserve a correct open reading frame and could encode BARD1 internally deleted proteins, while the remaining six variants display premature stop codons. Characterization of the relative expression of *BARD1* FL, *BARD1*δ, and *BARD1* ΔRIN using quantitative PCR analysis indicated that the mean expression levels of *BARD1* FL, *BARD1*δ, and *BARD1* ΔRIN were significantly higher in tumors than in morphologically normal tissues and lymphocytes. However, we were unable to identify either qualitatively or quantitatively tumor-specific expression patterns of the identified *BARD1* splicing variants. © 2007 Wiley-Liss, Inc.

INTRODUCTION

BARD1 has been shown to be the dominant binding partner of BRCA1 in vivo (Baer and Ludwig, 2002). A considerable body of evidence points to BARD1 as a key regulator of BRCA1 stability, localization, and function and indicates BARD1-BRCA1 heterodimer to be a physiologically relevant form of BRCA1, mainly involved in DNA repair (Irminger-Finger and Jefford, 2006). The *BARD1* gene has been reported to be mutated in subsets of both familial and sporadic breast and ovarian cancer (Thai et al., 1998; Ghimenti et al., 2002; Sauer and Andrulis, 2005).

Recent genome-wide analysis of alternative splicing indicates that up to 70% of human genes may have alternative splice forms, and spliceosomal errors have been shown to play a functionally significant role in tumorigenesis (Brinkman, 2004; Venables, 2004; Kalnina et al., 2005; Okumura et al., 2005). Occurrence of *BARD1* splicing variants (SVs) has been described in rat spermatocyte precursors (Feki et al., 2004) and rat NuTu-19 ovarian cancer cells (Feki et al., 2005). In human breast cancer cell lines, a deletion from exon 2 to exon 6 has been identified (Tsuzuki et al., 2006).

In the present study, we have characterized the pattern of *BARD1* SVs in vivo, using human lymphocytes as a reference tissue, and compared their relative expression in sporadic breast tumors versus morphologically normal adjacent tissues. Seven novel SVs were identified, together with one transcript (*BARD1* ΔRIN) that had already been described (Tsuzuki et al., 2006).

MATERIALS AND METHODS

Tissues and Cells

A total of 52 samples from 37 sporadic breast cancer patients who underwent surgery at the Pisa University Hospital between 1991 and 1993 were

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included in the study. Fifteen pairs of breast tumor/morphologically normal tissue samples were examined, together with 22 tumor tissue samples for which no peritumoral tissue was available. Samples with more than 50% of tumor cells were selected for analysis. Peripheral blood lymphocytes (PBLs) from five healthy donors were used as a reference tissue. Laser capture microdissection (LCM) was performed in 13 of 37 cases, as previously described (Lombardi et al., 2007).

RNA Extraction and cDNA Synthesis

Total RNA extraction and cDNA synthesis from PBLs and breast tissues were carried out as previously described (Lombardi et al., 2007).

PCR and Sequence Analysis

BARD1 cDNA (GenBank accession no.: NM_000465) was amplified by PCR, using ABI 9700 thermocycler (Applied Biosystems, Foster City, CA). Primer sequences and PCR conditions are available upon request.

DNA sequencing was carried out directly on gel-purified PCR products (Wizard SV Gel and PCR Clean-Up System Kit, Promega, Madison, WI) according to the manufacturer's instructions (BigDye terminator v 3.1 mix, Applied Biosystems).

SYBR Green Quantitative PCR

Relative quantification of *BARD1* expression was performed by *SYBR* Green quantitative PCR. Primer sequences are available upon request. Reactions were carried out in triplicates using 2× SYBR Green PCR Master Mix (Applied Biosystems) in 7900HT Fast Real-Time PCR Systems apparatus (Applied Biosystems). The comparative ΔCt method was used for quantification of expression (Livak and Schmittgen, 2001). Constitutively expressed TBP (Tata binding protein) was chosen for normalization as it gave amplification efficiencies similar to *BARD1* at all dilutions chosen for the standardization experiment. Data fit was calculated by linear regression analysis (data not shown).

RESULTS

Identification of BARD1 SVs in Human Lymphocytes

Numerous cDNAs products, ranging from 2.2 to 0.4 kb, were amplified using primers placed in exon 1 (1F3) and exon 11 (11R3) of *BARD1* (Fig. 1). Their number and relative amount varied among individuals. Nine cDNAs were successfully sequenced, including the 2.2-kb *BARD1* FL band and eight *BARD1* SVs. Two of the eight SVs, *BARD1*δ and *BARD1* ΔRIN, preserved the correct open reading frame while the remaining six featured premature stop codons (Table 1). To rule out the possibility of PCR artifacts, we performed "splice-specific PCR" (Okumura et al., 2005) by using combinations of a forward primer located across the new exon–exon junction created by the supposed splicing event and a reverse primer in an exon downstream the new junction. The analysis confirmed the presence of the alternatively spliced fragments (data not shown).

Identification of BARD1 SVs in Sporadic Breast Tumors

To investigate the role of *BARD1* alternative splicing in breast carcinogenesis, presence of *BARD1* FL transcript was assessed in tumoral and peritumoral tissues by RT-PCR using primers at exon 1 (1F3) and exon 11 (11R3) (Fig. 1b). All samples displayed several SVs (Fig. 1a). To exclude contamination from non-neoplastic cells, 13 randomly selected tumors were purified by LCM and the RT-PCR experiment repeated. No alternative translation start or stop codons were observed by using primers targeting *BARD1* 5′-UTR (1F2) and 3′-UTR (11R2) (Fig. 1b). However, we cannot fully exclude their existence upstream of nucleotide (NT) 45 or downstream of NT 2,438 (reference sequence NM_000465). All SVs identified in PBLs were also detected by splice-specific PCR, in breast tumoral and peritumoral tissues, with differences in number and relative amounts among individuals as well as between tumors and morphologically normal adjacent tissues (Table 1).

Quantitative Analysis of the SVs

To address the hypothesis that differential expression of *BARD1* FL and SVs would selectively occur in tumors, we performed relative quantification analysis. *BARD1*δ and *BARD1* ΔRIN SVs were chosen for their potential capability to encode BARD1 isoforms retaining functional BRCT domains. The analysis was performed in eight tumors and nine controls (four morphologically normal tissues and five healthy donor lymphocytes), using *SYBR* Green quantitative PCR. As a general trend, *BARD1* FL, *BARD1*δ, and *BARD1* ΔRIN SVs appeared to be overexpressed from 3- to 6-folds in tumors ($P = 0.006$, 0.018, and 0.090, respectively) (Fig. 2). No significant differences
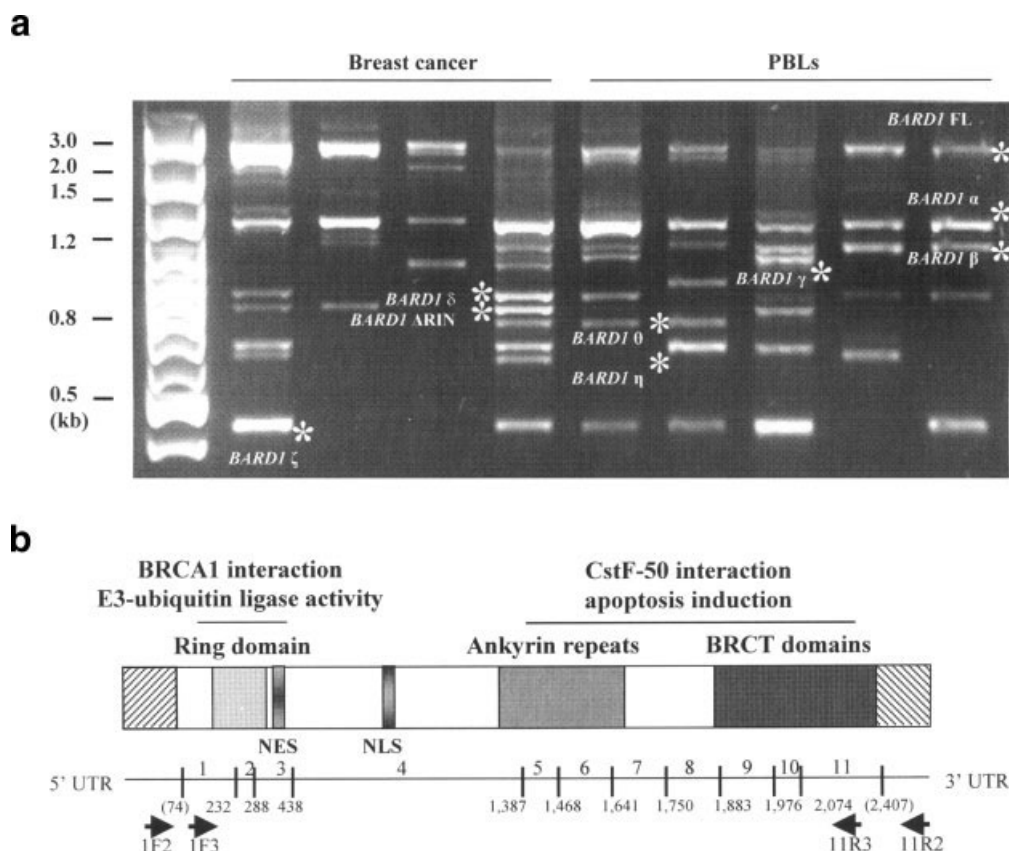
Figure 1. (a) Patterns of *BARD1* splicing variants (SVs) in sporadic breast cancer and PBLs. Molecular marker shown on the left. SV cDNA bands are marked by asterisks. (b) Schematic representation of BARD1 (sequence NM_000465): protein domains and open reading frame (ORF) (bottom) are indicated as modified from Sauer and Andrulis (2005). Positions of primers 1F2, 1F3, 11R2, and 11R3 are also shown.

were observed in the relative amounts of *BARD1*δ and *BARD1* ΔRIN SVs when compared with *BARD1* FL either in tumors or in morphologically normal tissues (data not shown).

DISCUSSION

In the present study we identified seven novel *BARD1* SVs in human lymphocytes and in sporadic breast cancers, as well as in morphologically normal tissues adjacent to the tumors. We also detected one transcript (*BARD1* ΔRIN), that had already been described (Tsuzuki et al., 2006). Only two of the eight SVs, *BARD1*δ and *BARD1* ΔRIN, would lead to internally deleted proteins. BARD1 ΔRIN has been shown unable to bind BRCA1, while retaining the capacity to compete with full-length BARD1 for CstF-50 interaction (Tsuzuki et al., 2006). The remaining six SVs displayed premature stop codons.

We performed quantitative expression analysis to address the hypothesis that differential expression of *BARD1* FL and SVs would selectively occur in tumors, as previously shown for *NF1* and *WT1*

(Skuse and Cappione 1997; Caballero et al., 2001). As a general trend all three transcripts analyzed (*BARD1* FL, *BARD1*δ, and *BARD1* ΔRIN) appeared to be overexpressed in tumors. We were unable to identify tumor-specific SV patterns, either qualitatively or quantitatively, to support a pathogenetically important role of *BARD1* alternative splicing in breast cancer. In particular, no differences in the expression levels of *BARD1* FL, *BARD1*δ, and *BARD1* ΔRIN were found among tumors. However, a potential role for *BARD1* alternative splicing in breast tumorigenesis cannot be excluded since an overall assessment of SVs has is to be performed. Interestingly, none of the *BARD1* SVs we identified appears to have lost the 5′ end, which differs from what recently has been described in ovarian cancer (Wu et al., 2006).

Preliminary BARD1 protein expression analysis by immunohistochemistry on a selection of 27 breast cancer samples and 13 peritumoral morphologically normal tissues confirmed BARD1 upregulation in breast tumors. Moreover, BARD1 showed a prominent cytoplasmic localization in tumor

TABLE 1. Alternative Splicing Variants of *BARD1* in Lymphocytes, Tumors, and Normal Adjacent Tissues

Splice variant	Missing NT (exon)	Missing AA	Domains retained	Position of stop codon (NT)	PBLs carriyng splice variant (%)	Tumors carrying splice variant	Peritumors carrying splice variant
BARD1 FL	None	None	All	2,407	100	100	100
h*BARD1* α	438–1,387 (4)	122–438	RING domain; NES	1,405	100	100	100
h*BARD1* β	288–1,387 (3,4)	71–438	RING domain (46–70)	1,494	100	100	67
h*BARD1* γ	232–1,387 (2,3,4)	53–438	RING domain (46–52)	1,494	40	87	53
h*BARD1* δ	288–1,641 (3,4,5,6)	71–523	RING domain (46–70); BRCTs	2,407	60	100	100
h*BARD1* θ	288–1,641 + 1,751–1,883 (3,4,5,6,8)	71–523 + 559–603	RING domain (46–70)	1,884	40	34	47
BARD1 ΔRIN	232–1,641 (2,3,4,5,6)	232–523	RING domain (46–52); BRCTs	2,407	20	100	100
h*BARD1* η	232–1,641 + 1,751–1,883 (2,3,4,5,6,8)	53–523 + 559–603	RING domain (46–52)	1,884	40	50	40
h*BARD1* ζ	232–1,976 (2,3,4,5,6,7,8,9)	53–634	RING domain (46–52)	2,008	80	100	80

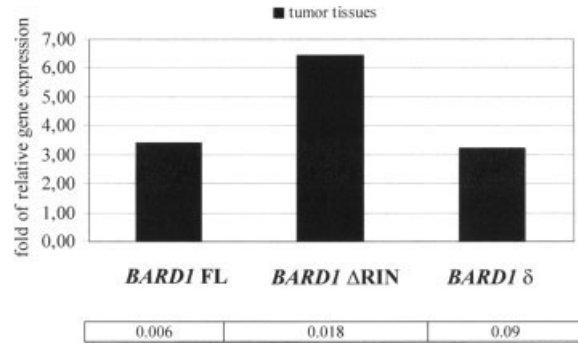NT, nucleotides; AA, aminoacids; NES, nuclear export signal.

Figure 2. SYBR Green relative quantification of *BARD1* FL, *BARD1*δ, and *BARD1* ΔRIN SVs. Expression levels in tumors were normalized to nontumoral tissue samples and compared by *t* test. *P* values are reported at the bottom.

cells when compared with a prevalent nuclear weak staining in the surrounding tissue (unpublished data) in line with recent literature data (Wu et al., 2006).

In summary, our results suggest that alternative splicing may be a relevant mechanism behind *BARD1* regulation. As such, this would represent a mechanism to generate protein isoforms endowed with novel physiological functions, providing an intriguing potential strategy to knock down the expression of functional BARD1 in tumors. However, further investigation in vivo and in vitro will have to be performed to clarify the expression pattern of BARD1 isoforms and their role in cancer pathogenesis.

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