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Expression of oncogenic BARD1 isoforms affects colon cancer progression and correlates with clinical outcome

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BACKGROUND: Colon cancer predisposition is associated with mutations in *BRCA1*. BRCA1 protein stability depends on binding to BARD1. In different cancers, expression of differentially spliced BARD1 isoforms is correlated with poor prognosis and decreased patient survival. We therefore suspected a role of BARD1 isoforms in colon cancer.

METHODS: We performed immunohistochemistry in 168 colorectal cancers, using four antibodies directed against differentially expressed regions of BARD1. We determined structure and relative expression of *BARD1* mRNA isoforms in 40 tumour and paired normal peri-tumour tissues. BARD1 expression was correlated with clinical outcome.

RESULTS: BARD1 isoforms were expressed in 98% of cases and not correlated with BRCA1. BARD1 mRNA isoforms were upregulated in all tumours as compared with paired normal peri-tumour tissues. Non-correlated expression and localisation of different epitopes suggested insignificant expression of full-length (FL) BARD1. Expression of N- and C-terminal epitopes correlated with increased survival, but expression of epitopes mapping to the middle of BARD1 correlated with decreased survival. Middle epitopes are present in oncogenic BARD1 isoforms, which have pro-proliferative functions. Correlated upregulation of only N- and C-terminal epitopes reflects the expression of isoforms BARD1δ and BARD1φ.

CONCLUSION: Our results suggest that BARD1 isoforms, but not FL BARD1, are expressed in colon cancer and affect its progression and clinical outcome.

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Survival and prognosis for colorectal cancer, the third leading cause of cancer-related death (WHO, February 2009), depend on the stage of the disease at the time of diagnosis (Hewitson *et al*, 2007; Figueredo *et al*, 2008). Therefore, a better understanding of the molecular events involved in colorectal cancer onset and metastatic progression is needed for early detection and treatment (Rudmik and Magliocco, 2005).

MLH1, MSH2, β-Catenin, and p53 are important markers for the prediction of outcome and response to chemotherapy for colorectal cancer (Markowitz and Bertagnolli, 2009). A number of studies suggest a role for *BRCA1* in colon cancer development. Loss of heterozygosity at chromosomal region 17q, comprising *BRCA1,* was found in 49% of colonic adenocarcinomas (Garcia-Patiño *et al*, 1998). Three-fold increase of colon cancer risk was reported for breast or ovarian cancer patients with *BRCA1* mutations when compared with non-carrier patients (Ford *et al*, 1994; Brose *et al*, 2002; Kadouri *et al*, 2007). However, increased colon cancer risk for *BRCA1* and *BRCA2* mutation carriers, was found to be age dependent (Lin *et al*, 1999; Suchy *et al*, 2010), and was not confirmed in studies that did not take the patients' age into account (Kirchhoff *et al*, 2004; Niell *et al*, 2004). BRCA1 and BARD1 also interact with *hMSH2*, a gene commonly associated with hereditary nonpolyposis colorectal cancer (HNPCC) (Lynch *et al*, 1997; Wang *et al*, 2001), and defects in the BRCA1-hMSH2 signalling pathway lead to the increased risk of cancer (Wang *et al*, 2001). These interactions might partially explain the high incidence of gynaecological tumours in HNPCC kindred, as well as the increased colon cancer susceptibility in *BRCA1* kindred (Easton *et al*, 1995; Lynch *et al*, 1997).

BRCA1 acts in pathways of DNA repair and maintenance of genetic stability, and its deficiency might provide ground for carcinogenesis. The BRCA1-associated protein, BARD1 is required for most tumour suppressor functions of BRCA1 (Wu *et al*, 1996; Fabbro *et al*, 2002). The BRCA1–BARD1 heterodimer has ubiquitin ligase functions (Hashizume *et al*, 2001; Baer and Ludwig, 2002; Oyake *et al*, 2002; Morris and Solomon, 2004), specifically important for G2/M checkpoint control and genetic stability (Ouchi *et al*, 2004; Starita *et al*, 2004). Individually, BRCA1 and BARD1 have low ubiquitin ligase activities *in vitro* (Meza *et al*, 1999; Joukov *et al*, 2001), which implies that BRCA1 activity can be compromised not only by *BRCA1* gene mutations but also by aberrant expression of *BARD1* (Irminger-Finger and Jefford, 2006).

Differentially spliced and highly upregulated BARD1 isoforms were identified in breast and ovarian cancer (Li *et al*, 2007; Zhang *et al*, 2012). Most BARD1 isoforms lack the RING finger,

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which is required for BRCA1 interaction, but retain the BRCT domains. Aberrant BARD1 isoforms are also expressed in non-small cell lung cancer (NSCLC) and significantly correlated with decreased patient survival (Zhang *et al*, 2012). Furthermore, BARD1 isoforms have been shown to encode functions essential for cancer cell viability antagonising the BRCA1-BARD1 ubiquitin ligase activity (Li *et al*, 2007; Ryser *et al*, 2009; Dizin and Irminger-Finger, 2010; Zhang *et al*, 2012).

BARD1 isoforms were recently reported in colorectal cancer (Gautier *et al*, 2000), and it was suggested, based on lack of expression of an N-terminal BARD1 epitope, that lack of full-length (FL) BARD1 is a prognostic marker for poor outcome (Sporn *et al*, 2011). In this study, we investigated *BARD1* mRNA and protein expression, by RT–PCR and immunodetection of epitopes from different regions of BARD1 in 168 colorectal cancer samples, and tested their correlation with clinical characteristics and patient outcome.

PATIENTS AND METHODS

Patients' characteristics

Pathological diagnoses were made by experienced pathologists based on WHO criteria and staged according to American Joint Committee on Cancer classification. All patients were informed and compliance was obtained as well as approval of the local ethical committees. A total of 168 cases with colorectal cancer containing 20 cases from Italy and 148 cases from Germany were examined (Table 1).

The sections used for immunochemical staining were tissue microarrays with tetramerous for each case. Of 148 cases, 75 had follow-up records and 73 patients had no survival data. Follow-up was from 1 to 72 months. Of the 75 patients with follow-up records, 22 were dead, 48 were lost, and 5 were still alive during last follow-up period.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded 5-μm tissue sections were immunostained as described previously (Wu *et al*, 2006). The primary antibodies used for BARD1 detection were N19 (sc-7373, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1 : 25), PVC (1 : 100), WFS (1 : 100), and C20 (sc-7372, Santa Cruz Biotechnology) (1 : 20), which recognise epitopes in exons 1, 3, 4, and 11, respectively; the BRCA1 antibody was C20 (sc-642, Santa Cruz Biotechnology) (1 : 100). P8 antibody specific for exon 11 was raised against oligopeptide ILSRKPKPDSDVTQC at GenScript (www.genscript.com). All BARD1 antibodies and the BRCA1 antibody have been used previously (Irminger-Finger *et al*, 1998; Wu *et al*, 2006; Li *et al*, 2007; Zhang *et al*, 2012).

A selection of cases ($N = 8$) was stained with a commercial antibody BL (A300-263, Bethyl Laboratories, Montgomery, TX, USA), mapping to exon 4, and a newly generated antibody (Zhang *et al*, 2012), directed against exon 11.

Expression levels of BARD1 and BRCA1 epitopes were measured semi-quantitatively. Staining was scored using intensity and percentage of the stained tumour cells as described before (Li *et al*, 2007; Zhang *et al*, 2012). The value of the staining intensity and positive cell percentage were multiplied to get the final staining score. The total staining score of each antibody is from 0 to 100. A score of 25 or less was defined as negative ('$-$'), more than 25 was defined as positive ('$+$' for >25, '$++$' for >50, and '$+++$' for >75). For statistical analysis, only positive *vs* negative cases were considered, except the correlation of different antibodies staining using staining score. Four different regions were chosen for each tumour section and scored independently by three observers (YQ Zhang, L Li, and J Wu) without knowledge of clinical data.

Table 1 Patient characteristics of colorectal cancer

Samples	Germany	Cagliari	Total
Cases	148	20	168
Gender			
Male	83	10	93
Female	65	10	75
Age			
Range	41–97	33–73	33–97
Median	73	60.5	71
Normal (peri-tumour)	0	20	20
Tumour	148	20	168
Histology			
Adenocarcinoma	148	20	168
Grade			
Well differentiated	10	0	10
Moderately differentiated	105	12	117
Poorly differentiated	32	6	38
Undifferentiated	0	0	0
Unspecified	1	2	3
Tumour			
T1	3	1	4
T2	32	4	36
T3	69	13	82
T4	42	2	44
TX	2	0	2
Node			
N0	74	7	81
N1	34	7	41
N2	36	6	42
N3	1	0	1
NX	3	0	3
Metastasis			
M0	101	15	116
M1	44	5	49
MX	3	0	3
Stage			
I	26	4	30
II	38	3	41
III	35	9	44
IV	44	4	48
Unknown	5	0	5

Total RNA extraction, reverse transcription and PCR

Matched pairs of colorectal cancers and non-tumoural surrounding tissues were obtained from patients who underwent surgical resection of the tumour. Immediately after surgical specimen extraction, the colon was opened and both, tumoural tissue and normal mucosa, were collected. To preserve only the mucosal layer, a mucosectomy was performed after injecting saline solution to separate it from the submucosal layer.

RNA was isolated from frozen tissue sections using TRIzol (Invitrogen, Lucerne, Switzerland) according to manufacturer's instruction. RT–PCR was performed to qualitatively show expression of different isoforms and to determine their structure.

Reverse transcription was performed using Promega (Madison, WI, USA) M-MLV reverse transcriptase according to manufacturer's guidelines. A total of 2 μl of the reversed transcription reaction mixture was used for amplification of various fragments of BARD1

mRNAs with Taq polymerase (Qiagen) in a 50-μl reaction mix, according to manufacturer's protocols.

PCR reactions were optimised semi-quantitatively for a cycle number that permits detection of all isoforms without reaching a plateau for the most abundant ones. BARD1 exon 1 to exon 11 amplification primers: forward (5′-GAGGAGCCTTTCATC CGAAG-3′), reverse (5′-CGAACCCTCTCTGGGTGATA-3′), 120 s elongation time, 56 °C annealing temperature, 35 cycles. BARD1 exon 1 to exon 4 amplification primers: forward (5′-GAGGAGCCTT TCATCCGAAG-3′), reverse (5′-ATTGCAGGCTGGGTTTGCACTG AAG-3′), 60 s elongation time, 56 °C annealing temperature, 35 cycles. PCR reactions were quantified as described (Zhang *et al*, 2012).

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was amplified as internal reference – forward primer (5′-AGCCACATCGCTC AGACACC-3′), reversed primer (5′-GTACTCAGCGCCAGCATCG-3′), 57 °C annealing temperature, 30 s elongation time, 25 cycles. ER-alpha PCR forward primer (5′-ACAAGCGCCAGAGAGATGAT-3′), reverse primer (5′-GATGTGGGAGAGGATGAGGA-3′), 57 °C annealing temperature, 60 s elongation time, 30 cycles.

DNA purification and sequencing

The QIAEX II kit (Qiagen, Hombrechtikon, Switzerland) was used for DNA purification of RT–PCR products according to the manufacturer's instructions, followed by sequencing.

Methylation-specific PCR (MSP)

The methylation status of *BARD1* was evaluated by MSP (methylation-specific PCR) as described previously (Herman *et al*, 1996; Schneider-Stock *et al*, 2003). Primers were used as described in a previous study on *BARD1* methylation (Li *et al*, 2007).

Statistical analysis

The Spearman's correlation coefficient ρ was used to assess the correlation between expression levels of distinct epitopes of BARD1 and BRCA1. The χ^2 test was used to compare the percentage of positive cases in tumour *vs* peri-tumour tissues and correlation of positive cases of BARD1 expression with clinical variables. Survival differences were estimated using Kaplan–Meier method compared by the log-rank test. For all calculations, the tests performed were two-sided, a value of $P<0.05$ was considered statistically significant. Analyses were performed using Statistical Package for the Social Sciences (SPSS) for Windows version 13 (SPSS Inc., Chicago, IL, USA).

RESULTS

BARD1 mRNA expression pattern in colorectal cancer

As in other cancers, we suspected that splice isoforms of *BARD1* were expressed in colon cancer tissues. We assessed *BARD1* mRNA expression in 20 tumour and peri-tumour tissues including 10 male and 10 female cases. We performed RT–PCR using forward primer specific to exon 1 and reverse primers in exons 4 or 11 to amplify the corresponding *BARD1* coding regions (see Patients and Methods). Human *GAPDH* cDNA was amplified as internal control (Figure 1A).

We have sequenced all isoforms from at least one patient sample and determined the presence of FL BARD1, beta, kappa, and pi in colon cancer, but not alpha, which is expressed in lung and gynaecological cancers. The isoforms labelled * and ** in Figure 1A, correspond to deletion of exons 3 and 4 (gamma del-3), and deletion of exons 2–4 (gamma del 2–3), respectively, which were reported previously (Sporn *et al*, 2011) (Supplementary Figure 1).

We have also investigated the use of alternative transcript start sites of BARD1 using 5′RACE from different exons (2, 3, and 6), but we did not observe any other start sites than those already described (Li *et al*, 2007).

Interestingly, all BARD1 isoforms that were recently identified in NSCLC (Zhang *et al*, 2012), including isoforms κ, lacking exon 3, and π with a partial deletion (408 bp) of exon 4, were expressed in colorectal cancer (Figure 1A and C).

BARD1 expression in colon cancer is not regulated by oestrogen or methylation

As oestrogen signalling was associated with colon cancer initiation and progression (Hogan *et al*, 2009), and expression of *BARD1* and *BARD1* isoforms can be modulated by oestrogen through ERα (Niell *et al*, 2004; Russo *et al*, 2009), we also examined the expression of ERα mRNA in colon peri-tumour and tumour tissues from males and females. MCF-7 cells were used as a positive control. We found no ERα expression in colorectal tissues in these samples (Figure 1B). Consistent with this result, similar profiles were observed for FL BARD1 and isoforms expression levels and frequency in colorectal tumours and corresponding peri-tumour tissue from males and females ($P>0.05$) (data not shown).

As methylation was reported for the *BRCA1* promoter (Esteller *et al*, 2001), we investigated whether the *BARD1* promoter was methylated in colon cancer. Methylation analysis of 109 tumour samples (Table 1) by MSP revealed only one methylation-positive case (data not shown). A similar negative result was obtained for *BARD1* promoter methylation in breast/ovarian cancer (Li *et al*, 2007).

BARD1 isoforms are upregulated in colon tumours and expression correlates with clinical variables

On the contrary to what was observed in NSCLC (Zhang *et al*, 2012), FL BARD1 and BARD1 isoforms were significantly more expressed in tumour than in peri-tumour tissues ($P<0.05$) (Figure 1A and 2A, B). Full-length BARD1 and isoforms were expressed in 90%, of the tumour samples (18 of 20), whereas in peri-tumour tissue BARD1 expression was much less frequent (35%, 7 of 20 cases). In seven samples, only FL BARD1 or FL BARD1 and few isoforms were expressed. The difference was statistically significant ($P=0.0003$) (Figure 2A). Similar results were obtained in males (8 out of 10 in tumour tissues *vs* 4 out of 10, in peri-tumour, $P=0.0679$) and in females (10 out of 10 *vs* 3 out of 10, respectively, $P<0.001$).

To determine whether BARD1 isoform expression correlated with patients' clinicopathological characteristics, we compared expression of FL BARD1 and isoforms with clinicopathological variables based on the presence or absence of their expression in tumour tissues. Full-length BARD1 and BARD1 isoforms were more frequently expressed in patients older than 60 years (Figure 2C). In particular, frequencies of *BARD1* isoforms φ, δ, and π expression were significantly associated with older age ($P<0.01$). The frequency of isoform BARD1κ expression was significantly associated with large tumour size or with nearby tissue invasion (T3 and T4; $P=0.0098$), lymph node involvement (N1 and N2, $P=0.0422$), and advanced tumour stages (stage III and IV, $P=0.0422$) (Figure 2D–F). No correlation was observed between *BARD1* expression and histopathological tumour grade.

Only few BARD1 isoforms are likely to influence tumorigenesis

We compared our BARD1 isoform expression pattern with 19 isoforms reported by Sporn *et al* (2011). Only those isoforms that were previously reported by us (Li *et al*, 2007; Zhang *et al*, 2012) contained an open reading frame (ORF) and are likely to be

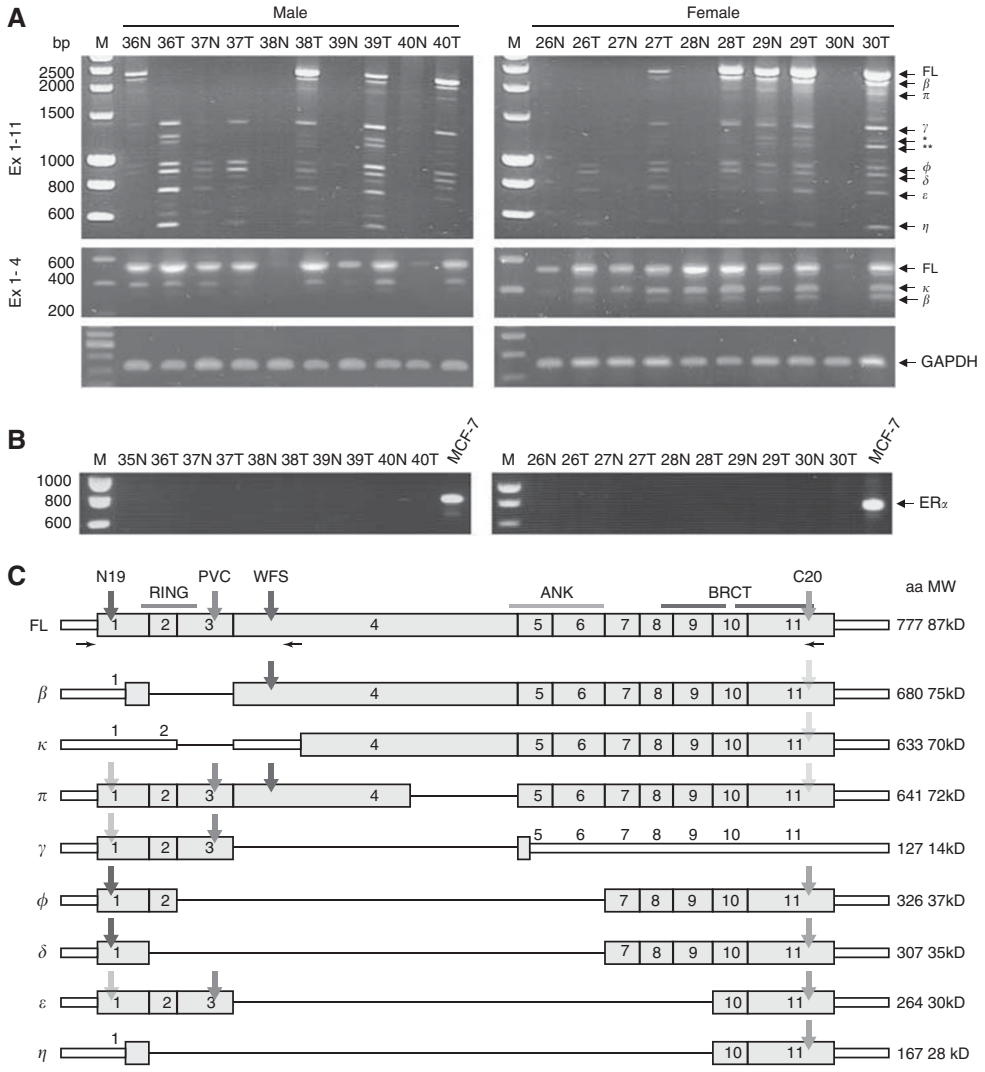


Figure 1 *BARD1* mRNA isoform expression in colorectal cancer. (**A**) Amplification of FL BARD1 and/or isoforms using forward primer in exon 1, and reverse primer in exon 11 (Ex 1–11) or exon 4 (Ex 1–4). As examples, pairs of peri-tumour (N) and tumour (T) tissues of five male and five female patients are shown. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression is shown for the same samples as standard. Molecular marker is shown on the left (M). Presumed FL BARD1 and truncated isoforms are indicated on the right. The isoforms labelled * and ** in correspond to the deletion of exons 3 and 4 (gamma del-3), and deletion of exons 2–4 (gamma del 2–3), respectively, which were reported previously (Sporn *et al*, 2011). Patterns of isoforms were different in peri-tumour and tumour tissues expressed and expression was less frequent in peri-tumour tissues. (**B**) Amplification of oestrogen receptor α (ERα) in the same samples. MCF-7 was used as positive control (right). No ERα expression was observed in colorectal tissues, neither in peri-tumour nor in tumour samples of males and females. (**C**) Schematic presentation of exon structure of BARD1 and isoforms. Exons with ORF are shown as light blue bars, non-coding sequences as white narrow bars and alternatively translated sequences are shown as yellow bars. The positions of the primers used for RT–PCR are shown as horizontal black arrows below BARD1 FL scheme. BARD1 epitopes recognised by N19, C20, PVC, WFS antibodies are indicated with coloured arrows. The antibody epitopes presumably hidden owing to the protein conformation are shown as pale arrows. RING domain (RING), Ankyrin repeats (ANK) and BRCT domains (BRCT) are shown. The color reproduction of this figure is available on the *British Journal of Cancer* online.

translated (Supplementary Figure 1). Oncogenic functions have been attributed previously to isoforms BARD1β, κ, and π (Li *et al*, 2007; Ryser *et al*, 2009; Zhang *et al*, 2012). Isoforms φ, δ, ε, and η were found in tumours with poor outcome in ovarian and breast cancer (Li *et al*, 2007). The oncogenic roles of BARD1β and δ are based on antagonising the functions of FL BARD1 (Ryser *et al*, 2009; Dizin and Irminger-Finger, 2010; Bosse *et al*, 2012).

Isoform γ could be translated in two ways: either translation of ORF common to FL BARD1 from exon 1 through exon 3, ending in a stop codon in exon 4, or an alternative ORF and translation start in exon 3 and translation of exons 4 through 11. We used γ-specific siRNA to repress γ expression in cell cultures and investigated the resulting BARD1 protein profile on western blots (Supplementary Figure 2). We thus identified BARD1γ as a protein translated from exons 1 through 3. BARD1γ comprises the RING domain and could potentially bind to BRCA1 as well as FL BARD1. Consistent with this view, the level of FL BARD1 dropped in the cells treated with γ-specific siRNA (Supplementary Figure 2). This suggests that a BARD1γ-encoded protein is able to interact and stabilise FL BARD1 and possibly BRCA1.

Our data suggest that only few of the *BARD1* mRNA isoforms are translated into stable proteins. These few protein isoforms therefore could be reflected in the antibody staining patterns of colon cancer tissues.

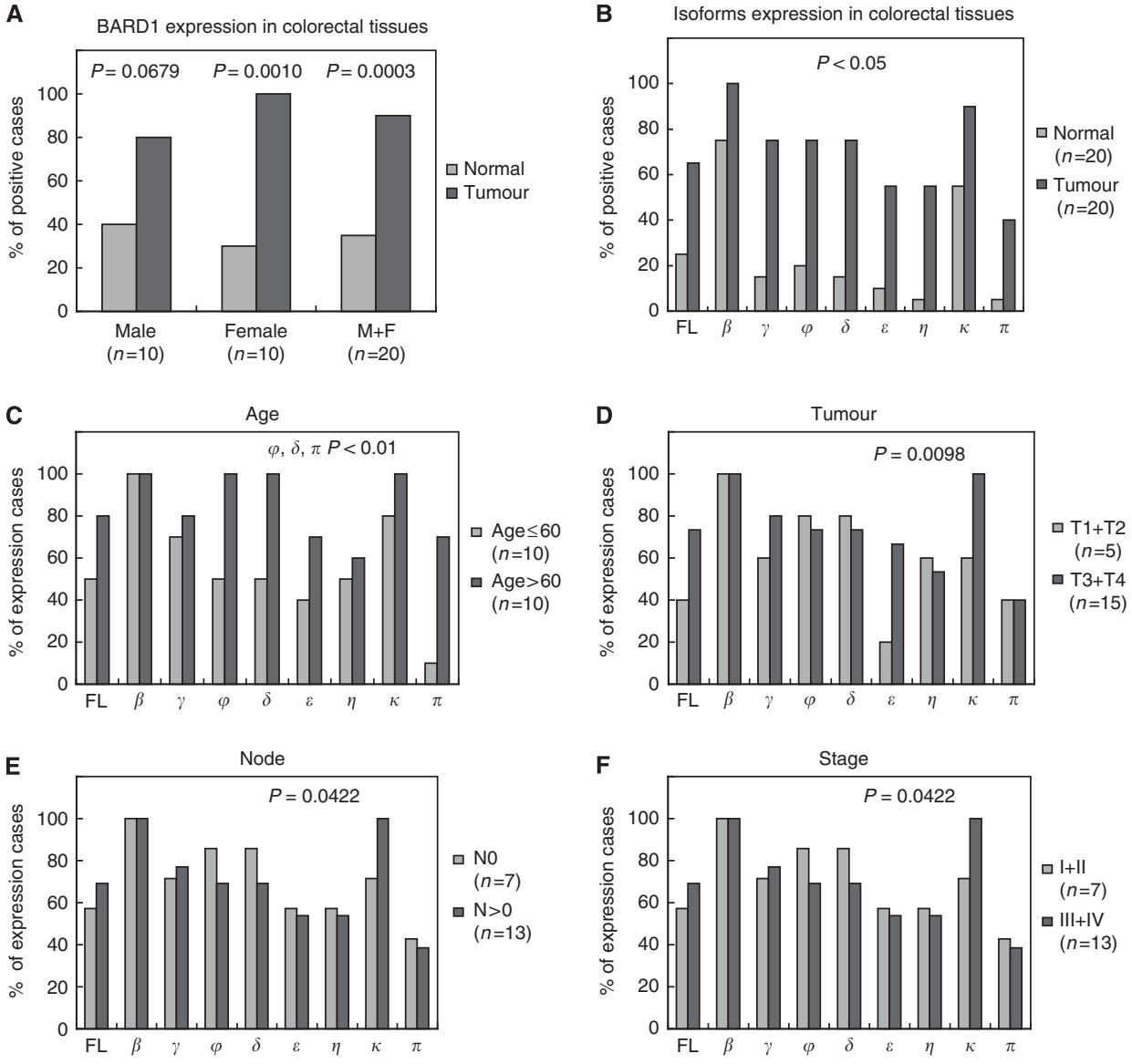
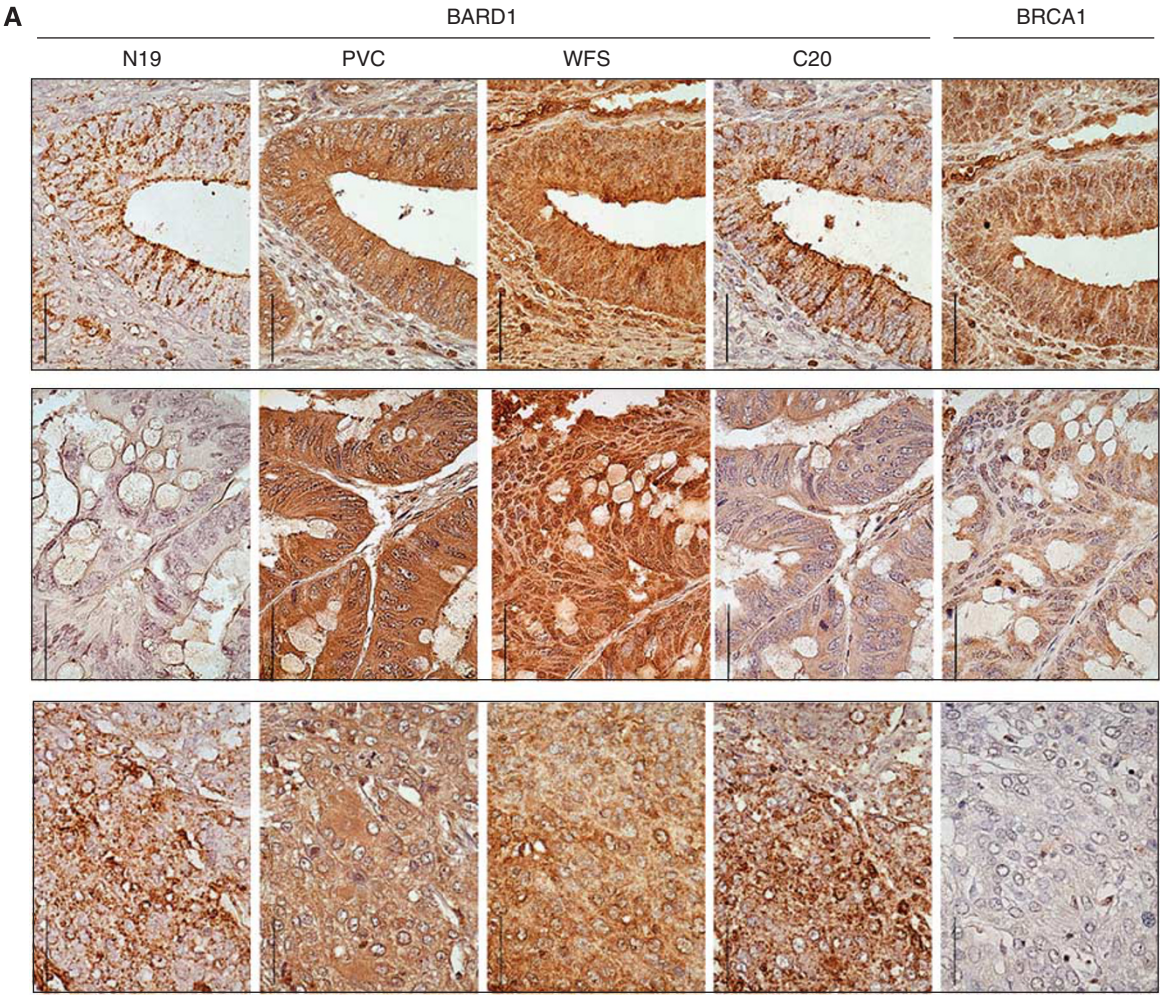


Figure 2 Correlation of BARD1 mRNA isoform expression with clinicopathological variables of the patients with colorectal cancer. (**A**) Comparison of BARD1 expression in peri-tumour and tumour tissues from males, females, or both, based on absence or presence of any form of BARD1. BARD1 expression significantly was more frequent and more abundant in tumours than peri-tumour tissues. The *P*-value was obtained by the χ^2 test. (**B**) Comparison of FL BARD1 and isoform expression in peri-tumour and tumour tissues. All forms were upregulated in tumours with statistical significance ($P<0.05$ for all). The *P*-value was obtained by the χ^2 test. (**C**) Comparison of FL BARD1 and isoform expression in younger ($\leqslant 60$ years) and older (>60 years) patients. Full-length BARD1 and all isoforms, except isoform β, were more upregulated in older than in younger patients. Specially, expression of isoforms φ, δ, and π were significantly associated with older patients ($P<0.01$). The *P*-value was obtained by the χ^2 test. (**D**–**F**) Comparison of FL BARD1 and isoforms expression with primary tumour and lymph node status, and tumour stage and grade. BARD1 isoform κ expression was significantly associated with large tumour size or nearby tissue invasion (**D**), lymph node involvement (**E**), and advanced stage (**F**) (stage III and IV). The *P*-value was obtained by the χ^2 test in all cases.

BARD1 expression in colorectal cancer samples

To investigate BARD1 expression in colorectal cancer, we performed IHC on 168 colon cancer cases, including 20 paired tumour and peri-tumoral normal tissue sections and 148 colorectal tumours presented as tissue microarray with tetramerous for each case (Table 1). To distinguish the expression of different exons of BARD1, we used four previously characterised antibodies (Irminger-Finger *et al*, 1998; Wu *et al*, 2006; Li *et al*, 2007; Zhang *et al*, 2012) (N19, PVC, WFS, and C20) recognising regions in exon 1 (N-terminus), end of exon 3 (after RING region), beginning of exon 4, and exon 11 (C-terminus) (Figure 1C), on adjacent tissue sections. We also investigated BRCA1 expression using an antibody against BRCA1. Staining was mostly cytoplasmic with all BARD1 antibodies. However, different antibodies stained different sub-cellular compartments and/or different tumour regions (Figure 3A). Typically, BARD1 N19 and C20 showed a granular staining, whereas PVC and WFS showed diffuse staining and they were co-localised to the same cells or the same regions, respectively.

The positivity was variable for each antibody (Figure 3B). BARD1 N19, PVC, WFS, and C20 staining were classified as positive in 36 (24.8%), 122 (84.1%), 129 (89%) and 61 (42.1%) cases, respectively. A total of 142 cases were positive for at least one antibody, and no expression of BARD1 was found in only 3 cases. Hence, 97.9% (142 of 145) of colorectal cancer samples expressed at least one epitope of BARD1.



B

BARD1 expression pattern in CRC

n=145	N19	PVC	WFS	C20
56	−	+	+	−
27	+	+	+	+
26	−	+	+	+
12	−	−	+	−
6	−	+	−	−
4	−	−	+	+
3	+	+	−	−
2	+	−	+	+
1	−	+	−	+
1	−	+	−	−
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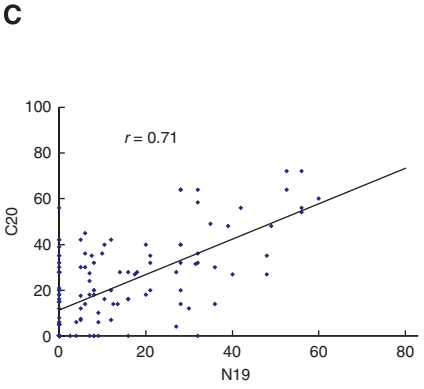
1	+	+	+	−
3	−	−	−	−



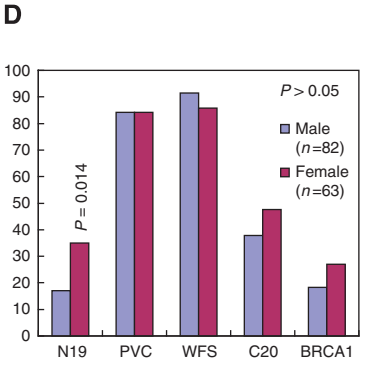


Figure 3 BARD1 and BRCA1 expression in colorectal cancer. Immunohistochemistry (IHC) was performed on samples of 168 colorectal cancer cases with BARD1 antibodies N19, C20, PVC, WFS, and BRCA1. Samples were presented as tissue microarray with tetramerous for each of the cases. A total of 145 samples were eligible for analysis after IHC assay. (**A**) Examples of IHC using BARD1 antibodies and BRCA1 antibody. BARD1 N19 and C20 showed cytoplasmic granular staining, and co-localised to the same cells or tissue regions. BARD1 PVC and WFS stainings were diffusely cytoplasmic. BRCA1 staining was granular in both cytoplasm and nucleus. Examples of the positive staining with BARD1 antibodies and BRCA1 antibody (upper panel), the weaker staining with N19, C20, and BRCA1 antibodies (middle panel) and BARD1-positive but BRCA1-negative staining (lower panel) are shown. Scale bars correspond to 50 μm. (**B**) BARD1 expression patterns in colorectal cancer. Expression patterns were obtained for four BARD1 antibodies based on positive (+) and negative (−) staining for each case. PVC- and WFS-positive, but N19- and C20-negative staining was the most frequent expression pattern, 'all four antibodies positive' staining was the second, N19-negative but PVC-, WFS-, and C20-positive staining was the third most frequently observed expression pattern. (**C**) The correlation of BARD1 N19 and C20 antibodies staining. (**D**) BARD1 N19-positive staining was significantly associated with female gender ($P = 0.014$). The *P*-value was obtained by the χ^2 test.

Table 2 Correlation of distinct epitopes of BARD1 and BRCA1 expression with survival in 75 colorectal cancer patients

			1-year survival		2-year survival		3-year survival	
Expression pattern	**No. of patients**	**Median survival**	**%**	***P*-value**	**%**	***P*-value**	**%**	***P*-value**
N neg *vs* pos	55 *vs* 20	11 *vs* 26	47.3 *vs* 85.0	0.0035	21.8 *vs* 50.0	0.0178	12.7 *vs* 40.0	0.009
P neg *vs* pos	14 *vs* 61	14 *vs* 15	57.1 *vs* 57.4	0.9873	28.6 *vs* 29.5	0.9446	21.4 *vs* 19.7	0.8822
W neg *vs* pos	4 *vs* 71							
C neg *vs* pos	42 *vs* 33	9 *vs* 17	45.2 *vs* 72.7	0.0169	21.4 *vs* 39.4	0.0898	11.9 *vs* 30.3	0.048
BRCA1 neg *vs* pos	60 *vs* 15	16 *vs* 12	60.0 *vs* 46.7	0.3504	31.7 *vs* 20.0	0.3747	23.3 *vs* 6.7	0.1489

Note: 'neg', negative staining; 'pos', positive staining. Antibody abbreviations: N - N19, P - PVC, C - C20, W - WFS. For WFS, negative staining cases were not enough for further analysis.

Table 3 Correlation of BARD1 expression patterns with survival in 75 colorectal cancer patients

			1-year survival		2-year survival		3-year survival	
Expression pattern	**No. of patients**	**Median survival**	**%**	***P*-value**	**%**	***P*-value**	**%**	***P*-value**
+ + + + *vs* − + + −	17 *vs* 31	27 *vs* 9	88.2 *vs* 41.9	0.0019	52.9 *vs* 16.1	0.0073	41.2 *vs* 6.5	0.0032
+ + + + *vs* − + + +	17 *vs* 11	27 *vs* 12	88.2 *vs* 45.5	0.0144	52.9 *vs* 18.2	0.0659	41.2 *vs* 9.1	0.0664
+ + + + *vs* others	17 *vs* 58	27 *vs* 12	88.2 *vs* 48.3	0.0034	52.9 *vs* 22.4	0.0151	41.2 *vs* 13.8	0.0131
− + + − *vs* − + + +	31 *vs* 11	9 *vs* 12	41.9 *vs* 45.5	0.8390	16.1 *vs* 18.2	0.8750	6.5 *vs* 9.1	0.7702
− + + − *vs* others	31 *vs* 44	9 *vs* 16.5	41.9 *vs* 68.2	0.0236	16.1 *vs* 38.6	0.0350	6.5 *vs* 29.5	0.0138
− + + + *vs* others	11 *vs* 64	12 *vs* 15.5	45.5 *vs* 59.4	0.3885	18.2 *vs* 31.3	0.3792	9.1 *vs* 21.9	0.3274

Note: + + + +, four antibodies positive staining; − + + −, only PVC and WFS positive staining; − + + +, only N19-negative staining; others, other than the expression pattern that is compared.

Although there are 16 possible combinations for the expression of four BARD1 epitopes, only 3 combinations were observed (Figure 3B): simultaneous expression of epitopes PVC and WFS (after RING domain and exon 4, respectively) was the most frequent pattern (38.6%), positive staining for all four antibodies was the second most frequent (18.6%), and loss of the N-terminal epitope but expression of PVC, WFS, and C20 (17.9%) the third most frequent.

To investigate this further, we quantified and compared the expression patterns obtained for each antibody. Strong correlation was observed between expression levels of N19 and C20 ($\rho = 0.71$, $P = 0.001$) (Figure 3C). Other comparisons showed only weak correlations, namely for PVC and WFS ($\rho = 0.39$, $P = 0.001$), PVC and C20 ($\rho = 0.36$, $P = 0.001$), and WFS and C20 ($\rho = 0.27$, $P = 0.001$), or no correlation for N19 and PVC, and N19 and WFS staining (data not shown).

Correlated expression of N19 and C20 is consistent with expression of isoforms δ, φ, and ε. All other patterns of expression might reflect a combination of expression of isoforms β, κ, and π. Based on these analyses, none of the observed expression patterns is compatible with expression of FL BARD1.

Non-coordinate expression of BARD1 epitopes and BRCA1

As mentioned, BARD1 is important for stability and subcellular localisation of BRCA1. Unlike BARD1, BRCA1 showed both cytoplasmic and nuclear granular staining within the same cell (Figure 3A). BRCA1-positive staining was observed in 22.1% (32 of 145) of colorectal cancer cases, similar to BARD1 N19-positive staining, which was observed in 24.8% (36 of 145) of the cases. However, BRCA1 expression was not correlated with expression of any BARD1 epitope (data not shown).

BARD1 expression pattern correlates with patients' prognosis but not with other clinicopathological characteristics

Immunohistochemistry analysis of BARD1 N19, PVC, WFS, C20, and BRCA1 expression was compared with clinical variables of 145 cases eligible for the statistical analysis.

No significant correlation was observed between the expression of BRCA1 or BARD1 epitopes and clinicopathologic variables, such as tumour grade, primary tumour, lymph node and distant metastasis status, or tumour stage. We also analysed the correlation between the three major expression patterns of BARD1 (Figure 3B) with clinicopathological variables. No significant correlation was observed in this case either (data not shown).

To assess the correlation of BARD1 and BRCA1 expression with survival, we compared the individual expression of the four BARD1 epitopes, as well as BRCA1 epitope, and the different BARD1 expression patterns (Figure 3B) with survival data for 75 colorectal cancer cases with follow-up data (Tables 2 and 3). For the individual BARD1 epitopes, we found that patients with BARD1 N19 (N-terminal epitope)-positive staining had significantly higher 1-year, 2-year, and 3-year survival rates, while the BARD1 C20 (C-terminal epitope)-positive patients had significantly higher 1-year and 3-year survival rates when compared with the patients with the negative staining for the corresponding epitopes (Table 2). Interestingly, the frequency of N19-positive staining was also significantly associated with female sex ($P = 0.014$, Figure 3D). No conclusion could be made from the comparison of BARD1 PVC- and WFS-positive patients' survival rates as the number of negative staining cases was not sufficient for the analysis. No significant difference was observed for the comparison of BRCA1-positive and -negative cases with corresponding survival rates (data not shown).

When BARD1 epitope expression patterns were used for the correlation studies, we found that the simultaneous expression of all four BARD1 epitopes correlated with higher 1-, 2-, and 3-year survival rates, when compared with the expression of only two middle epitopes or other expression patterns. However, the expression of only two middle epitopes (PVC and WFS) was correlated with lower 1-, 2-, and 3-year survival rates as compared with all other expression patterns including the all four antibodies positive staining pattern (Table 3).

Taken together our data suggest that the simultaneous expression of all four BARD1 epitopes is a positive prognostic factor, as well as the expression of BARD1 N and C-terminal epitopes. Inversely, the simultaneous expression of only two middle epitopes (PVC and WFS) is a negative prognostic factor. These data can

only be explained with the simultaneous expression of a combination of isoforms (Figure 1C). Isoforms expressing PVC and WFS epitopes are clearly correlated with poor prognosis in colon cancer, and were correlated with decreased survival in lung cancer (Zhang *et al*, 2012). Which isoforms are contributing to a positive prognosis has to be determined.

DISCUSSION

In the present study, we demonstrate that *BARD1* is differentially spliced in colon cancer, that protein products of splice isoforms might affect BRCA1 localisation, and that the splice isoforms might have oncogenic functions by themselves. Alternative splicing of tumour suppressor genes can produce proteins with dominant negative functions, which are often found associated with cancer (Srebrow and Kornblihtt, 2006). Antagonistic functions were reported for BARD1 isoform β (Li *et al*, 2007; Bosse *et al*, 2012) and for isoform δ (Dizin and Irminger-Finger, 2010).

By using antibodies against the differentially expressed regions, we found at least one of the respective epitopes expressed in each of 168 samples of the colorectal cancer. The pattern of positive epitopes excluded any relevant expression levels of FL BARD1. Overexpression of oncogenic forms rather than repression of FL BARD1 is consistent with lack of promoter methylation of *BARD1*, as observed in all colorectal cancer samples tested ($N=98$). Similarly, no methylation of *BARD1* promoter was found in ovarian cancer (Li *et al*, 2007). *In vitro* repression experiments demonstrated that BARD1 isoform expression is essential for cell proliferation (Li *et al*, 2007; Ryser *et al*, 2009; Bosse *et al*, 2012). Our results therefore suggest that rather than loss of BARD1 expression, it is the expression of at least one form of BARD1 that might be essential for tumour growth.

There is evidence for a role of *BRCA1* in hereditary as well as sporadic colon cancer (Garcia-Patiño *et al*, 1998; Lin *et al*, 1999; Mohamad and Apffelstaedt, 2008; Russo *et al*, 2009; Suchy *et al*, 2010). *BRCA1* expression in colon cancer might be affected by the aberrant expression of *BARD1*. The un-coordinated expression of BARD1 epitopes excludes expression of FL BARD1 and suggests that the E3 ubiquitin ligase functions of the BRCA1–BARD1 heterodimer (Baer and Ludwig, 2002) are jeopardised in colorectal cancer. Dysfunction of the BRCA1-BARD1 ubiquitin ligase can affect repair functions and lead to genomic instability.

There is evidence that differentially spliced BARD1 isoforms might be themselves drivers of tumorigenesis. Their expression was correlated with poor prognosis in breast, ovarian, and lung cancer (Wu *et al*, 2006; Li *et al*, 2007; Zhang *et al*, 2012) and as shown here, in colon cancer. Especially, isoforms that express epitopes mapping to exons 3 and 4, present on BARD1 β, κ, π, are correlated with short survival in colorectal cancer, as well as lung cancer (Zhang *et al*, 2012). *In vitro* experiments support the notion that BARD1 isoforms may be drivers of tumorigenesis, as they have transforming activity (Bosse *et al*, 2012) and are required for cancer cell proliferation (Li *et al*, 2007; Ryser *et al*, 2009; Bosse *et al*, 2012).

We found that *BARD1* mRNA isoforms were generally more expressed in females (Figure 1A), and N19-positive staining was significantly associated with female gender in colorectal cancer ($P=0.014$). However, their expression cannot be driven by oestrogen and ERα, as no ERα mRNA expression was found in 20 cancer cases that we analysed.

Alternatively spliced BARD1 isoforms in colon cancer have also been reported by others (Sporn *et al*, 2011), based on 15 and 99 colon tumours, analysed by RT–PCR and IHC, respectively. Of 19 mRNA isoforms that were characterised, only few are protein coding and likely to affect tumorigenesis; all of these have been reported previously by us and others (Supplementary Figure 1). Immunohistochemistry analysis of 99 colon tumours was only based on one monoclonal antibody directed against an undefined epitope within the first 300 amino acids of BARD1 (Sporn *et al*, 2011). The expression of other regions and isoforms lacking the N-terminus were not investigated with this method. Thus, the conclusion of the authors, that lack of FL BARD1 is a negative prognostic and prospective marker is only partially true. The N-terminal epitope detected with this antibody could, in addition to FL BARD1, detect isoforms π, γ, δ, φ, and ε. Thus, the Sporn *et al* (2011) study identified isoforms on the mRNA level, but their relevance for tumorigenesis was not completely addressed at the protein level.

We found that a positive staining pattern for four antibodies was significantly associated with longer survival in colorectal cancer, so were N19- and C20-positive staining. The expressions of N- and C-terminal epitopes are significantly correlated and are consistent with isoform δ or φ expression, suggesting an inhibitory effect of isoform δ or φ on tumorigenesis. The positivity for four antibodies most likely reflects a combination of isoforms and not FL BARD1.

We found that PVC- and WFS-positive staining was strongly correlated with shorter survival in colon cancer. This staining is consistent with isoforms β, κ, and π expression. N19 and C20 epitopes might be blocked in these isoforms, as they are located in structured regions of the RING and BRCT domains, respectively (Figure 1C). To support the hypothesis that the C20 epitope is present, but not accessible in most isoforms, we have used an antibody against a different sequence in exon 11, as used in a study of lung cancer (Zhang *et al*, 2012) and compared it to the C20 staining pattern (Supplementary Figure 3). Similarly, we have performed IHC with a commercial antibody BL (exon 4) and compared its staining pattern with that of WFS (exon 4) on a selected number of colon cancer tissue samples (Supplementary Figure 3). These staining patterns demonstrate that WFS, BL, and P8 antibodies show the identical signal distribution indicating their specificity, whereas C20 staining is much weaker supporting our hypothesis that the C20 epitope may be present but not accessible in some isoforms.

Comparison of mRNA isoform expression in colon tumour tissues showed that specifically the expression of *BARD1* isoforms κ was significantly correlated with advanced tumour stage and invasiveness (Figure 2D–F). Upregulation of the same mRNA isoform was also observed by Sporn *et al* (2011) in colon cancer.

In summary, our data strongly suggest that BARD1 isoforms κ, β, and π are involved in colon cancer tumorigenesis and progression and might be promising specific prognostic markers, while isoforms δ and φ might have an inhibitory effect. Further studies are needed for defining the use of BARD1 isoforms as prognostic markers for response to treatment regimens.

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Conflict of interest

IIF and YQZ have a conflict of interest. Both have authored a patent on BARD1 isoforms. The other authors declare no conflict of interest.

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