

BARD1 Expression Predicts Outcome in Colon Cancer

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Abstract

Purpose: BARD1 is a BRCA1-binding partner with tumor suppressive properties. Aberrant splice variants of BARD1 have been detected in various cancers, and it has been postulated that the presence of some splice variants is cancer specific. This is the first study assessing BARD1 expression patterns and correlation with clinical outcome in colon cancer.

Experimental Design: We analyzed colon cancer samples for the occurrence of BARD1 splice variants, characterized novel BARD1 splice variants, and quantified the mRNA expression levels of these isoforms in primary colon cancers and their corresponding normal tissue. We tested the correlation of full-length BARD1 protein expression and clinical outcome in primary colon cancer samples.

Results: In addition to the full-length BARD1 mRNA, we now find 19 distinct BARD1 splice variants in colon cancer. Contrary to previous assumptions, these splice variants also occur in the adjacent normal colon tissue. Although BARD1 splice variants account for a considerable amount of BARD1 mRNA in both cancer and normal colon samples, distinct variants show a cancer-specific regulation pattern. Consistent with its role as tumor suppressor, we further find that the expression of the full-length BARD1 protein predicts outcome in colon cancer and that loss of full-length BARD1 protein is associated with a poor prognosis ($P = 0.0002$).

Conclusion: Taken together, this is the first report to suggest that BARD1 regulation is an important pathway in colon cancer and that the BARD1 full-length protein may be a useful marker to improve risk stratification in colon cancer patients. *Clin Cancer Res*; 17(16); 5451–62. ©2011 AACR.

Introduction

The association of truncating *BRCA1* germline mutations and breast cancer is well established. Mutations of the *BRCA1* gene are responsible for a highly increased risk of breast and ovarian cancer (1). A recent study showed that carriers of the *BRCA1* founder mutation also have a 4-fold increased risk of developing colon cancer (2), thus suggesting that loss of *BRCA1* function may also be an important mechanism for colon cancer development.

Tumor suppressor functions of *BRCA1* are thought to be mediated by a heterodimer formed by *BRCA1* and BARD1, the *BRCA1*-associated RING domain protein 1 (3). BARD1

was identified as one of the main-binding partners of *BRCA1 in vivo* (3) and shares homology with 2 highly conserved regions of *BRCA1*: the *N*-terminal RING domain, required for the formation of the BARD1/*BRCA1* heterodimer, and the *C*-terminal BRCT domains (Fig. 1A). Tumorigenic amino acid substitution in *BRCA1* was shown to disrupt the heterodimer suggesting that the *BRCA1*/BARD1 complex formation is important for the tumor suppressor function of *BRCA1* (3). Conditional inactivation of *BARD1* in mice induces mammary carcinomas that are indistinguishable from carcinomas induced by conditional knock-out of *BRCA1*, which establishes BARD1 itself as a tumor suppressor (4). Mutation analyses of *BRCA1/2* wild-type gynecologic cancers reveal a few rare *BARD1* germline mutations that are associated with an increased breast cancer risk (5, 6). Somatic *BARD1* mutations have also been described in breast cancer, but are uncommon as well (7).

Alternative splice variants of the BARD1 transcript, however, are very common and surprisingly numerous. Occurrence of BARD1 splice variants has been described in rat spermatocyte precursors (8), rat Nu-Tu-19 ovarian cancer cells (9), breast cancer cell lines (10) and cytotrophoblast cells (11). Several studies assessed the occurrence of BARD1 splice variants in breast and ovarian cancer samples, in which 13 different splice variants were described (12, 13).

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Translational Relevance

In this study, we show that the BRCA1 associated-binding protein, BARD1, plays an important role in colon cancer. We characterize 19 distinct BARD1 splice variants and show that the expression of full-length BARD1 protein predicts survival in colon cancer patients. Loss of full-length BARD1 protein is associated with a poor prognosis, which is consistent with the role of BARD1 as a tumor suppressor and identifies BARD1 full-length protein as a novel tool for risk stratification in colon cancer patients. This is another step toward clinical utilization of markers that help to define individual risk signatures in patients, leading to a better targeting of therapeutic strategies and ultimately improving the overall survival in cancer patients.

Two BARD1 isoforms were associated with advanced stage in ovarian carcinoma and it was speculated that BARD1 splice variants are specifically expressed in gynecologic cancers (12).

In our study, we show that the expression of BARD1 can predict outcome in colon cancer patients. This identifies BARD1 as a novel tool for risk stratification of colon cancer patients and suggests an important role of the BARD1/BRCA1 pathway in colon cancer. Moreover, we analyzed the occurrence of BARD1 splice variants in colon cancer, characterized novel splice variants and quantified the mRNA expression of BARD1 isoforms in colon cancer samples and matched normal colon tissue. We find 19 distinct BARD1 splice variants, of which 11 have not been described before. These splice variants account for a significant amount of the total BARD1 mRNA either in colon cancer and normal colon samples

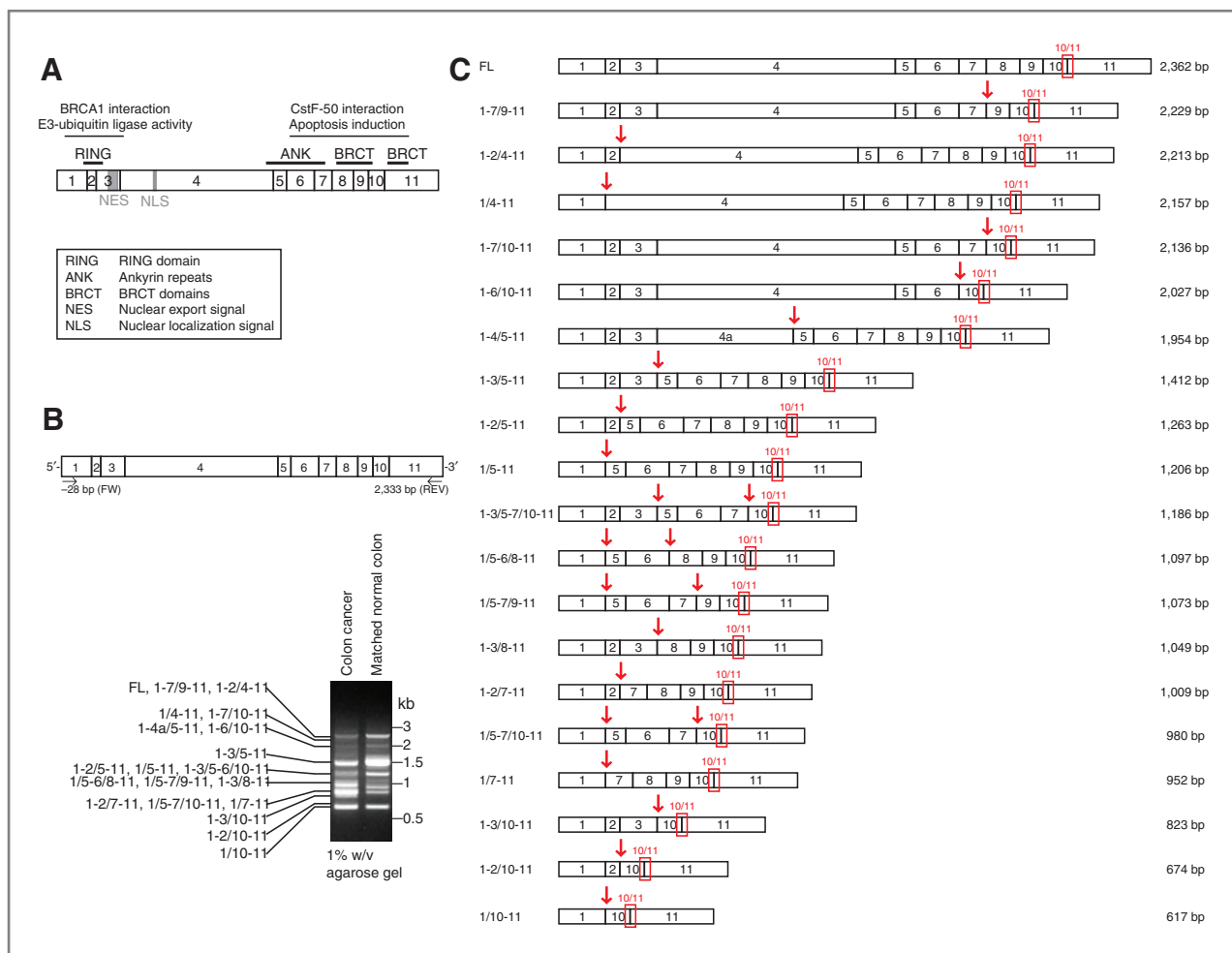


Figure 1. Characterization of 19 BARD1 splice variants in colon cancer. A, schematic representation of BARD1 (NM_000465.2). Position and function of structural protein domains are indicated as modified from Lombardi and colleagues (13) and Sauer and Andrulelis (29). B, PCR on human colon cancer and matched normal colon cDNA samples results in several PCR products. Primer pair targeting the first and last exon of the BARD1 transcript (arrows) was used for PCR. One representative matched sample pair is shown. C, in addition to the full-length BARD1 (FL) transcript, 19 distinct BARD1 splice variants are found and characterized. Red arrows indicate the new exon/exon boundaries. Exon boundary 10/11 is marked in red and found in full-length BARD1 and all BARD1 isoforms.

and distinct splice variants show a cancer-specific regulation pattern.

Materials and Methods

Reverse transcription and PCR

RNA from 15 human colorectal cancer samples and 15 matched normal colon samples was acquired from Biochain. RNA quality was assessed with the Agilent Bio-Chip (RIN > 6.5). One microgram of RNA of each sample was reverse-transcribed using the Superscript III First-Strand Synthesis SuperMix and Oligo(dT)₂₀ primers by Invitrogen according to the manufacturer's instruction. Reverse transcription was followed by RNase H digest (New England Biolabs). cDNA served as the PCR template. BARD1-specific primers (12) initiating in the first and last exon of the BARD1 transcript were used at a final concentration of 200 nmol/L: 5'-GAGGAGCCTTTCATCCGAAG-3' (FW) and 5'-CAGCTGTC AAGAGGAAGCAAC-3' (REV). To ensure specificity, PCR was done as a Touchdown-PCR (annealing temperature gradually decreased from 65°C to 56°C) using a hot start enzyme, the HotStarTaq Plus DNA Polymerase (Qiagen). The PCR was set up according to the manufacturer's instructions and run on a standard thermocycler. PCR products were visualized on a 1% w/v agarose gel stained with ethidium bromide.

In addition, we collected 5 colorectal mucosa samples from noncancer patients under Institutional Review Board (IRB) approval at University of Chicago (IRB# 10-209-A), assessed the RNA quality and did reverse transcription as described above. cDNA from noncancer patients served as a template for quantitative real-time PCR.

Purification of PCR products and subcloning

PCR bands were cut out and purified using the QIAquick Gel Extraction Kit (Qiagen). One aliquot of each purification was submitted for direct sequencing, a second aliquot was subcloned using the Invitrogen TOPO TA Cloning Kit (pCR2.1-TOPO vector and TOP10 One Shot competent cells) according to the manufacturer's instruction. Bacterial suspensions from single colonies were purified with the PureLink Quick Plasmid Miniprep Kit (Invitrogen) and plasmids were subsequently sequenced.

Sequence analysis

Resulting sequences were aligned against the full-length BARD1 mRNA (NM_000465.2) using the NCBI Spidey program. Splice variants were characterized and analyzed with Geneious Pro software.

Quantitative real-time PCR

To directly quantify the expression of BARD1 splice variants, we conducted SYBR Green quantitative real-time PCR assays. Primer 3 (14) and Primer Express software V3.0 (Applied Biosystems) were used to design specific qPCR primer pairs with one primer designed to target the new variant-specific exon junction, the second primer posi-

tioned in one of the adjacent exons to yield a product of 50 to 150 bp. qPCR reactions were carried out in triplicates using the Fast SYBR Green Master Mix (Applied Biosystems), a total reaction volume of 20 µL and primer concentrations of 300 nmol/L. The experiments were run on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) following the standard protocol and conditions for the Fast SYBR Green Master Mix. A dissociation stage was added to the run protocol. PCR efficiency was established by calibration curves using cDNA from CaCo₂ cells as a template. CaCo₂ cells were tested and authenticated by STR profiling using the PowerPlex 1.2 System (Promega). Control reactions were done using genomic DNA as a template and no template, both resulted in no amplification. Dissociation curves were analyzed to ensure the specificity of the detected signal. A common threshold was determined for all splice variant runs. The values of C_q for each sample were transformed to relative quantities, corrected for efficiency, and normalized to the relative quantity of the total BARD1 transcript of the same sample, as detected by a primer pair spanning exon boundary 10/11, thus targeting the full-length transcript and all described splice variants. The ratio for each splice variant was averaged among cancer samples and among normal samples. The average percentage expression and the expression range found for each splice variant was plotted on a graph. In a separate graph, we plotted the percentage expression of cancer versus normal samples for the 6 most abundant splice variants side by side, indicating the SD within the 15 samples and significant (*P* < 0.05) expression differences as determined by *t* test. In addition, we conducted quantification on pooled cDNA from 16 different human tissues using the Human MTC Panel I and Human MTC Panel II from Clontech Laboratories. The percentage expression for the 6 most abundant splice variants (>1% of total BARD1 transcript) was plotted on a graph.

For normalization purposes, we quantified the expression of 5 reference genes in the 15 matched pair samples: L19, GAPDH, B2M, RPLPO, HPRT1. L19 was quantified in a SYBR Green assay according to the method described above, using the following primers at a concentration of 200 nmol/L: (5'-ACCCAATGACCAATGAAAT-3' and 5'-CAGCCCATCTTTGATGAGCTT-3'). The other 4 reference genes were quantified using Pre-Developed TaqMan Assay Reagents (Applied Biosystems, 4333764F, 4333766F, 4333761F, 4333768F) and the TaqMan Gene Expression Master Mix (Applied Biosystems). The experiments were run on the same instrument following the standard protocol and conditions as outlined for the TaqMan Gene Expression Master Mix. PCR efficiency was established by calibration curves and control reactions were done. The values of C_q for each sample were transformed to relative quantities (corrected for efficiency) and the geometric mean of the 5 reference genes was calculated for each sample. The relative quantity of each sample determined in the splice variant runs was normalized to the geometric mean of the reference genes. The ratio of the

normalized relative quantity of each cancer sample and its matched normal colon sample was calculated and plotted on a graph with logarithmic scale.

As a further quality control, we conducted a 3':5' assay to ensure the integrity of the RNA/cDNA and used the Solaris qPCR control kit (Thermo Scientific) to exclude any relevant inhibition of the reactions (data not shown).

Immunohistochemistry

Paraffin-embedded tissue multiarrays containing 99 primary colorectal cancer samples were purchased from Imgenex and assessed for BARD1 protein expression. Slides were probed with a mouse monoclonal anti-BARD1 antibody (E11) raised against the N-terminus of full-length BARD1 (Santa Cruz Biotechnology), detected with diaminobenzidine (DAB; Vector Laboratories) and counterstained with Mayer's hematoxylin (Sigma-Aldrich). Slides were scanned using an Aperio Scanscope XT instrument at 20 \times . Tumor cells within each core were selected for analysis using the pen tool within the WebScope viewing software. For the analysis, the Aperio Nuclear tool (Nuclear Analysis v9.1 algorithm, Supplementary Table S2) was used to measure the nuclear staining intensity of the cancer cells within each core. For quality reasons (no tissue or no tumor tissue in particular cores) only 81 patient samples could be included in the analysis (Supplementary Table S3). Three intensity levels were discerned: 3 (strong nuclear staining), 2 (intermediate nuclear staining), 1 (weak or no staining).

A second cohort consisting of 43 single section slides from a study done under IRB approval at the University of California and Veterans Administration Medical Centers in San Diego, California (15), was analyzed as above. Data for this cohort includes treatment information on adjuvant chemotherapy with 5-FU (Supplementary Table S3).

Statistical analysis

Patient data for the first cohort was provided by Imgenex, patient data for the second cohort was gathered from the respective study. The impact of BARD1, staging, and treatment on overall survival was assessed using the log-rank test in a univariate analysis.

A multivariate model for BARD1, controlling for age, sex, and staging of the patient, was estimated by Cox regression. All pairwise comparisons of the tree BARD1 expression levels under multiplicity control were done by the Tukey posthoc procedure described by Hothorn and colleagues (16). In addition, a prognostic risk model based on age, sex, staging, and BARD1 expression was derived by a conditional inference survival tree (17). The model partitions the patients into risk groups based on a logical combination of patient characteristics useful for clinical prognosis (18). For the patients of the second cohort the multivariate models also controlled for treatment effects.

Association between BARD1 and staging or treatment was assessed using mosaic plots with residual-based shadings along with a permutation test for independence (19). Overall, *P* values less than 0.05 were considered significant.

All analyses were done using R (R Development Core Team, 2011; ref. 20) and the add-on packages survival (21), party (22), and multcomp (23).

Results

BARD1 splice variants occur in primary colon cancer samples and matched normal colon tissue

To investigate the role of BARD1 splice variants in colon cancer, we analyzed RNA from 15 colon cancer samples and 15 matched normal colon samples for the occurrence of isoforms. RNA was reverse transcribed and cDNA used as a template for PCR with primer targets in the first and last exon of the BARD1 transcript (Fig. 1B). Although the expected PCR product is 2362 bp (Fig. 1B, top panel), we find several additional bands ranging between 2.3 and 0.6 kb in size (Fig. 1B, bottom panel). Interestingly, we find these bands not only in the colon cancer samples but also in adjacent matched normal colon tissue (Fig. 1B, bottom panel and Supplementary Fig. S1).

Characterization of novel BARD1 splice variants

To characterize the PCR products, we purified and sequenced them by 2 different approaches: (i) direct sequencing after gel purification and (ii) subcloning with subsequent sequencing. Although direct sequencing was used in previous publications (13), many splice variants are very similar in length and putative single bands may contain multiple isoforms which require subcloning for full characterization. We then analyzed the obtained sequences by using the NCBI Spidey program, an mRNA to genomic alignment program, and aligned the sequences of the directly purified and subcloned splice variants against the full-length BARD1 mRNA (NM_000465.2). We characterized 19 distinct splice variants (Fig. 1C), 11 of which have not been previously described. Eighteen of the 19 splice variants lack 1 or more complete exons and 1 variant lacks part of an exon, thus creating new and specific exon boundaries in each case.

As the designation of the BARD1 splice variants has thus far been inconsistent (Table 1), we created a systematic approach using the structure of the splice variant. For example, BARD1_1-7/9-11 is a splice variant that consists of exon 1 to exon 7, directly followed by exon 9 to exon 11, hence missing exon 8 (Table 1). Thus, a new and variant-specific exon boundary of exons 7 and 9 (7/9) is created, which we then exploited for quantification of splice variant expression by qPCR. Table 1 summarizes the structure of the BARD1 isoforms in colon cancer.

BARD1 splice variants account for a considerable amount of the total BARD1 transcript in colon cancer

To quantify the above described BARD1 splice variants in normal colon and colon cancer, we conducted SYBRGreen qPCR assays. We designed specific qPCR primer pairs, with one primer designed to target the new variant-specific exon junction and the second primer positioned in one of the adjacent exons to yield a product of 50 to 150 bp suitable

Table 1. Structural characterization of BARD1 splice variants

Splice variant	Alternate name (previously published by Lombardi and colleagues, 2007)	Alternate name (previously published by Li and colleagues, 2007)	Missing exon(s)	Product length (bp)	Lost domains	Detected by qPCR primer targeting the new exon boundary	Detected by primer pair spanning exon junction 10/11
hBARD1 FL	hBARD1 FL	hBARD1 FL	None	2362	–	–	+
hBARD1_1-7/9-11			8	2229	1st BRCT domain (partial)	7/9	+
hBARD1_1-2/4-11			3	2213	RING domain (partial), NES	2/4	+
hBARD1_1/4-11		hBARD1β	2, 3	2157	Most of RING domain, NES	1/4	+
hBARD1_1-7/10-11			8, 9	2136	Most of 1st BRCT domain	7/10	+
hBARD1_1-6/10-11			7, 8, 9	2027	ANK (partial), most of 1st BRCT domain	–	+
hBARD1_1-4a/5-11			Part of exon 4	1954	–	4a/5	+
hBARD1_1-3/5-11	hBARD1α	hBARD1γ	4	1412	NLS	3/5	+
hBARD1_1-2/5-11	hBARD1β		3, 4	1263	RING domain (partial), NES, NLS	2/5	+
hBARD1_1/5-11	hBARD1γ		2, 3, 4	1206	Most of RING domain, NES, NLS	1/5	+
hBARD1_1-3/5-7/10-11			4, 8, 9	1186	NLS, 1st BRCT domain (partial)	3/5	+
hBARD1_1/5-6/8-11			2, 3, 4, 7	1097	Most of RING domain, NES, NLS, ANK (partial)	1/5, 6/8	+
hBARD1_1/5-7/9-11			2, 3, 4, 8	1073	Most of RING domain, NES, NLS, 1st BRCT (partial)	1/5, 7/9	+
hBARD1_1-3/8-11			4, 5, 6, 7	1049	NLS, ANK	–	+
hBARD1_1-2/7-11	hBARD1δ	hBARD1φ	3, 4, 5, 6	1009	Most of RING domain, NES, NLS, ANK (partial)	2/7	+
hBARD1_1/5-7/10-11			2, 3, 4, 8, 9	980	Most of RING domain, NES, NLS, most of 1st BRCT	1/5, 7/10	+
hBARD1_1/7-11	hBARD1ΔRIN	hBARD1δ	2, 3, 4, 5, 6	952	Most of RING domain, NES, NLS, ANK (partial)	1/7	+
hBARD1_1-3/10-11		hBARD1ε	4, 5, 6, 7, 8, 9	823	NLS, ANK, most of 1st BRCT domain	–	+
hBARD1_1-2/10-11			3, 4, 5, 6, 7, 8, 9	674	RING domain (partial), NES, NLS, ANK, most of 1st BRCT	2/10	+
hBARD1_1/10-11	hBARD1ζ	hBARD1η	2, 3, 4, 5, 6, 7, 8, 9	617	Most of RING domain, NES, NLS, ANK, most of 1st BRCT	1/10	+

Abbreviations: FL, full length; NES, nuclear export signal; NLS, nuclear localization signal.

for qPCR. Therefore, in the majority of cases, primers could not be designed as conventional exon spanning primers. Consequently, there was a risk that the primer that was supposed to target the new exon junction would anneal only with the primer portion lying within the same exon as the reverse primer, resulting in the amplification of an intraexonic fragment. To ensure that our primers specifically recognized the splice variants only, we conducted exhaustive primer testing and optimization. We tested all primer pairs on cDNA and genomic DNA to rule out amplification of intraexonic fragments. We also tested varying primer concentrations to ensure saturation of all reactions and optimal performance and used No Template Controls to assure that the results were not affected by primer artifacts. Only primer pairs that fulfilled all above requirements were included in the analysis. With this stringent approach, we were not able to design primer pairs for 3 of the variants (BARD1_1-3/8-11, BARD1_1-3/10-11, BARD1_1-6/10-11) that would fulfill all these criteria, which is why we excluded them from the analysis. Because some of the exon junctions were found in more than 1 splice variant (Table 2), this led to a total of 13 primer pairs targeting a total of 16 splice variants included in the analysis (Supplementary Table S1). For example, primer pair 3/5—targeting the new and splice variant-specific exon boundary 3/5—detects splice variant BARD1_1-3/5-11 and splice variant BARD1_1-3/5-7/10-11. As exon junction 10/11 is the only exon junction that is found in all splice variants and in the full-length BARD1 transcript (Fig. 1C), we used a primer pair spanning exon junction 10/11 to quantify the total BARD1 transcript.

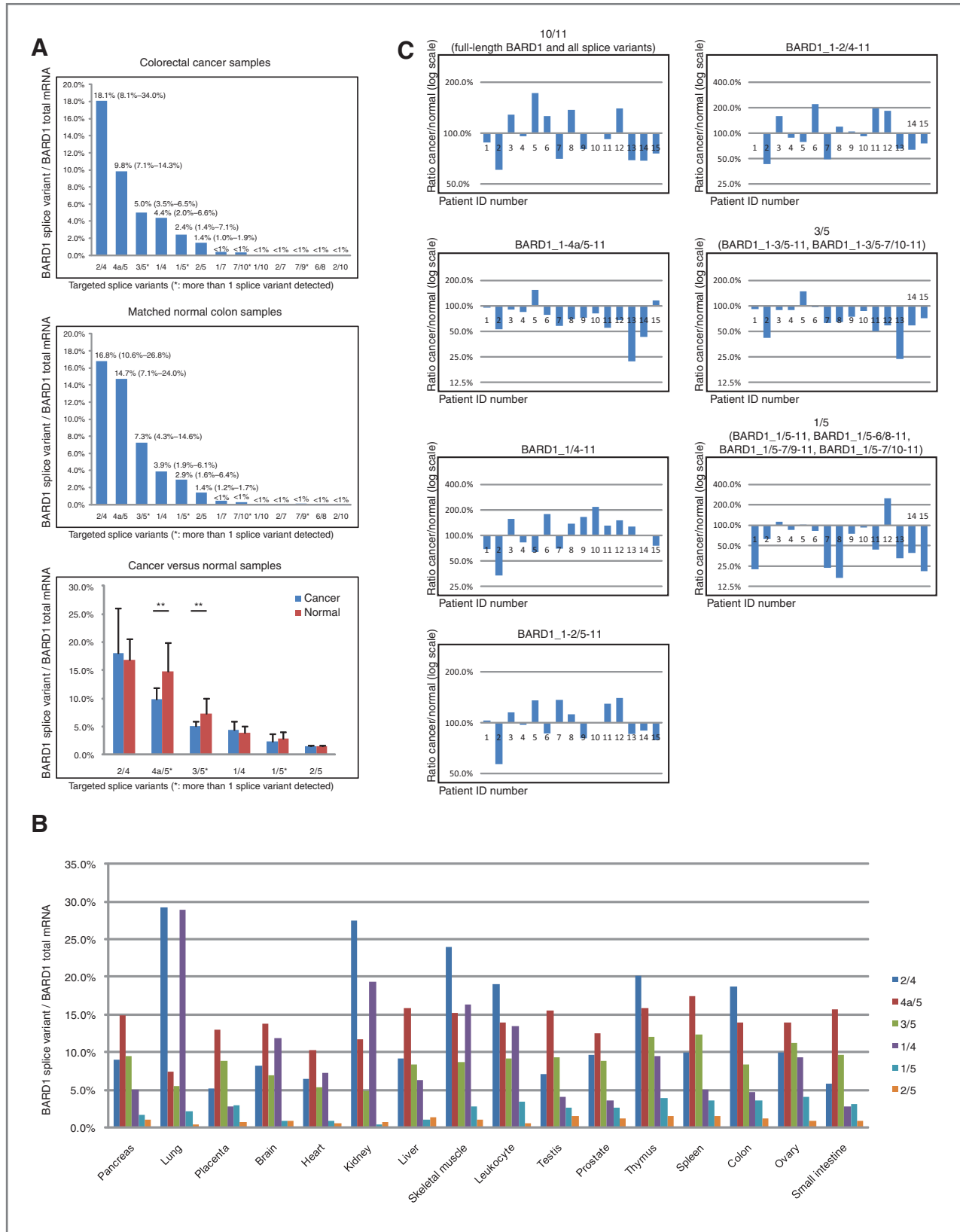
To evaluate the relative abundance of each variant, we calculated the efficiency-corrected relative quantity of each splice variant and normalized it to the relative quantity of the total BARD1 transcript for each of the 30 samples, also corrected for efficiency. We find that BARD1 splice variants account for a considerable percentage of the total BARD1 transcript (Fig. 2A) either in colon cancer and matched normal colon samples. In both groups, BARD1_1-2/4-11 is the most abundant splice variant and accounts for 18.1% (8.1%–34.0%) of the

Table 2. Overview of BARD1 qPCR primers

Targeted exon boundary	Detected splice variants
1/4	1/4-11
1/5	1/5-11
	1/5-6/8-11
	1/5-7/9-11
	1/5-7/10-11
1/7	1/7-11
1/10	1/10-11
2/4	1-2/4-11
2/5	1-2/5-11
2/7	1-2/7-11
2/10	1-2/10-11
3/5	1-3/5-11
	1-3/5-7/10-11
4a/5	1-4/5-11
6/8	1/5-6/8-11
7/9	1-7/9-11
	1/5-7/9-11
7/10	1-7/10-11
	1-3/5-7/10-11
	1/5-7/10-11
10/11	Full-length BARD1
	All splice variants

BARD1 transcript in colon cancer samples and 16.8% (10.6%–26.8%) in the matched normal colon samples (Fig. 2A). BARD1_1-4a/5-11 is the second most abundant splice variant with a relative expression of 9.8% (7.1%–14.3%) in cancer samples and 14.7% (7.1%–24.0%) in matched normal colon tissue. Splice variants detected by primer pair 3/5 (BARD1_1-3/5-11 and BARD1_1-3/5-7/10-11) account for an average of 5.0% and 7.3%, respectively. Expression of splice variant BARD1_1/4-11 amounts to 4.4% of the total BARD1 transcript in cancer and 3.9% in normal colon. Splice variants detected by primer pair 1/5 (BARD1_1/5-11, BARD1_1/5-6/8-11,

Figure 2. Quantitative real-time PCR analysis of BARD1 splice variants. A, direct quantification of BARD1 splice variants shows that BARD1 variants account for a considerable percentage of the total BARD1 transcript in colon cancer and matched normal colon samples. Expression of splice variants were quantified by qPCR, transformed to relative quantities (corrected for efficiency), and normalized to the total BARD1 transcript of the same sample, as detected by a primer pair spanning exon boundary 10/11. The ratio for each splice variant was averaged among cancer samples (top) and among normal samples (middle). The average percentage expression and the expression range found for each splice variant was plotted on a graph. Percentage expression of cancer versus matched normal samples for the 6 most abundant splice variants was plotted side by side, indicating the SD within the 15 samples and significant ($P < 0.05$) expression differences (**) as determined by t test (bottom). BARD1_4a/5 and the variants detected by primer pair 3/5 are significantly downregulated in colon cancer samples compared with matched normal samples. B, direct quantification of BARD1 splice variants using pooled cDNA from 16 different human tissues shows a tissue-specific expression pattern of BARD1 splice variants. The percentage expression for the 6 most abundant splice variants (>1% of total BARD1 transcript) was plotted on a graph. C, BARD1 splice variants show distinct expression patterns in colon cancer compared with normal tissue. The 6 most abundant splice variants were addressed in further detail (4a/5, 2/4, 3/5, 2/5, 1/4, 1/5). For normalization, the expression of 5 reference genes was quantified: L19, GAPDH, B2M, RPLPO, HPRT1. The relative quantity of each sample determined in the splice variant runs was normalized to the geometric mean of the reference genes. The ratio of the normalized relative quantity of each cancer sample and its matched normal colon sample was calculated and plotted on a graph with logarithmic scale. Two different regulation patterns can be described. The first group of splice variants shows no change or downregulation in all except one of the cancer samples compared with the matched normal samples (1-4a/5-11, 3/5, and 1/5), the second group shows varying levels among different patients from upregulation to downregulation in cancer samples compared with normal samples (1-2/4-11, 1-2/5-11, and 1/4-11).



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BARD1_{1/5-7/9-11}, BARD1_{1/5-7/10-11}) account for 2.4% of the transcript in cancer samples and 2.9% in normal tissue, BARD1_{1-2/5-11} for 1.5% and 1.4%. The quantities detected with the 7 remaining primer pairs (1/7, 7/10, 1/10, 2/7, 7/9, 6/8, 2/10) each comprise less than 1% of the total transcript for both cancer samples and matched normal tissue. The relative expression levels of the splice variants are quite similar among cancer and normal samples, with the exception of BARD1_{1-4a/5-11} and the variants detected by primer pair 3/5 (BARD1_{1-3/5-11} and BARD1_{1-3/5-7/10-11}) which are lower in cancer than in normal colon tissue (9.8% vs. 14.7% and 5.0% vs. 7.3%), whereas levels of BARD1_{1-2/4-11} and BARD1_{1/4-11} are slightly elevated (18.1% vs. 16.8% and 4.4% vs. 3.9%, respectively). Comparing cancer and normal samples by *t* test analysis shows a significant downregulation of BARD1_{1-4a/5-11} and the variants detected by primer pair 3/5 (BARD1_{1-3/5-11} and BARD1_{1-3/5-7/10-11}; Fig. 2A). As a control, we analyzed the expression of BARD1 splice variants in 5 colorectal mucosa samples from noncancer patients. The percentage expression of the variants from noncancer patients lies within the range of the matched normal samples from cancer patients suggesting a tissue-specific expression profile that is altered in cancer. To further analyze the tissue-specific expression pattern of the BARD1 variants, we conducted quantitative assays on pooled cDNA from 16 human tissues and calculated the percentage expression for each variant in each tissue (Fig. 2B). The splice variants can be detected at varying levels in different tissues creating a tissue-specific profile. This finding again shows that BARD1 splice variants are also a common feature of normal cells and suggests a complex function in health and disease.

Distinct BARD1 splice variants show a cancer-specific regulation pattern

To focus on the differences between cancer samples and normal control samples, we calculated the ratio of the normalized relative quantity for each matched pair (Fig. 2C). We included all splice variants with a relative expression of more than 1%. As the results are greatly influenced by the way of normalization and as no single perfect normalization gene is available to compare colon cancer and normal colon tissue, we carefully selected 5 genes that were successfully used in expression studies of other cancer types (24): L19, GAPDH, B2M, RPLPO, HPRT1. We conducted normalization runs and calculated the geometric mean of the relative quantities of these reference genes for each sample, all corrected for efficiencies (25). We normalized all samples to the geometric mean of the reference genes and calculated the ratio of the normalized relative quantity of each cancer sample and its corresponding normal colon tissue. We depicted the ratio using a logarithmic scale. Using this approach, we find 2 different regulation patterns: One group, consisting of 4a/5, 3/5, and 1/5, shows a tendency of downregulation, as for each of the 3 primer pairs only one of the 15 matched pairs

shows a slight upregulation, while the other 14 matched pairs show similar expression levels or downregulation (Fig. 2C). The second group consists of 2/4, 1/4, and 2/5. These variants show more varying levels. Some of the matched pairs indicate an upregulation of the splice variant in cancer versus normal, some have similar expression levels and some show a downregulation of the splice variant in cancer versus normal.

Interestingly, the second group contains isoforms BARD1_{1-2/4-11} and BARD1_{1/4-11}, that show a higher percentage expression (when normalized to the total BARD1 transcript) in the cancer group than in the normal colon tissue group, even though the difference is rather small for BARD1_{1/4-11} (4.4% in cancer vs. 3.9% in normal tissue; Fig. 2A). BARD1_{1-2/4-11} and BARD1_{1/4-11} are also the splice variants that show the highest variation in percentage expression among different human tissues (Fig. 2B), which may hint toward an important tissue-specific function that could be altered in cancer. Notably, BARD1_{1/4-11} was previously published as BARD1 β and proposed to be associated with advanced stage of ovarian cancer and a negative prognostic factor in ovarian cancer (12). Due to the lack of patient data associated with our samples and the limited number of good quality RNA samples, we could not test for a similar relationship in colon cancer. It would be interesting to see whether the differential expression of BARD1_{1/4-11} and/or BARD1_{1-2/4-11} also correlates with advanced colon cancer in a future larger cohort.

We cannot exclude that there are other splice variants using a different transcription initiation, as we focused in our study on the splice variants detected by primers in exon 1 and exon 11. In previous studies splice variants initiating in the 5' end could not always be detected (12). As the lack of detection in the 5' end could also be explained by poor quality RNA and cDNA rather than the absence of splice variants initiating in the 5' end, we conducted careful quality testing of all samples by bioanalyzer and 3':5' assays, before we used them in our experiments. As for our study, we can detect splice variants initiating in exon 1 in all samples, cancer, and normal.

Expression of the full-length BARD1 protein correlates with outcome in colon cancer samples

To quantify full-length BARD1 expression, we conducted immunohistochemical stainings on 81 colorectal cancer samples using a BARD1-specific antibody. Quantitative PCR does not allow for quantification of full-length BARD1 expression, as there is no sequence, which is specific for full-length BARD1 only. All exon boundaries of full-length BARD1 are found in at least some of the splice variants. To quantify the full-length BARD1 expression, we conducted immunohistochemistry with a mouse monoclonal antibody raised against the N-terminus of BARD1. We ascertained the specificity of the antibody by Western blot (Supplementary Fig. S3). The

antibody was used on multitissue arrays containing 81 colorectal cancer samples of different stages, detected with DAB and counterstained with hematoxylin. To strengthen the reliability of the expression analysis, we scanned the slides using the Aperio Slide Scanner and analyzed the staining intensity using the Aperio ImageScope software (Fig. 3A). Three staining intensities were discriminated: 3 (strong nuclear staining), 2 (intermediate expression), 1 (weak or no staining). We assessed the relationship between BARD1 expression and overall survival by a log-rank test. Interestingly, we find that the expression levels of BARD1 correlate significantly with survival ($P = 0.0002$). High expression of full-length BARD1 predicts a favorable outcome, whereas loss of expression is associated with a worse prognosis (Fig. 3B). We also find that BARD1 expression significantly correlates with staging with over half of the patients with stage IV showing a weak expression (Expr. 1) of full-length BARD1 ($P = 0.043614$; Fig. 3C). This is in line with the expected finding that stage is also a good predictor of survival ($P < 0.0001$; Fig. 3C). To control for possible effects of age, sex, and staging, a multivariate model for BARD1 was estimated by Cox regression. Pairwise comparisons show a significant difference in survival probability for patients with low expression of BARD1 (Expr. 1) versus patients with intermediate or high expression of BARD1 (Expr. 2, Expr. 3) supporting the potential usefulness of BARD1 as a predictive biomarker. In a second approach a prognostic risk model based on age, sex, staging, and BARD1 expression was derived by a conditional inference survival tree (Fig. 3D). Stage IV versus stages II, III shows the highest correlation with outcome ($P < 0.001$). As expected, stage IV patients have a worse prognosis than patients with stages II and III. There is no significant difference in survival between stages II and III patients. However, among patients with stages II and III disease, expression of full-length BARD1 protein shows a significant difference in survival comparing weak (Expr. 1) and intermediate/strong expression (Expr. 2, Expr. 3) with weak expression predicting a worse prognosis. Interestingly, there is a significant difference in survival comparing stages II and III ($P = 0.022$) within the group of intermediate/strong BARD1 expression (Fig. 3D).

These findings are consistent with the role of BARD1 as a tumor suppressor and identify full-length BARD1 as a novel and potentially useful biomarker for an improved risk stratification of colon cancer patients.

To further consolidate these findings and to test for a possible dependence on treatment, we analyzed a second cohort of 43 patients from a previous study that determined the effect of 5-FU treatment on overall survival. This group consists only of stages II and III patients eliminating stage as a predictor of survival. The analyses were done as with the first cohort and the multivariate analyses were also adjusted for treatment. Survival analysis again shows a significant correlation between survival and expression of full-length BARD1 (Supplemen-

tary Fig. S4A), whereas neither 5-FU treatment (Supplementary Fig. S4B) nor stage (Supplementary Fig. S4C) is a significant predictor of survival in this cohort. Accordingly, there is no significant correlation between expression of BARD1 and stage (Supplementary Fig. S4D) or 5-FU treatment (Supplementary Fig. S4E), which strengthens the usefulness of BARD1 as additive marker for risk stratification. These findings are supported by the results of the Cox regression analysis and the conditional interference survival tree (Supplementary Fig. S4F), that both show a statistical significance in survival between colon cancer patients with low expression of full-length BARD1 protein (Expr. 1) and patients with intermediate or high expression of full-length BARD1 protein (Expr. 2, Expr. 3).

Discussion

Alterations of the BARD1/BRCA1 pathway have been shown to play a significant role in gynecologic cancers and their importance in other cancer types has been suggested. Studies among *BRCA1* mutation carriers revealed an increased risk for colon cancer (2), which suggests an important role of the BARD1/BRCA1 pathway in colon cancer.

We now find that BARD1 protein expression correlates significantly with survival in colon cancer patients. Patients with low levels of BARD1 have a worse outcome than patients with high expression of full-length BARD1. This is consistent with the role of BARD1 as a tumor suppressor and substantiates the importance of the BARD1/BRCA1 pathway in colon cancer. We also show that full-length BARD1 is a useful predictor of survival in subgroups that could not be distinguished previously which substantiates the potential of BARD1 as a novel predictive marker that could ultimately improve the risk stratification of colon cancer patient.

Colon cancer is among the most common cancer types in both men and women and associated with high mortality, particularly at advanced stages (26). Markers that can help to define individual risk signatures in colon cancer patients are of great clinical value, as they might allow for a better targeting of therapies to attain improved outcomes in colon cancer patients.

Alternative splicing is a common but poorly understood phenomenon. Aberrant splicing has been associated with carcinogenesis (27) typically attributing oncogenic function to the aberrant variants (28). BARD1 splice variants have been found to be an important feature of gynecologic cancers, why we analyzed colon cancer samples for the occurrence of BARD1 splice variants. We now find 19 distinct BARD1 splice variants in colon cancer that account for more than 40% of the total BARD1 transcript. Eighteen of these isoforms lack 1 or more complete exons, one lacks part of an exon, thus creating new and specific exon junctions in each case. Contrary to previous assumptions (12), we find these splice variants also occur in adjacent normal tissue, suggesting that alternative splicing of the BARD1 transcript is a common feature in human cells. The sheer number and abundance of BARD1 splice

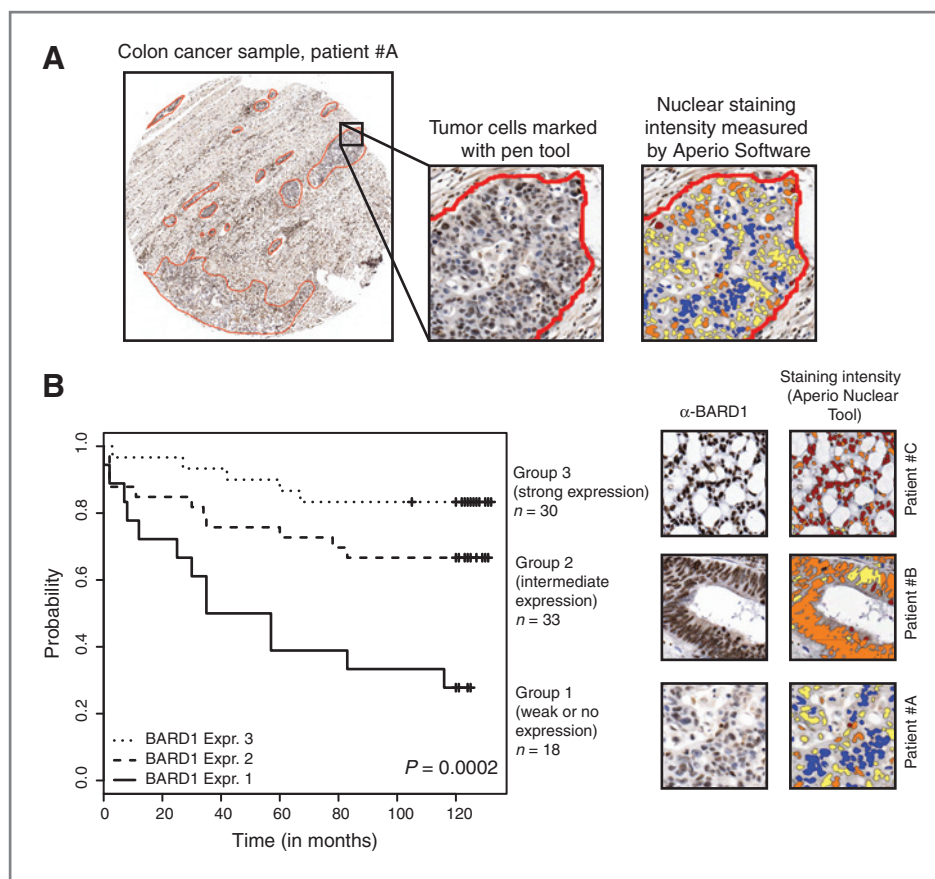


Figure 3. Full-length BARD1 protein expression predicts survival in colon cancer. **A**, paraffin-embedded tissue multi-arrays containing colorectal cancer samples (Imgenex) were assessed for BARD1 protein expression. Slides were probed with a mouse monoclonal anti-BARD1 antibody (E11) raised against the *N*-terminus of full-length BARD1 (Santa Cruz Biotechnology), detected with DAB (Vector Laboratories), and counterstained with Mayer's hematoxylin (Sigma-Aldrich). Slides were scanned using an Aperio Scanscope XT instrument at 20 \times . Tumor cells within each core (left) were selected for analysis using the pen tool within the WebScope viewing software (middle). For the analysis, the Aperio Nuclear tool was used to measure the nuclear staining intensity of the cancer cells within each core (right). Eighty-one patient samples were included in the analysis. **B**, BARD1 expression predicts survival in colon cancer. Three intensity levels were discerned: 3 (strong nuclear staining), 2 (intermediate nuclear staining), 1 (weak or no staining). Representative patient samples for each group are shown with IHC and nuclear staining intensity side by side. Expression levels of full-length BARD1 protein show a significant correlation with survival in colon cancer samples ($P = 0.0002$).

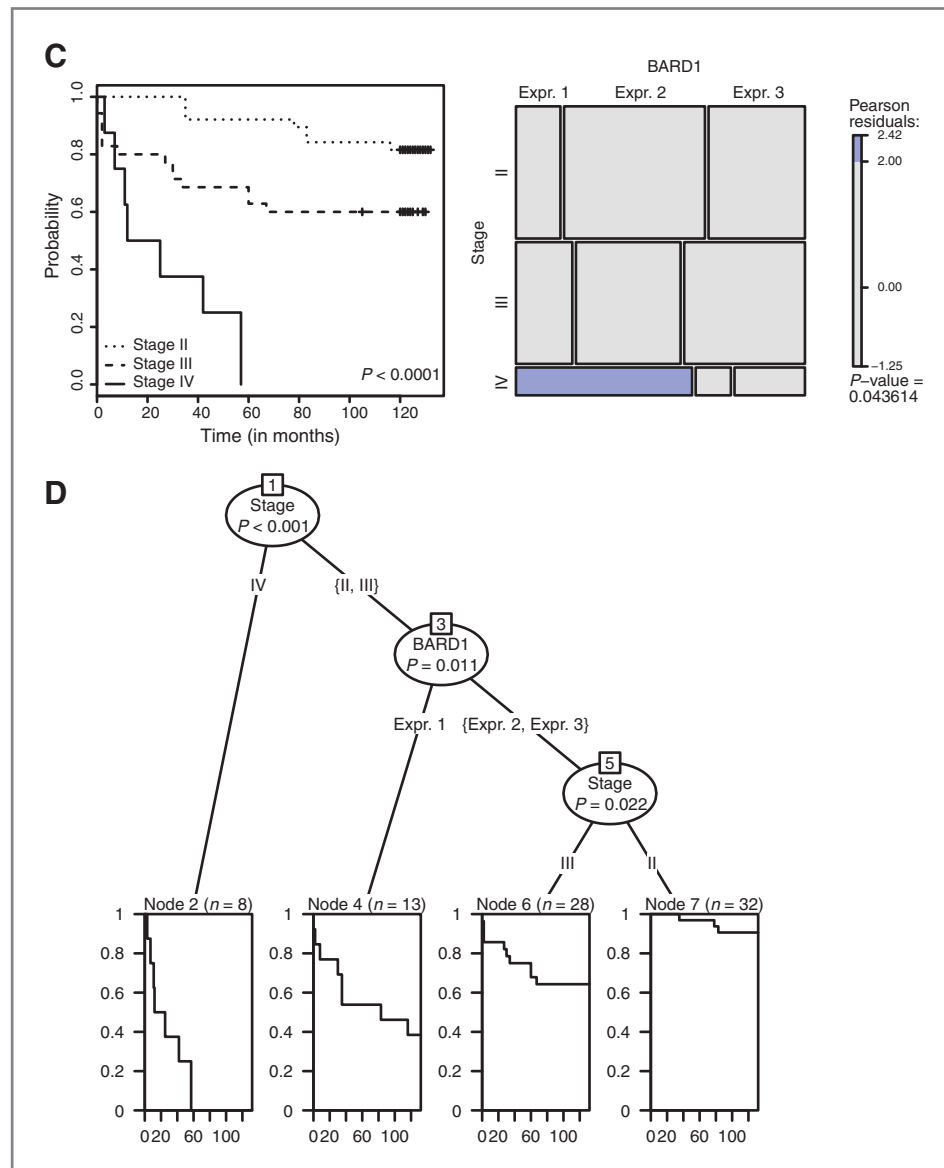
variants is striking and points to a complex function in health and disease. The mechanism underpinning the regulation of the splice variant expression remains to be elucidated. It is thus far unclear, if regulation of the BARD1 splice variants directly influences the full-length protein expression or function, as previously postulated (12).

Currently available patient information for the RNA samples is insufficient to assess correlations between the expression level of splice variants and outcome. On the basis of our data from 15 matched colon cancer/normal colon samples we can observe 2 regulation patterns: One group of splice variants is downregulated in cancer compared with normal, whereas another group shows mRNA expression levels that vary from patient to patient and range from upregulation to downregulation. It is notable that the splice variants that form the second group, BARD1_2/4-11, BARD1_1/4-11, and

BARD1_2/5-11, all lack exon 3 and thus part of the RING domain, which is crucial for the interaction with BRCA1 and the function of the BARD1/BRCA1 heterodimer. In a previous publication BARD1_1/4-11, BARD1 β , was found to be upregulated in advanced stage ovarian cancer and proposed as a negative prognostic factor in ovarian cancer (12). The varying expression levels that we see in colon cancer samples compared with normal colon tissue suggest a varying expression in distinct colon cancer patients that might be of similar prognostic value as in gynecologic cancer types. The question whether upregulation of BARD1_1/4-11 also correlates with advanced colon cancer will require a future larger cohort.

In summary, we find that BARD1 splice variants are abundant and complexly, but not exclusively, expressed in colon cancer. We characterized 19 distinct splice variants that account for more than 40% of the total BARD1

Figure 3. (Continued) C, BARD1 expression and staging correlate significantly. Correlation between stage and expression levels of full-length BARD1 was visualized in a mosaic plot (right). Over half of the patients with stage IV show a weak expression (Expr. 1) of full-length BARD1 ($P = 0.043614$). Staging by itself is a good predictor of outcome for this cohort ($P < 0.0001$; left). D, a prognostic risk model based on age, sex, staging, and BARD1 expression was derived by a conditional inference survival tree. Stage IV versus stages II, III shows the highest correlation with outcome ($P < 0.001$). Stage IV patients have a worse prognosis than patients with stages II and III. There is no significant difference in survival between stages II and III patients. However, among patients with stages II and III disease, expression of full-length BARD1 protein shows a significant difference in survival comparing weak (Expr. 1) and intermediate/strong expression (Expr. 2, Expr. 3) with weak expression predicting a worse prognosis. Interestingly, there is a significant difference in survival comparing stages II and III ($P = 0.022$) within the group of intermediate/strong BARD1 expression.



transcript. One group of splice variants is downregulated in cancer compared with normal, whereas another group shows varying expression levels from patient to patient. We also find that expression of full-length BARD1 significantly correlates with outcome in colon cancer patients and that loss of BARD1 predicts a worse prognosis. Taken together, this is the first report to show that the BARD1/BRCA1 pathway is an important pathway in colon cancer and that the BARD1 full-length protein may be a useful marker for improved risk stratification in colon cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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