Expression and subcellular localization of the bromodomain-containing protein 7 is a prognostic biomarker in breast cancer

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Bromodomain-containing protein 7 (BRD7) is a member of the bromodomain-containing protein family. Previous studies suggest that BRD7 is predominantly localized in the nucleus, wherein it functions as a transcriptional regulator. Several lines of evidence imply a tumour suppressor function for BRD7. However, the importance of BRD7 in the pathogenesis of breast cancer is not well understood. We have investigated the expression, CpG island methylation and subcellular localization of BRD7 in breast cancer cell lines and clinical cases and thereby assessed its prognostic significance by correlating with clinical-pathological features and time-dependent clinical outcomes. We show that nuclear exclusion of BRD7 occurs commonly in breast cancer and is strongly associated with cases expressing wild-type p53. Moreover, clinical outcomes are significantly less favourable in cases with nuclear exclusion or loss of expression than those in which there is nuclear expression of BRD7. Methylation of the CpG island of *BRD7* increases in breast cancer relative to normal breast tissue, but there is not an obvious correlation between methylation and reduced expression or between methylation and clinical outcomes. Overall, our results suggest that nuclear exclusion, rather than transcriptional silencing, is a common mechanism by which the tumour suppressor function of wild-type p53 is inhibited in breast cancer, and show that *BRD7* is a promising candidate biomarker in breast cancer.

Keywords: biomarker, breast cancer, bromodomain-containing protein 7, expression, localization, methylation

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Introduction

Breast cancer is the most frequently diagnosed invasive cancer in females affecting approximately 12% of women [1]. Despite obvious improvements in the systemic management of the disease, metastatic breast cancer remains an essentially incurable disease which is a major source of female morbidity and mortality.

Molecular genetic platforms such as OncotypeDX have improved risk stratification for a subset of patients with early breast cancer [2]. However, clinical decision-making with regard to postoperative (adjuvant) chemotherapy for many patients continues to be based on clinical-pathological indices such as the Nottingham Prognostic Index and the St. Gallen criteria. As such, there is an obvious need for simple, cost-effective prognostic and predictive biomarkers which allow accurate future risk-assessment to inform optimal risk-reducing postoperative systemic therapy. Given the frequency of breast cancer diagnoses, such biomarkers should be measurable from routine histo-pathological samples and of sufficient simplicity to be performed and analysed in local laboratories.

Bromodomain-containing protein 7 (BRD7) is a member of the bromodomain-containing protein family [3] mainly localized in the nuclear compartment of the cell [4].

A number of studies have shown that BRD7 is involved in transcriptional regulation and in modulation of chromatin remodelling through interactions with acetylated histone H3 [5–9].

BRD7 was originally proposed as a candidate tumour suppressor gene nasopharyngeal carcinoma (NPC) via negative regulation of the beta-catenin and ERK pathways [10,11]. More recently, accumulating evidence suggests that BRD7 suppresses tumoringenesis by recruiting chromatin-remodelling complexes to the promoters of target genes, affecting histone acetylation, p53 acetylation and promoter activity [12].

Moreover, BRD7 directly interacts with p85α to negatively regulate PI3K signalling, which maintains the homeostatic cell growth [13].

BRD7 participates in the recruitment of BRCA1 and Oct-1 to the oestrogen receptor 1 (ESR1) promoter and may be involved in the regulation of approximately 30% of BRCA1 targets [14]. Loss of BRCA1 or BRD7 resulted in loss of ERα expression in breast cancer cells and resistance to the antiproliferative effects of fulvestrant [14].

Several reports have shown that BRD7 expression is downregulated in cancer and that loss of the protein correlates with clinical outcomes in a broad range of malignant tumours, such as osteosarcoma, prostate cancer, colorectal cancer and epithelial ovarian carcinoma [15–20]. These findings clearly imply a tumour suppressor function for *BRD7* and in the prognosis of patients with cancer.

In breast cancer, BRD7 was downregulated and even deleted on chromosome 16q12, in p53-wild-type but not mutant breast cancer cells [12]. Other studies proved that *BRD7* was not a frequent high-penetrance susceptibility gene in breast cancer because its variants represented only rare polymorphisms [21] and no pathogenic *BRD7* germ-line mutations were found in Triple Negative Breast Cancer patients with familial bC, ruling out the role of BRD7 in the genetic predisposition to breast cancer [22].

Despite the apparent importance of BRD7 in tumour suppression, mechanisms of inactivation of BRD7 in breast cancer remain suboptimally defined and the utility as a biomarker in this disease unexplored.

In the present study, we have investigated the expression of BRD7 in a well annotated series of breast cancer cases from our clinical practice and evaluated its prognostic significance by correlating BRD7 protein expression and subcellular localization with clinical-pathological variables and patient outcomes.

Materials and methods

Cell lines

A panel of 13 breast cancer cell lines (BT-549; HCC1937; Hs 578T; JIMT-1; MCF7; MDA-MB-231; MDA-MB-361; MDA-MB-436; MDA-MB-453; MDA-MB-468; SK-BR-3; T-47D; ZR-75-1) of human origin documented by the American Type Culture Collection archives was used to analyse BRD7 expression and methylation in the CpG island of the gene. Cells were routinely cultured in the appropriate culture medium supplemented with 10% heat-inactivated foetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, in a fully humidified AutoFlow Nu-4750 water Jacket incubator (Nuaire, Plymouth, Minnesota, USA) at a 37°C in an atmosphere containing 5% CO_2.

Patients and tumour tissue samples

A series of 50 human primary breast cancer tissues and paired metastases from 14 patients in our clinical practice were analysed. Patients underwent surgical breast resection (mastectomy/quadrectomy) at the S. Croce & Carle Teaching Hospital between 1998 and 2011. The median follow-up was of 94.05 months and time ranged from 2.2 and 203.5 months, because we updated patients until June 2017. The clinical-pathological characteristics are reported in Table 1. Routine analysis of expression of the oestrogen receptor and the progesterone receptor was performed according to standard protocols of clinical care. Analysis of *TP53* sequence has been reported previously [23]. All primary tissues samples were collected before that patients received any preoperative chemotherapy or radiotherapy. When, possible, patients signed a written informed consent, approved by local Ethical Committee, allowing the retrospective analysis of their biological material.

Analysis of bromodomain-containing protein 7 expression

Expression of BRD7 was studied at the protein level in all 64 clinical cases by immunohistochemistry (IHC). Tissue sections were deparaffinized, rehydrated and subjected

Table 1 Clinical parameters of the 50 patients affected by breast cancer

Characteristics	*N* (%)
Age (years)	
Median 51.4 (range 27.2–80.0)	50
Nodal status	
Negative	20 (40%)
Positive	29 (58%)
Unknown	1 (2%)
Grading	
G1	6 (12%)
G2	20 (40%)
G3	15 (30%)
Unknown	9 (18%)
T classification	
T0/T1	29 (58%)
T2/T3/T4	21 (42%)
Oestrogen-receptor status	
Negative	9 (18%)
Positive	38 (76%)
Unknown	3 (6%)
Progesterone-receptor status	
Negative	20 (40%)
Positive	26 (52%)
Unknown	4 (8%)
HER2 status	
Negative	25 (50%)
Positive	15 (30%)
Unknown	10 (20%)
Ki-67	
≤20%	16 (32%)
>20%	29 (58%)
Unknown	5 (10%)
Neoadjuvant CT	
Yes	33 (66%)
No	16 (32%)
Unknown	1 (2%)
Adjuvant CT	
Yes	32 (64%)
No	17 (34%)
Unknown	1 (2%)
Adjuvant HT	
Yes	37 (74%)
No	12 (24%)
Unknown	1 (2%)
Herceptin administration in HER2 pos (*N*=15)	
Yes	11 (73%)
No	4 (27%)

CT, computed tomography; HT, hormone therapy.

to antigen retrieval using standard protocols. Endogenous peroxide blocked tissue sections were incubated with the anti-BRD7 rabbit polyclonal antibody (1:250) against the C-terminal portion (aa 361-651) of human BRD7 for 1 hour and 30 minutes [12]. The sections were developed with diaminobenzidine tetrahydrochloride on a Ventana Benchmark ULTRA automated Immunostainer (Ventana Medical System Inc., Tucson, Arizona, USA) using standard procedures. The degree and localization of immunostaining were reviewed and scored by a pathologist who was blinded to the clinical data of patients. For each slide, at least 1000 tumour cells were analysed and the percentage of positively stained cells recorded. Cases were classified according to nuclear or non-nuclear BRD7 antigen localization and then stratified for high and low BRD7 expression according to Wu *et al.* [16] and Chen *et al.* [24]. The proportion of BRD7-expressing cells varied from 0 to 100% and the intensity of nuclear staining from weak to strong.

Methylation analysis of bromodomain-containing protein 7 CpG island

Aberrant CpG methylation was analysed using Pyrosequencing (Biotage, Uppsala, Sweden). A large CpG island is located in the 5′ regulatory sequences of *BRD7* and in its first exon and IVS I region (http://genome.ucsc.edu). PCR primers were designed to amplify a fragment of 124 bp in IVS I. This amplicon contains 14 CpG dinucleotides and has been optimized for software dedicated to methylation analysis (PyroMark CpG Software 1.0.11).

Primer sequences were as follows:

Forward: 5′ -BIOT TTTTTTTTTAAGGTAGGTT TAGGT- 3′

Reverse: 5′ - CTCCCCACCAAAAACCCAC- 3′

Genomic DNA was extracted both from cell lines and FFPE tissues using proteinase K. Five hundred nanograms of genomic DNA were subjected to bisulfite conversion using the EZ methylation Kit (Zymo research, Irvine, California, USA) according to the manufacturer's instructions. Fifty nanograms of bisulphite modified DNA wereused for PCR with the following conditions: 95°C for 10 minutes; 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 40 seconds (40 cycles); 72°C for 7 minutes.

PCR products were resolved on 2% agarose gels, visualized on a transilluminator and then analysed by pyrosequencing using the PyroMark ID System PyroMark Q96 ID (Biotage) using the Biotage Sample Prep kit with the specific sequencing nonbiotinylated forward primer. After the Pyrosequencing run, analysis of methylation percentage at each CpG dinucleotide was performed using QCpG Software (Qiagen). As unmethylated (unmet) controls, we used DNA obtained from three healthy breast tissues (average methylation: 3%) and from placenta (average methylation 0%), while as methylated (met) control we used a universal commercial human DNA (CpGenome Universal Methylated DNA, Millipore Corporation, Billerica, Massachusetts, USA) (average methylation 98%). On the basis of this consideration, as in our previously works, we established a methylation cutoff of 10%.

Real-time quantitative reverse transcriptase PCR

RNA extraction from fresh cell lines, reverse transcription and real-time PCR was performed according to standard methods as described previously [23]. RNA concentration and purity were measured by absorbency at 260 nm on a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA). Real-time quantitative reverse transcriptase PCR (qRT-PCR) analysis was performed with the resulting cDNAs, to assess the relative mRNA expression levels of *BRD7* and beta-2-microglobulin (an internal control) on the 7000 Sequence Detection System using the Hs00832493_sH and Hs99999907_m1 assays, respectively. Relative expression level of *BRD7* mRNA was calculated and normalized to that of beta-2-microglobulin using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

Statistical analysis

Overall survival (OS) analysis was based on the time from diagnosis to death or last contact in which the survivors were censored. Progression-free survival (PFS) analysis was based on the time from diagnosis to first event (locoregional recurrence or distant metastasis); patients without an event were censored at their last follow-up. Patients that died from treatment-associated toxicities were considered as not progressed. Survival curves were plotted by the Kaplan–Meier method and compared by the log-rank test for statistical significance. Relationships between BRD7 expression and clinical parameters (site, age, tumour size, histological grade and nodal status) and molecular characteristics (HER2, oestrogen receptor, progesterone receptor and Ki67) were analysed by cross-tabulation.

The effects of BRD7 expression and breast cancer clinical characteristics on survival were evaluated using univariate and multivariate Cox regression analyses (proportional-hazard model).

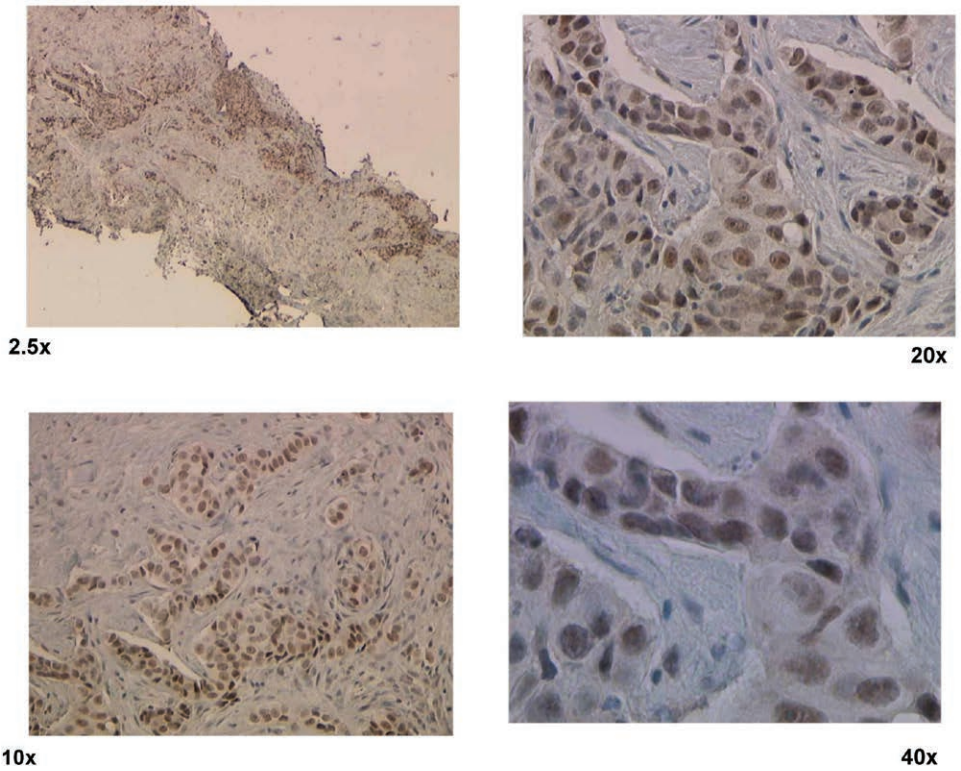
Significant variables ($P < 0.30$) were entered into Cox multivariable logistic regression with morbidity as the dependent variable and significance set at the 0.05 level. Significant independent predictors of morbidity were used to construct a prognostic index using the coefficients from the multivariable analysis. Statistical analyses were performed using R software, version 3.3.1.

Results

Bromodomain-containing protein 7 is expressed and the CpG island unmethylated in breast cancer cell lines

BRD7 was expressed at levels comparable to normal breast epithelium in all 13 breast cancer cell lines

Fig. 1



Representative IHC analysis on tumoral breast cancer tissue scored as 3+ using specific anti-BRD7 antibody. 2,5-10-20-40x indicate different level of imagine magnification. BRD7, bromodomain-containing protein 7; IHC, immunohistochemistry.

analysed. Consistent with expression analysis, the *BRD7* CpG island was unmethylated in all 13 breast cancer cell lines (average CpG methylation of 2.1%, which is comparable to negative control DNA (data not shown).

Bromodomain-containing protein 7 expression and methylation in clinical cases of breast cancer

Notwithstanding the relatively uniform expression levels of BRD7 mRNA in breast cancer cell lines, we proceeded to perform IHC analysis of BRD7 in a series of 50 clinical cases. The clinical-pathological details of the cases are shown in Table 1. Representative examples of IHC are shown in Fig. 1. BDR7 expression was localized in the nucleus of tumour cells in 34/50 (68%) of cases. In the remaining 16 cases, there was either diffuse staining in the cytoplasm and nucleus ($N=4$) or only in the cytoplasm ($N=6$) and six cases were entirely negative (BRD7–). Because the majority of the samples were biopsies, nonneoplastic tissue adjacent to cancer cells was frequently scanty and difficult to compare. Where it was feasible, we observed a lower BRD7 immunoreactivity in adjacent noncancerous compare with tumour tissues. Genomic DNA was isolated from 42/50 cases and analysed for CpG island methylation using Pyrosequencing. The CpG island was methylated in 11/42 cases.

Subcellular localization of bromodomain-containing protein 7 correlates with specific clinical-pathological variables

Next, we compared clinical-pathological characteristics in samples with nuclear and nonnuclear or absent staining. There was no difference between the two groups in nodal status, grade, clinical stage, histological subtype or in receptor expression status (oestrogen receptor, progesterone receptor and HER2) and Ki-67 expression. However, cases with nuclear BRD7 expression ($N=34$) were smaller ($P=0.012$), of a better vital status ($P=0.002$) and exhibited a lower clinical relapse rate ($P=0.03$). Moreover, cases with nuclear BRD7 expression were more likely to be of wild-type *TP53* status ($P=0.04$) and unmethylated *BRD7* ($P=0.04$) compared to cases with nonnuclear expression.

When we combined nuclear and nonnuclear BRD7 localization with low and high expression, we still observed differences in tumour size and outcome. Of particular note, patients with both nuclear localization and high BRD7 expression presented the smallest tumours (10/12 = 83%), are all alive at the time of censor and only 25% relapsed (3/12) (Table 2).

Table 2 Comparison between clinical-pathological characteristics of breast cancer patients with bromodomain-containing protein 7 nuclear immunohistochemistry localization versus diffuse localization or negative immunohistochemistry

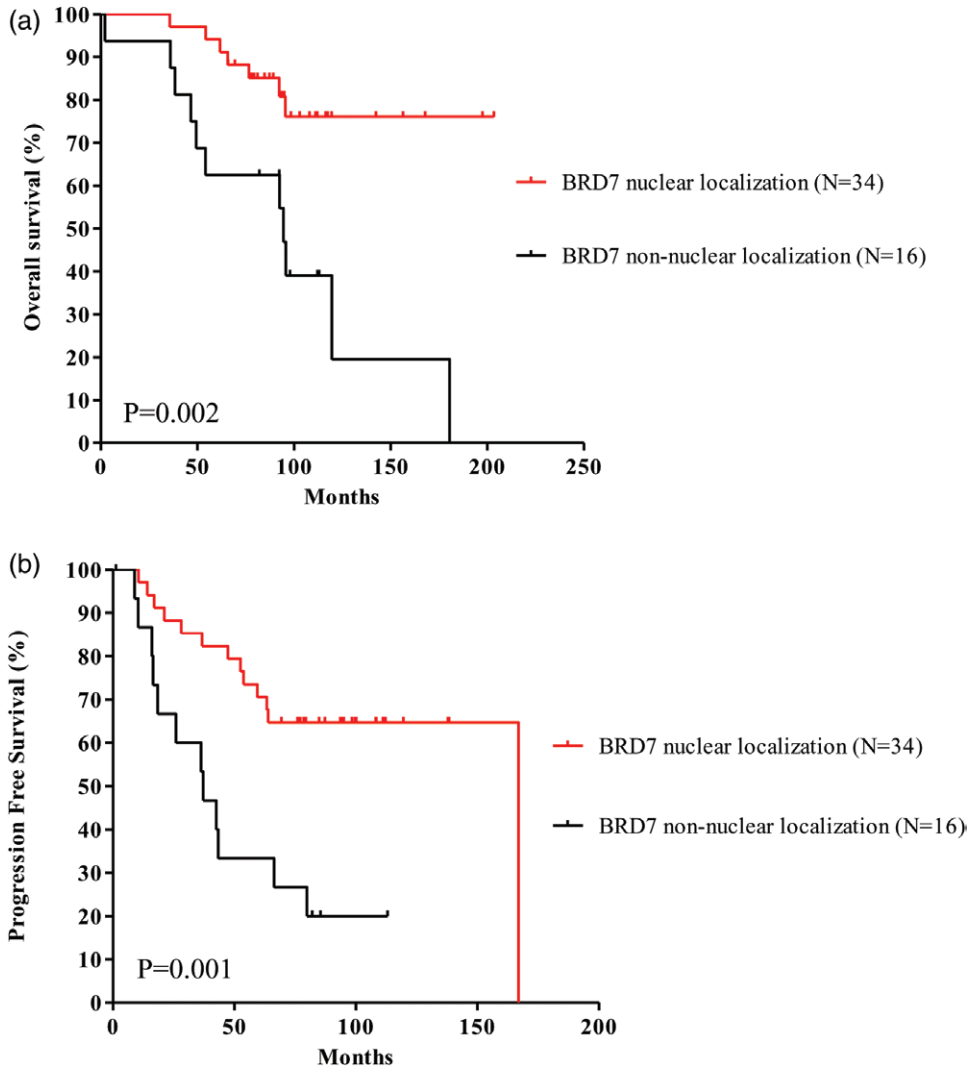
	BRD7 nuclear IHC localization (*N*=34)	%	BRD7 diffuse localization or negative IHC (*N*=16)	%	Chi-square test *P*-value
Age (years)					
<51.4	19	55.9	6	37.5	0.22
>51.4	15	44.1	10	62.5	
T classification					
T0/T1	24	70.6	5	31.3	**0.01**[a]
T2/T3/T4	10	29.4	11	68.8	
Nodal status					
Negative	16	47.1	4	25.0	0.227
Positive	17	50.0	12	75.0	
Unknown	1	2.9	0	0.0	
Grading					
G1	3	8.8	3	18.8	0.32
G2	12	35.3	8	50.0	
G3	11	32.4	4	25.0	
Unknown	8	23.5	1	6.3	
Clinical stage					
II	8	23.5	7	43.8	0.19
III	26	76.5	9	56.3	
Histological type					
Ductal carcinoma	31	91.2	15	93.8	1
Lobular carcinoma	3	8.8	1	6.3	
Oestrogen-receptor status					
Negative	6	17.6	3	18.8	0.4
Positive	27	79.4	11	68.8	
Unknown	1	2.9	2	12.5	
Progesterone-receptor status					
Negative	13	38.2	5	31.3	0.16
Positive	20	58.8	8	50.0	
Unknown	1	2.9	3	18.8	
HER2 status					
Negative	19	55.9	6	37.5	0.5
Positive	9	26.5	6	37.5	
Unknown	6	17.6	4	25.0	
Ki-67					
≤20%	9	26.5	7	43.8	0.4
≥20%	22	64.7	7	43.8	
Unknown	3	8.8	2	12.5	
TP53 status					
Wild-type	17	50.0	3	18.8	**0.04**
Mutated	15	44.1	13	81.3	
Unknown	2	5.9	0	0.0	
Methylation status					
Unmethylated	24	70.6	7	43.8	**0.04**
Methylated	4	11.8	7	43.8	
Unknown	6	17.6	2	12.5	
Vital status					
Alive	27	79.4	5	31.3	**0.002**[a]
Dead	7	20.6	11	68.8	
Relapsed status					
Nonprogressed	21	61.8	4	25.0	**0.03**[a]
Progressed	13	38.2	12	75.0	

BRD7, bromodomain-containing protein 7; IHC, immunohistochemistry.
[a]Fisher exact test.
0.01 **
0.04*
0.03*
0.002**

Bromodomain-containing protein 7 localization predicts time-dependent outcomes in breast cancer

Next, we asked whether the subcellular localization of BRD7 has prognostic value in breast cancer. In cases with nuclear BRD7 localization, median OS had not been reached at the time of censor and median PFS was 166.9 months, compared with 94.5 months (median OS) and 37.1 months (median PFS) in patients with nonnuclear BRD7. OS and PFS rates at 5 years were 88.2 and 64.7%, respectively, in patients with nuclear BRD7 compared to 66.7 and 26.7%, respectively, in patients with nonnuclear BDR7 ($P=0.002$ and $P=0.001$, respectively; log-rank test) (Fig. 2). Combining nuclear and nonnuclear BRD7 localization with low and high expression, we found that patients ($N=12$) showing both nuclear localization and high BRD7 expression presented both a longer OS ($P=0.005$) and PFS ($P=0.001$) (Fig. 3). Because the localization of BRD7 strongly correlates with OS and PFS, we extended our analysis to assess the impact on outcomes in patients at differing clinical stage. Patients with advanced tumours (stage III) performed better when BRD7 was nuclear ($P=0.02$ for OS and $P=0.001$ for

Fig. 2



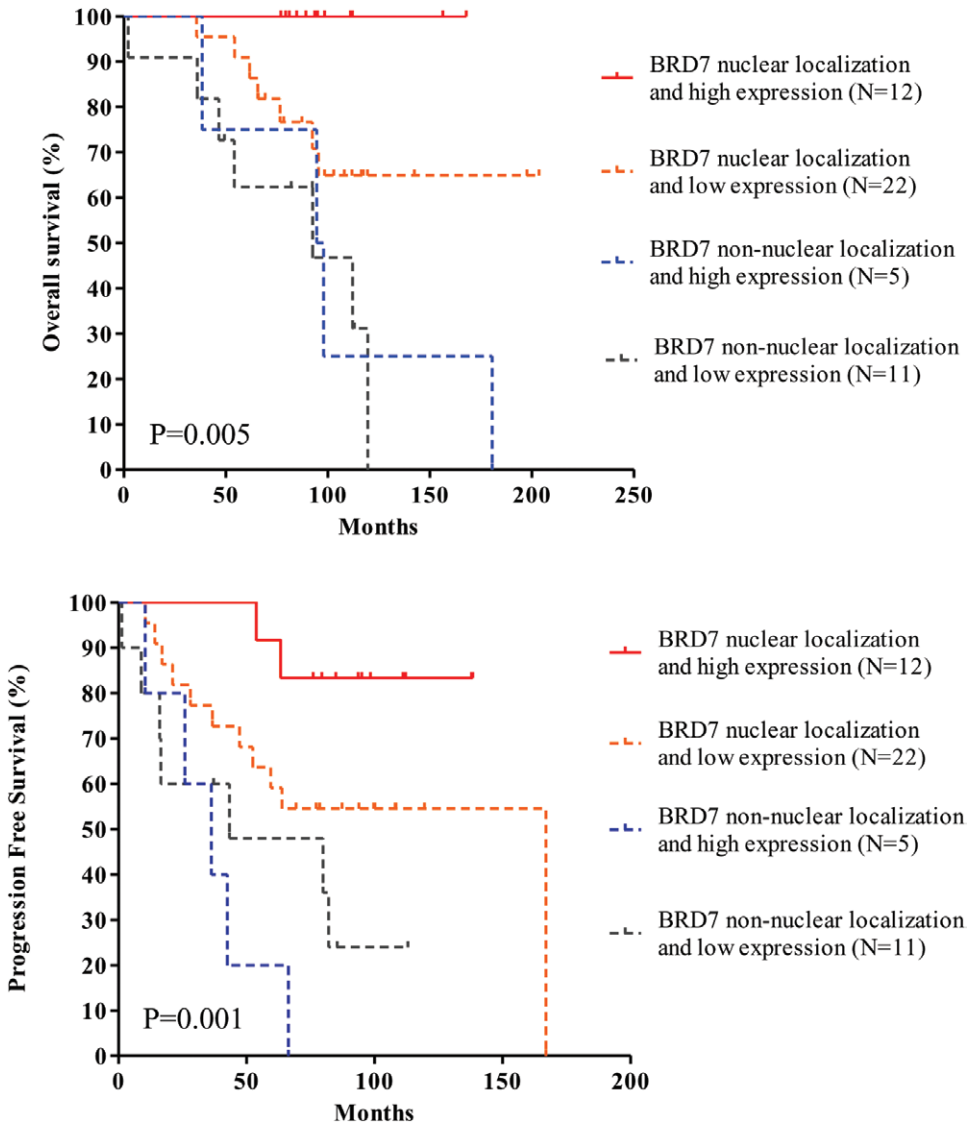
Kaplan–Meier survival curves for breast cancer patients according to BRD7 localization. (a) Overall survival (OS) and (b) progression-free survival (PFS) curves are shown. Significantly improved OS and PFS were observed in breast cancer patients whose tumours exhibited a nuclear BRD7 localization versus those whose tumours showed a nonnuclear BRD7 localization (P=0.002 and P=0.001). BRD7, bromodomain-containing protein 7.

PFS). No significant difference in OS and PFS was found in stage II patients with nuclear versus nonnuclear BRD7 (Supplementary Fig. 1a, Supplemental digital content 1, *http://links.lww.com/ACD/A328*). Finally, we analysed the association between BRD7 localization and outcomes in patients with oestrogen receptor positive cancers receiving endocrine therapy in the adjuvant setting. Median OS (P=0.003) and PFS (P=0.001) were significantly longer in cases with nuclear localisation of BRD7 (N=24) than in cases with nonnuclear BRD7 (N=9) (Supplementary Fig. 1b, Supplemental digital content 1, *http://links.lww.com/ACD/A328*). The effect of BRD7 localization and expression on patient survival was further evaluated by univariate and multivariate analyses. In univariate analysis, BRD7 was significantly associated with improved OS and PFS patients (Table 2). Multivariate analysis further suggested that BRD7 expression is associated with improved OS, although BRD7 expression was not an independent predictor of PFS in breast cancer patients.

Discussion

The bromodomain-containing protein BRD7 is a transcriptional coactivator for p53, modulating histone acetylation, p53 acetylation and promoter activity in a subset of p53 target genes. Here, we have analysed the expression and subcellular localization of BRD7 in a well annotated series of breast cancer cases from our clinical practice. We have used IHC with a high quality, validated antibody

Fig. 3



Kaplan–Meier survival curves for breast cancer patients according to BRD7 localization and stratified for high and low BRD7 expression. (a) Overall survival (OS) and (b) progression-free survival (PFS) curves are shown. Significantly improved OS and PFS were observed in breast cancer patients whose tumours showed the combination of nuclear localization and high BRD7 expression ($N=12$) ($P=0.005$ and $P=0.001$). BRD7, bromodomain-containing protein 7.

to evaluate both expression and subcellular localisation of BRD7 and sought correlations with clinical-pathological variables and time-dependent patient outcomes. Notwithstanding the relatively small size of our series, we show that BRD7 may have utility as a prognostic biomarker in early breast cancer.

Perhaps, most noteworthy from our study is the observation that cases with nuclear localization of BRD7 have better clinical outcomes than those with nonnuclear. A highly significant difference was seen in median OS and PFS. This implies not only that immunohistochemical analysis of BRD7 may be a useful biomarker in predicting prognosis and outcome. BRD7 did not reach significance in multivariate analysis, but this may reflect the relatively small size of the patient population in our study. Analysis of a much larger number of cases to validate the candidacy of BRD7 is necessary.

Cases with nonnuclear localization of BRD7 were more likely to have wild-type *TP53*. These results are consistent with a previous study in which a subset of p53 wild-type tumours expressed very low levels of BRD7 and imply that nuclear exclusion of BRD7 may represent a

mechanism by which the ability of BRD7 to coactivate a subset of p53 target genes is inhibited in breast carcinogenesis and by which the tumour suppressor function of p53 is attenuated in the absence of inactivating mutations. Others have proposed a similar hypothesis [12,25]. Of note, six cases did not express detectable BRD7 and all were wild-type for *TP53*. Although we did not detect a clear correlation between expression of BRD7 and aberrant CpG island methylation, methylation was clearly higher in cancers than in normal breast tissue. The *BRD7* CpG island is large, and the possibility of methylation-dependent transcriptional silencing of *BRD7* requires additional study by analysis of a wider area of the island.

Overall, our data are fully consistent with a tumour suppressor function for *BRD7* in breast cancer. They suggest that nuclear exclusion rather than transcriptional silencing is the principal mechanism of inactivation in breast cancer and demonstrate utility as a prognostic biomarker. Larger studies to develop the biomarker potential of BRD7 are necessary.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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