ANTICANCER RESEARCH *41*: 3597-3606 (2021)
doi:10.21873/anticanres.15148

Bromodomain-containing Protein 4 Is a Favourable Prognostic Factor in Breast Cancer Patients

CHIHO SUZUKI[1], AKIMITSU YAMADA[1], SHOKO ADACHI[1], HIDETAKA SHIMA[1], KUMIKO KIDA[1], MASANORI OSHI[1,2], SADATOSHI SUGAE[1], SHINYA YAMAMOTO[3], KAZUTAKA NARUI[3], MIKIKO TANABE[4], KAZUAKI TAKABE[2], YASUSHI ICHIKAWA[5], TAKASHI ISHIKAWA[6] and ITARU ENDO[1]

[1]*Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine, Yokohama, Japan;*
[2]*Division of Breast Surgery, Department of Surgical Oncology, Roswell Park Cancer Institute, Buffalo, NY, U.S.A.;*
[3]*Department of Breast and Thyroid Surgery, Yokohama City University Medical Center, Yokohama, Japan;*
[4]*Division of Diagnostic Pathology, Yokohama City University Medical Center, Yokohama, Japan;*
[5]*Department of Oncology, Yokohama City University Hospital, Yokohama, Japan;*
[6]*Department of Breast Surgery and Oncology, Tokyo Medical University Hospital, Tokyo, Japan*

Abstract. *Aim: To evaluate the association between bromodomain-containing protein 4 (BRD4) expression and clinicopathological factors and prognosis in human breast cancer specimens. Patients and Methods: We used tissue microarrays constructed from samples of patients (n=183) who underwent surgery. We validated the association between BRD4 expression and prognosis in solid tumours, including breast cancer, using The Cancer Genome Atlas (TCGA) database. Results: Immunohistochemical staining showed that BRD4 was widely distributed in breast cancer tissues. BRD4 was strongly expressed in 19.7% of patients but BRD4 staining intensity was not correlated with other clinicopathological factors. Most importantly, patients with a strong BRD4 expression had a significantly longer disease-specific survival than those with a weak BRD4 expression (100.0% vs. 91.3% at 5 years, p=0.027). mRNA expression analysis showed similar results (91.2% vs. 80.2% at 6 years, p=0.047). Conclusion: Strong BRD4 expression was associated with a significantly better prognosis in breast cancer tumours.*

Correspondence to: Akimitsu Yamada, MD, Ph.D., Assistant Professor, Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ward, Yokohama, Kanagawa 236-0004, Japan. Tel: +81 457872650, Fax: +81 457829161, e-mail: ayamada@yokohama-cu.ac.jp

Key Words: BRD4, breast cancer, cancer stem cell, tissue microarray, TCGA.

Breast cancer is the most common malignancy in women worldwide. In recent years, there has been a significant advancement in subtype-based systemic therapeutic methods and strategies for breast cancer treatment (1). Although the mortality rate is still high owing to disease metastases and treatment-resistant recurrences, recent advances have resulted in the improvement of survival outcomes.

Cancer stem cells (CSCs) comprise a small population of cells within a tumour and have characteristics similar to those of normal stem cells, in that they can self-renew and differentiate (2). Owing to these characteristics, CSCs are considered to be involved in the metastasis and recurrence after initial treatment, chemotherapy, and/or radiation therapy (3). In breast cancer, ESA+, CD44+/CD24–, and ALDH1A1+ are known CSC markers (4). We previously reported that patients with ALDH1A1-positive breast cancer have poor prognosis and are less likely to achieve pathological complete response with preoperative chemotherapy (5).

In a previous study, we used network analysis and identified 10 genes, namely, *SIRT2*, *PCAF*, *LXR*, *BRD4*, *SMAD4*, *Pitx3*, *RARα*, *MUC1*, *HASH1*, and *C/EBPβ,* that are believed to directly control ALDH1 expression (6). In this study, we focused on one of these genes, bromodomain-containing protein 4 (*BRD4*), which has multiple functions as an epigenetic factor. BRD4 is a member of the bromo- and extra-terminal domain (BET) protein family that contains two tandem bromodomains (BDI and BDII) and an extra-terminal domain (7). It interacts with the hyperacetylated histone region and acts at both the transcription initiation and elongation steps, promoting gene transcription (8-10). BRD4 binds to the acetylation region of histones, cooperates with RNA polymerase and acts as an

enhancer to promote transcription of various genes. Among them, cancer-related genes such as *Myc*, *FOSCL*, and *RUNX2* are thought to promote tumour growth (8, 11, 12).

There are several reports on BRD4 in haematological and solid tumours, including breast cancer, and it is being developed as a new target for cancer treatment (13, 14). Several *in vitro* and *in vivo* studies have also shown that inhibition of BRD4 reduces the tumour size in breast cancer (15, 16). However, the association between BRD4 and clinicopathological factors in breast cancer remains unclear. Therefore, in this study, we aimed to evaluate the association between BRD4 expression and the clinicopathological factors and prognosis in human breast cancer specimens.

Patients and Methods

Patient sample collection. Tissue samples were obtained from 183 patients who underwent surgery without presurgical treatment at Yokohama City University Medical Center, between April 2006 and December 2008. The median follow-up period was 120 months (5-154 months). This study was approved by the Institutional Review Board of Yokohama City University (190700023) and informed consent was obtained from all individual participants included in the study. The primary outcomes of this study were disease-free survival (DFS) and disease-specific survival (DSS). The DFS interval was defined as the period from diagnosis to the relapse of breast cancer or metastasis, and any cause of death; DSS was defined as the period from the diagnosis to breast cancer-related death.

To quantify the expression of BRD4 protein, we used tissue microarrays (TMAs) constructed from formalin-fixed, paraffin-embedded tumour blocks obtained from the surgical specimens of primary breast cancer, as previously described (5, 17). Two board-certified pathologists performed the pathological diagnosis and immunohistochemical (IHC) evaluation in a blinded manner. Oestrogen receptor (ER) and progesterone receptor (PgR) status were evaluated using the Allred score (18). Human epidermal growth factor receptor 2 (HER2) status was examined using the Dako HercepTest™ (K5204; Dako, Santa Clara, CA, USA) or PathVysion™ (Abbott, Abbott Park, IL, USA). HER2 positivity (overexpression or amplification) was scored according to the American Society of Clinical Oncology/College of American Pathologists guidelines (19). IHC staining was performed to examine BRD4 protein expression and localisation by using a monoclonal anti-human BRD4 [EPR5150(2), Abcam, Cambridge, UK]. The slides were incubated at 95˚C for 40 min and deparaffinised in xylene four times for 5 min, followed by 5 min each in 95, 90 and 70% ethanol and washed twice for 1 min in distilled water. Antigen retrieval was performed by autoclaving the slides in citrate buffer (pH 6.0) at 121˚C. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide/methanol solution for 15 minutes. The tissue sections were incubated with the primary antibody at 4˚C for 8 h and with the secondary antibody for 30 min. 3,3-Diaminobenzidine (DAB; Dako) was used as a chromogenic substrate. The slides were lightly counterstained with Mayer's haematoxylin. Immunohistochemically stained sections were examined for BRD4 expression and quantified using the Allred scoring method (18) by a pathologist who had a subspecialty training in breast pathology. The score consisted of two components: 1) the average intensity of BRD4 staining (negative: 0; weak: 1; medium: 2; and strong: 3) and 2) the percentage of BRD4-stained nuclei (none: 0; <1%: 1; 1%-10%: 2; 11%-33%: 3; 34%-66%: 4; and 67%-100%: 5). The sum of the two component scores formed the overall score with a possible value of 0 or 2-8. We evaluated the average intensity of BRD4 staining, and a cut-off score of 2 was determined by assessing the nuclear BRD4 expression levels. We evaluated the association between BRD4 expression and clinicopathological findings.

Data of The Cancer Genome Atlas cohort. Expression levels of the tumour gene in patients with breast (n=1082), pancreatic (n=176), gastric (n=375), lung (n=510), and ovarian (n=300) cancers in The Cancer Genome Atlas (TCGA) database were obtained from cBioPortal (20). DSS was defined as the period from the diagnosis to breast, pancreatic, gastric, lung, or ovarian cancer-related death. For breast cancer, we selected female patients whose *BRD4* gene expression data (n =1069) were available. To evaluate the DFS and DSS, we used data from the curated and filtered pan-Cancer Clinical Data Resource of survival endpoints for TCGA cases(21). To categorise the patients into two groups based on their tumour *BRD4* expression, we used the intra-cohort median value of gene expression.

Statistical analysis. The data were analysed using the Statistical Package for the Social Sciences (SPSS) Statistics v.24 software (IBM SPSS Statistics for Windows, Version 24.0. IBM Corp., Armonk, NY, USA) and R software (version 4.0.1). SPSS was used for data analysis of patient data by TMA, and R was used for TCGA data analysis. We used the Mann-Whitney *U*-test was used for the association of BRD4 expression with patient age and tumor size, and Fisher's exact test was used for the association with other biomarkers and clinicopathological factors. Survival data were evaluated using the Kaplan–Meier method and log-rank test. For survival analyses in R, by group comparison of the survival times, the log-rank test, and Cox proportional hazards regression models, the survival package was used. For the boxplot of BRD4 mRNA expression, we used Mann-Whitney *U* and Fisher's exact test to compare two groups, and Kruskal-Wallis test to compare multiple groups. Statistical significance was determined at $p<0.05$.

Results

BRD4 expression in human breast cancer. IHC staining was performed on TMAs to examine the differential expression of BRD4. The results are shown in Figure 1. BRD4 was expressed in the nucleus of most tumour cells but not in stromal cells. In all cases, BRD4-positive cells accounted for one-third or more of the tumour cells, and the proportion score was 4 or more in all cases. As the staining intensity was different for each case, cases with an intensity score of 2 or less were considered the weak group (147 cases) and those with a score of 3 were considered the strong group (36 cases).

Association of BRD4 expression with clinicopathological factors. Patient characteristics and histopathological data are shown in Table I. The median age was 58 (30-69) years, and 81.4% of the patients (n=149) were ER-positive, 9.3%

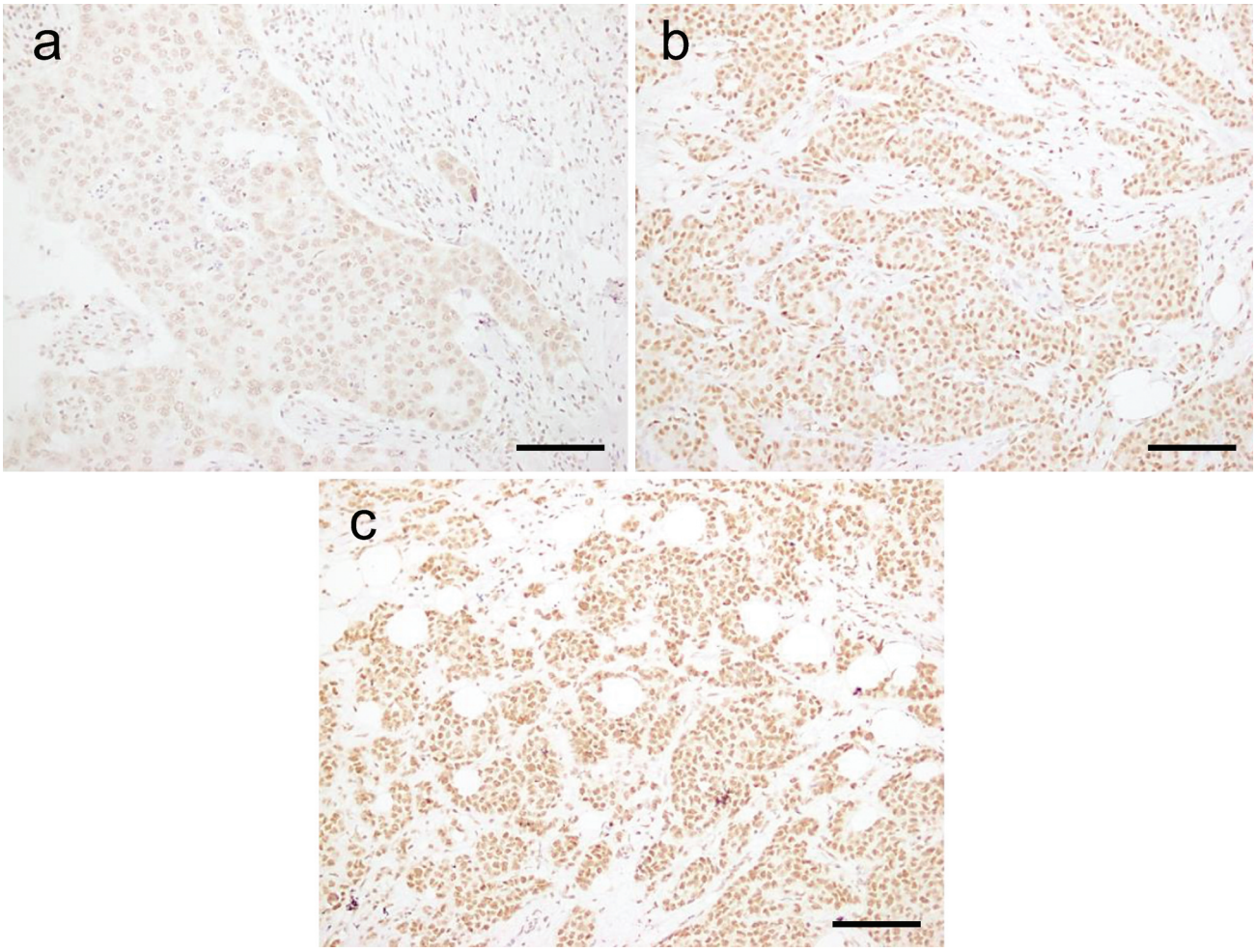

Figure 1. *Immunohistochemical staining of bromodomain-containing protein 4 (BRD4) in human breast cancer specimens. Representative images according to the intensity scoring (IS) are shown. (a) Weak (IS=1), (b) Intermediate, and (c) Strong (IS=3). Scale bar=100 μm.*

(*n*=17) were HER2-positive, and 11.4% (*n*=21) were triple-negative (TN: ER-negative, PgR-negative, and HER2-negative). We compared the weak and strong groups. The association of BRD4 expression with clinicopathological factors is shown in Table II. There were no significant differences between the two groups in terms of age, hormone status, operative procedure, adjuvant therapy, or axillary lymph node metastasis.

Survival analyses according to BRD4 expression. Survival data according to the BRD4 expression are shown in Figure 2. The median period of observation was 120 months. Although not significantly different, the strong group had a lower rate of breast cancer recurrence than the weak group (18.4% *vs*. 8.3%, *p*=0.182) and no breast cancer-related deaths (13.6% *vs*. 0.0%, *p*=0.041). The strong group tended to have a higher rate of DFS (96.9% *vs*. 86.5% at 5 years, *p*=0.207, Figure 2a) and significantly better DSS (100.0% *vs*. 91.3% at 5 years, *p*=0.027, Figure 2b) than the weak group.

Prognostic analysis using the TCGA cohort. Next, we validated the clinical relevance of BRD4 using TCGA datasets. There was no significant difference in DFS between the groups with high and low expression of BRD4 (78.4% *vs*. 77.1 % at 6 years, *p*=0.854, Figure 3a); however, the DSS was significantly more prolonged and better in the high expression group compared to the low expression group (91.2% *vs*. 80.2% at 6 years, *p*=0.047, Figure 3b), which was consistent with the results of our IHC analysis. The association between BRD4 expression and the clinicopathological factors in the TCGA cohort is shown in Table III. In this cohort, 54.3% (*n*=581) of the cases were ER-positive/HER2-negative, 16.5% (*n*=176) were HER2-positive and 14.2% (*n*=152) were triple-negative breast cancer (TNBC). A significantly greater number of TNBC cases (18.5% *vs*.

Table I. *Patient characteristics*.

Median age, years (min-max)	58 (30-89)
Menopausal status, No. (%)	
Premenopausal	47 (25.7)
Postmenopausal	107 (58.5)
N/A	29 (15.8)
ER status, No. (%)	
Positive	149 (81.4)
Negative	34 (18.6)
HER2 Status, No. (%)	
Positive	17 (9.3)
Negative	159 (86.9)
N/A	7 (3.8)
Histological grade, No. (%)	
1	77 (42.1)
2	39 (21.3)
3	67 (36.6)
Breast surgery, No (%)	
Bp	125 (68.3)
Bt	41 (22.4)
N/A	17 (9.3)
Axillary surgery, No (%)	
SNB	110 (60.1)
Ax	58 (31.7)
N/A	15 (8.2)
Adjuvant therapy received, No. (%)	
Yes	158 (86.3)
No	9 (4.9)
N/A	16 (8.8)
Endocrine therapy received, No. (%)	
Yes	131 (71.6)
No	36 (19.7)
N/A	18 (9.7)
Chemotherapy received, No. (%)	
Yes	68 (37.2)
No	99 (54.1)
N/A	18 (9.7)

ER: Oestrogen receptor; HER2: human epidermal growth factor receptor2; Bp: lumpectomy; Bt: mastectomy; SNB: sentinel lymph node biopsy; Ax: axillary dissection; N/A: data not applicable.

11.2%, p=0.003) and fewer lymph node metastatic cases (47.6% *vs*. 53.6%, p=0.026) were observed in the BRD4-high expression group than in the BRD4-low expression group. The expression of BRD4 mRNA was significantly higher in TNBC than in the other subtypes (Figure 4). Nonetheless, the subtype-based analysis showed no significant difference in prognosis between the high and low BRD4 expression groups in any of the subtypes (data not shown).

To analyse the association between BRD4 expression and prognosis in solid tumours, we used the data on breast, pancreatic, gastric, lung and ovarian cancers. A significantly prolonged DSS was observed in breast cancer with high BRD4 expression [hazard ratio (HR)=0.57, 95% confidence interval (CI): 0.36-0.91, p=0.019], whereas a high BRD4 expression was associated with a poor prognosis in ovarian cancer (HR=1.28, 95% CI=1.02-1.61, p=0.034). There were no significant differences between lung and gastric cancers; however, a tendency for high BRD4 expression leading to poor prognosis was observed (Figure 5).

Discussion

We evaluated the association between BRD4 expression determined by IHC staining, clinicopathological features, and clinical outcomes in breast cancer patients who underwent surgery. We found that the BRD4 protein was universally observed in breast cancer cases; however, the intensity of its expression varied between cases. Breast cancer with strong expression of BRD4 [intensity score (IS)=3 points] was associated with a favourable outcome. We validated the association between BRD4 expression and breast cancer prognosis using TCGA cohort data and found that the results were consistent with our protein analysis. Although the cut-off values for protein and mRNA expression were different, we would like to emphasise that the two analyses produced the same result, such that breast cancers with high BRD4 expression had a better prognosis than those with low BRD4 expression. Notably, BRD4 expression was associated with poor prognosis in other solid tumours, including ovarian, lung, and gastric cancers.

BRD4 promotes oncogene transcription (22-24). It has also been suggested that BRD4 is associated with cancer cell migration and invasion through epithelial-mesenchymal transition (EMT). In breast cancer cells, inhibition of BRD4 rapidly reduces the expression of Snail, a potent EMT transcription factor (EMT-TF) (25); hence, we hypothesised that BRD4 expression was related to poor prognosis in breast cancer. In a previous study, breast cancers with strong BRD4 expression were shown to have a large tumour size and a high Ki-67 index, as well as a high number of premenopausal patients (26). Moreover, it has been reported that breast cancers with high BRD4 expression have a poor prognosis (27). The discordance between these studies and our study may stem from differences in probes used for BRD4 detection and sample size of the database. In the current study, BRD4 expression did not correlate with poor prognostic factors, such as the tumour stage and grade. Contrary to our hypothesis, BRD4 expression was identified as a favourable prognostic factor in breast cancer and the results were validated in the TCGA cohort. Notably, BRD4 in other solid tumours was associated with poor prognosis, suggesting that BRD4 might have a different function in breast cancer from that in other solid tumours.

In this study, there was no significant difference in the recurrence rate of breast cancer according to BRD4 expression; however, no breast cancer-related death was observed in patients with high BRD4 expression. Thus, we speculate that BRD4 may modify the nature of metastatic

Table II. *Correlation between BRD4 expression and clinicopathological factors*

		Weak group (IS=1,2) 147 cases	Strong group (IS=3) 36 cases	*p*-Value
Median age, years (min-max)		57 (32-84)	59 (30-89)	0.703
Menopausal status, No. (%)	Premenopausal	40 (27.2)	7 (19.4)	
	Postmenopausal	86 (58.5)	21 (58.3)	0.635
	N/A	21 (14.3)	8 (22.2)	
ER status, No. (%)	Positive	127 (86.3)	28 (77.8)	
	Negative	20 (13.7)	8 (22.2)	0.302
HER2 status, No. (%)	Positive	14 (9.5)	3 (8.3)	
	Negative	126 (85.7)	33 (91.7)	>0.999
		N/A	7 (4.8)	
Histological grade, No. (%)	1	66 (44.9)	11 (30.5)	0.079
	2	33 (22.4)	6 (16.7)	
	3	48 (32.7)	19 (52.8)	
Pathological node status, No. (%)	Positive	43 (29.2)	10 (27.8)	
	Negative	103 (70.1)	26 (72.2)	>0.999
	N/A	1 (0.7)	0 (0.0)	
Median tumour size, mm	(min-max)	18.5 (5-80)	17.5 (3-78)	0.347
Lymph vascular invasion, No. (%)	Present	54 (36.7)	12 (33.3)	0.851
	Absent	93 (63.3)	24 (66.7)	
Breast surgery, No. (%)	Bp	100 (68.0)	25 (69.4)	
	Bt	32 (21.8)	9 (25.0)	0.963
	Na	15 (10.2)	2 (5.6)	
Axillary surgery, No. (%)	SNB	94 (63.9)	23 (63.9)	
	Ax	37 (25.2)	11 (30.5)	0.795
	N/A	16 (10.9)	2 (5.6)	
Adjuvant therapy received, No. (%)	Yes	127 (86.4)	33 (91.7)	>0.999
	No	6 (4.1)	2 (5.6)	
	N/A	14 (9.5)	1 (2.7)	
Endocrine therapy received, No. (%)	Yes	107 (72.8)	24 (66.7)	
	No	29 (19.8)	8 (22.2)	0.83
	N/A	11 (7.4)	4 (11.1)	
Adjuvant chemotherapy received, No. (%)	Yes	50 (34.0)	18 (50.0)	
	No	81 (55.1)	16 (44.4)	0.172
	N/A	16 (10.9)	2 (5.6)	

IS: Intensity score; ER: estrogen receptor; HER2: human epidermal growth factor receptor2; Bp: lumpectomy; Bt: mastectomy; SNB: sentinel lymph node biopsy; Ax: axillary dissection; N/A: data not applicable.

tumours, reducing their invasiveness, decreasing the tumour grade and making them chemosensitive. Activation of BRD4 reduces both the invasiveness and mobility of highly metastatic cell lines without affecting cell proliferation rates. Nevertheless, the activation of BRD4 has been shown to result in a significant decrease in tumour proliferation and metastatic capacity in *in vivo* experiments (28). Furthermore, BRD4 mediates resistance to neoplastic transformation in normal cells by altering genome-wide binding patterns, leading to the inhibition of oncogenic dedifferentiation (29).

BRD4 plays a dual role in cancer, suggesting that it exerts either antitumor or protumor functions, depending on the intracellular context. Wu *et al*. showed that BRD4 has two isoforms, BRD4-L and BRD4-S, which exert contrasting functions in breast cancer (30, 31); BRD4-L has an extended disordered proline-rich region and a P-TEFβ interaction domain, which are absent in BRD4-S (7). BRD4-S deficiency suppresses the proliferation, invasion, and migration of breast cancer cells. Ectopic BRD4-S expression in the mammary gland significantly promotes primary tumour growth and lung metastasis, while ectopic BRD4-L expression suppresses tumour growth *in vivo* (30). The antibody used in this study could not distinguish between the two isoforms. However, the same study reported that BRD4-L accounts for a large proportion of the isoforms, which is consistent with our findings.

Inhibitors against BETs, including BRD4, have received much attention as potential therapeutic agents for cancer (15, 32-35). However, this study showed that BRD4 has contrasting functions in breast and other cancers. Furthermore, the detailed functions of BRD4 in breast cancer proliferation and prognosis remain unclear and controversial.

Table III. *Correlation between BRD4 expression and clinicopathological factors in the TCGA cohort.*

		BRD4-low 534 cases	BRD4-high 535 cases	*p*-Value
Median age, years (min-max)		59 (49-68)	58 (48-66)	0.303
Subtype, No. (%)	ER+/HER2–	307 (57.4)	274 (51.2)	0.003
	HER2+	88 (16.4)	88 (16.5)	
	TN	60 (11.2)	99 (18.5)	
	N/A	79 (14.8)	74 (13.8)	
AJCC Stage, No. (%)	I	88 (16.4)	90 (16.8)	0.815
	II	296 (55.4)	308 (57.6)	
	III	128 (24.0)	115 (21.5)	
	IV	9 (1.7)	9 (1.7)	
	N/A	13 (2.5)	13 (2.4)	
T-category, No. (%)	I	143 (26.8)	130 (24.3)	0.262
	II	300 (56.2)	316 (59.1)	
	III	65 (12.1)	72 (13.5)	
	IV	24 (4.5)	14 (2.6)	
	N/A	2 (0.4)	3 (0.5)	
N-category, No. (%)	Positive	287 (53.7)	254 (47.5)	0.026
	Negative	233 (43.6)	273 (51.0)	
	N/A	14 (2.7)	8 (1.5)	
M-category, No. (%)	Positive	9 (1.7)	11 (2.0)	0.823
	Negative	434 (81.3)	453 (84.7)	
	N/A	91 (17.0)	71 (13.3)	
Histological grade, No. (%)	1	38 (7.1)	38 (7.1)	0.316
	2	138 (25.8)	124 (23.1)	
	3	106 (19.9)	125 (23.4)	
	N/A	252 (47.2)	248 (46.4)	
Race, No. (%)	White	374 (70.0)	366 (68.4)	0.919
	Black	88 (16.5)	91 (17.0)	
	Asian	29 (5.4)	31 (5.8)	
	N/A	43 (8.1)	47 (8.8)	

ER: Oestrogen receptor; HER2: human epidermal growth factor receptor2; TN: triple negative; N/A: data not applicable; AJCC: American Joint Committee on Cancer.

Therefore, for the safe and appropriate use of BET inhibitors, further investigations regarding the signalling pathways and complementary effects of BRD4 are warranted.

Nonetheless, this study has several limitations. First, as this study used TMA without presurgical treatment from a single institution, there is a selection bias. We had some patients who were ER-positive/HER2-negative, and a few other subtypes. Second, it was a retrospective study with a small number of patients. Third, because the BRD4 antibody used in this study could not distinguish between the different BRD4 isoforms, we were unable to address the association between the expression of BRD4-S/L isoforms and breast cancer prognosis. Similarly, for the TCGA cohort, we could not distinguish between the BRD4 isoforms in breast and other cancers. Nonetheless, this study suggests that the prognostic function of BRD4 may differ across malignancies, and further analysis of the distribution and functions of BRD4 isoforms is warranted.

In conclusion, BRD4 was widely distributed in human breast cancer specimens; nonetheless, its expression varied between cases. Breast cancer with a high BRD4 expression had a better prognosis than that with a low BRD4 expression; however, this was not noted in other solid tumours. Further investigations are warranted to further examine the antitumour function of BRD4.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Conception and design of study: C.S, A.Y, T.I; Acquisition of data: C.S, S.A, H.S, M.T; Analysis and/or interpretation of data: K.K, M.O, S.S ; Drafting the manuscript: C.S, A.Y, S.Y, K.N; Revising the manuscript critically for important intellectual content: K.T, Y.I, T.I, I,E.

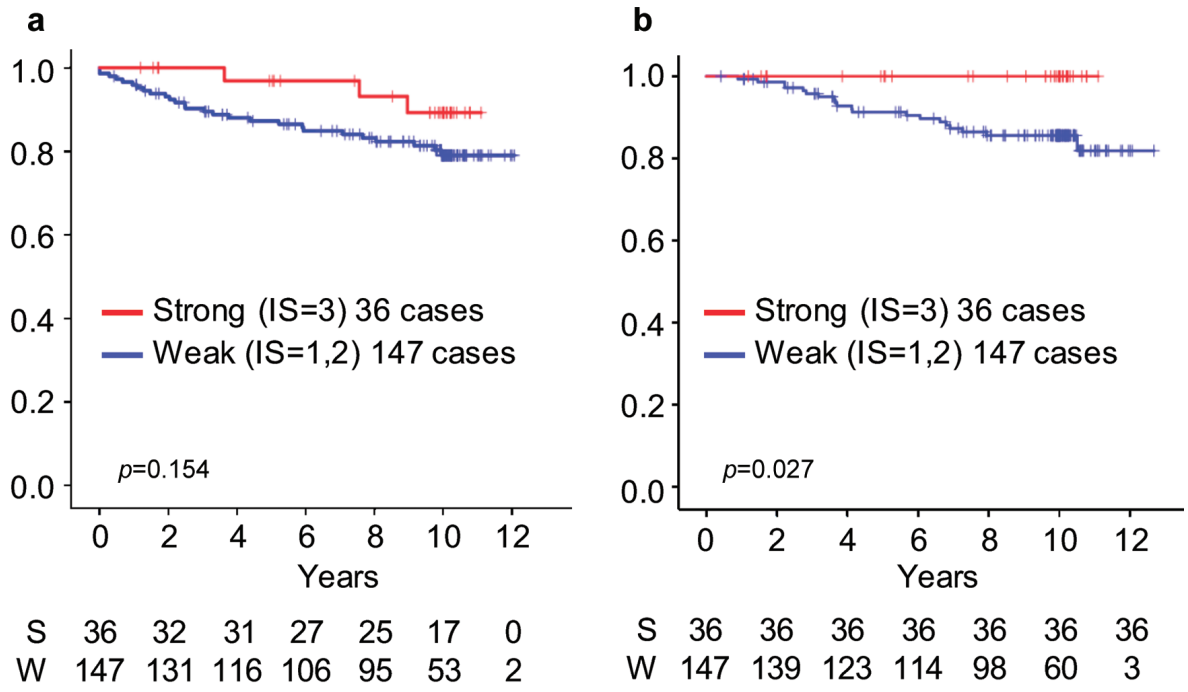

Figure 2. *Survival analysis according to bromodomain-containing protein 4 (BRD4) expression in patients with breast cancer. (a) Kaplan–Meier disease-free survival (DFS) and (b) disease-specific survival (DSS) in patients with strong BRD4 expression [intensity score (IS)=3; red] in comparison to those with weak BRD4 expression (IS=1, 2; blue). Breast cancer patients with high BRD4 expression with an IS of 3 have a significantly longer DSS and better prognosis than those with an IS of 2 or less.*

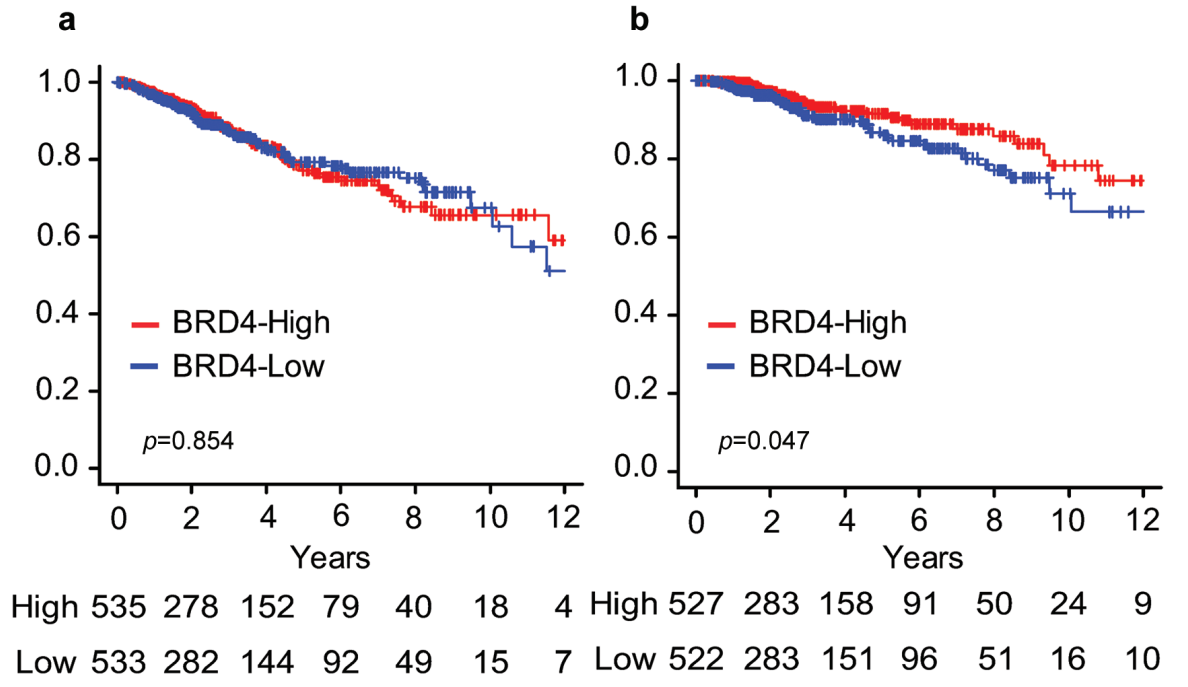

Figure 3. *Survival analysis according to bromodomain-containing protein 4 (BRD4) expression in The Cancer Genome Atlas (TCGA) cohort. (a) Kaplan–Meier disease-free survival (DFS) and disease-specific survival (DSS) (b) in patients with high BRD4 expression (red) compared with those with low BRD4 expression (blue) in the TCGA cohort. Classification by mRNA; median cut-off values are used.*

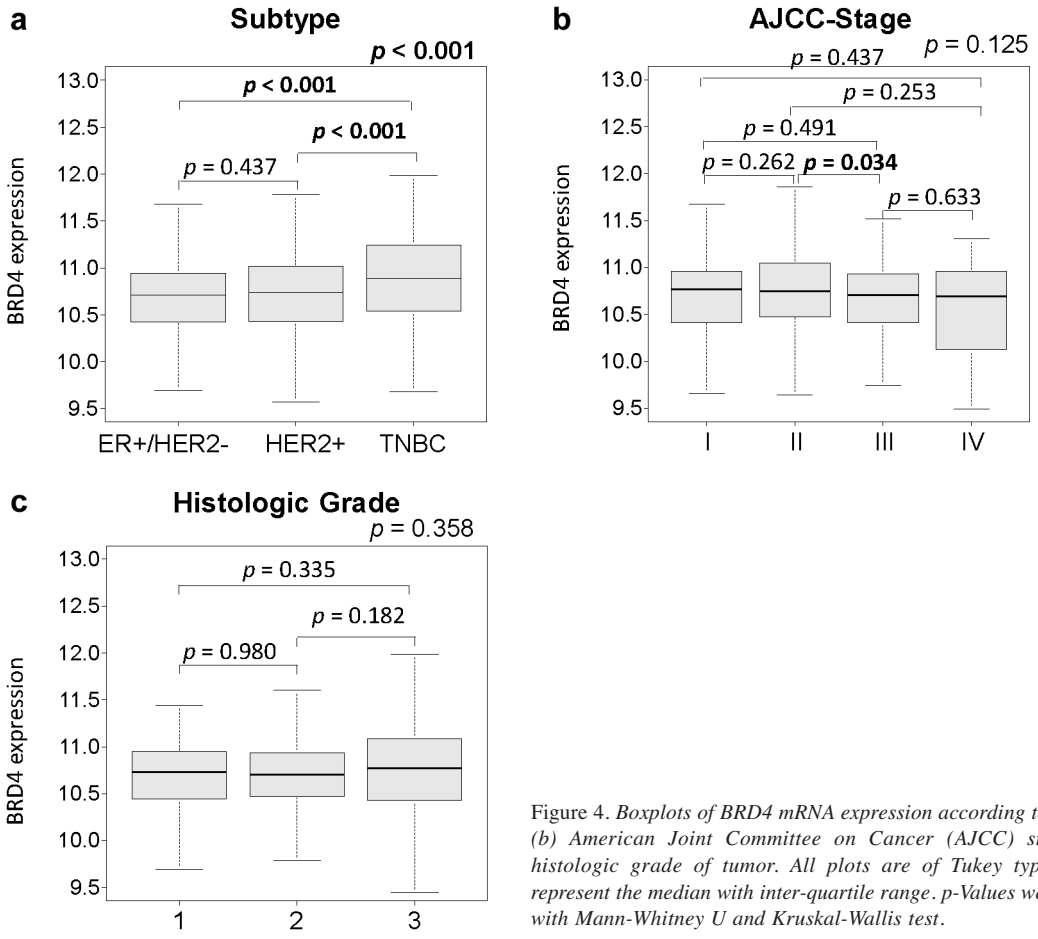

Figure 4. *Boxplots of BRD4 mRNA expression according to (a) subtype, (b) American Joint Committee on Cancer (AJCC) stage and (c) histologic grade of tumor. All plots are of Tukey type and boxes represent the median with inter-quartile range. p-Values were calculated with Mann-Whitney U and Kruskal-Wallis test.*

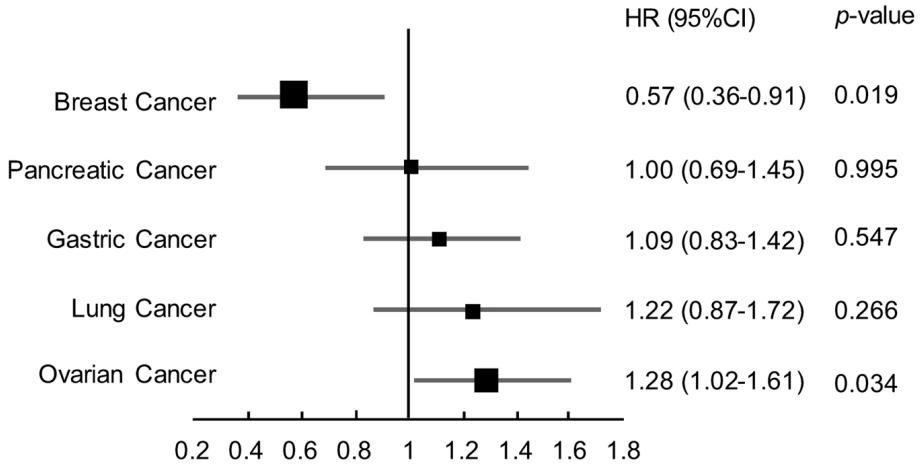

Figure 5. *Significance of tumour bromodomain-containing protein 4 (BRD4) expression for survival in various solid cancers. The association of gene expression (as continuous variable) with disease-specific survival is examined with univariate Cox proportional hazards regression for five types of cancers in The Cancer Genome Atlas cohort. The hazard ratio (HR) for mortality associated with high gene expression and its 95% confidence interval (CI) are plotted. Wald test p-values are shown.*

Acknowledgements

This work was supported by the National Institutes of Health (NIH) grant R01CA160688 to K.T. We would like to thank Editage (www.editage.com) for English language editing.

References

1 Caswell-Jin JL, Zimmer AD, Stedden W, Kingham KE, Zhou AY and Kurian AW: Cascade genetic testing of relatives for hereditary cancer risk: Results of an online initiative. J Natl Cancer Inst *111(1)*: 95-98, 2019. PMID: 30239769. DOI: 10.1093/jnci/djy147

2 Dawood S, Austin L and Cristofanilli M: Cancer stem cells: implications for cancer therapy. Oncology (Williston Park) *28(12)*: 1101-7, 1110, 2014. PMID: 25510809.

3 Batlle E and Clevers H: Cancer stem cells revisited. Nat Med *23(10)*: 1124-1134, 2017. PMID: 28985214. DOI: 10.1038/nm.4409

4 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A *100(7)*: 3983-3988, 2003. PMID: 12629218. DOI: 10.1073/pnas.0530291100

5 Kida K, Ishikawa T, Yamada A, Shimada K, Narui K, Sugae S, Shimizu D, Tanabe M, Sasaki T, Ichikawa Y and Endo I: Effect of ALDH1 on prognosis and chemoresistance by breast cancer subtype. Breast Cancer Res Treat *156(2)*: 261-269, 2016. PMID: 26975188. DOI: 10.1007/s10549-016-3738-7

6 Yamada A, Suzuki C, Shima H, Kida K, Adachi S, Yamamoto S, Narui K, Tanabe M, Shimizu D, Taniguchi R, Oshi M, Takabe K, Miyagi Y, Ichikawa Y, Ishikawa T and Endo I: Aldehyde dehydrogenase 1-related genes in triple-negative breast cancer investigated using network analysis. Anticancer Res *40(12)*: 6733-6742, 2020. PMID: 33288566. DOI: 10.21873/anticanres.14696

7 Wu SY and Chiang CM: The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. J Biol Chem *282(18)*: 13141-13145, 2007. PMID: 17329240. DOI: 10.1074/jbc.R700001200

8 Donati B, Lorenzini E and Ciarrocchi A: BRD4 and Cancer: going beyond transcriptional regulation. Mol Cancer *17(1)*: 164, 2018. PMID: 30466442. DOI: 10.1186/s12943-018-0915-9

9 Filippakopoulos P, Picaud S, Mangos M, Keates T, Lambert JP, Barsyte-Lovejoy D, Felletar I, Volkmer R, Müller S, Pawson T, Gingras AC, Arrowsmith CH and Knapp S: Histone recognition and large-scale structural analysis of the human bromodomain family. Cell *149(1)*: 214-231, 2012. PMID: 22464331. DOI: 10.1016/j.cell.2012.02.013

10 Taniguchi Y: The Bromodomain and Extra-Terminal Domain (BET) family: Functional anatomy of BET paralogous proteins. Int J Mol Sci *17(11)*: 1849, 2016. PMID: 27827996. DOI: 10.3390/ijms17111849

11 Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastritis E, Gilpatrick T, Paranal RM, Qi J, Chesi M, Schinzel AC, McKeown MR, Heffernan TP, Vakoc CR, Bergsagel PL, Ghobrial IM, Richardson PG, Young RA, Hahn WC, Anderson KC, Kung AL, Bradner JE and Mitsiades CS: BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell *146(6)*: 904-917, 2011. PMID: 21889194. DOI: 10.1016/j.cell.2011.08.017

12 Sancisi V, Manzotti G, Gugnoni M, Rossi T, Gandolfi G, Gobbi G, Torricelli F, Catellani F, Faria do Valle I, Remondini D, Castellani G, Ragazzi M, Piana S and Ciarrocchi A: RUNX2 expression in thyroid and breast cancer requires the cooperation of three non-redundant enhancers under the control of BRD4 and c-JUN. Nucleic Acids Res *45(19)*: 11249-11267, 2017. PMID: 28981843. DOI: 10.1093/nar/gkx802

13 Ferri E, Petosa C and McKenna CE: Bromodomains: Structure, function and pharmacology of inhibition. Biochem Pharmacol *106*: 1-18, 2016. PMID: 26707800. DOI: 10.1016/j.bcp.2015.12.005

14 Shimamura T, Chen Z, Soucheray M, Carretero J, Kikuchi E, Tchaicha JH, Gao Y, Cheng KA, Cohoon TJ, Qi J, Akbay E, Kimmelman AC, Kung AL, Bradner JE and Wong KK: Efficacy of BET bromodomain inhibition in Kras-mutant non-small cell lung cancer. Clin Cancer Res *19(22)*: 6183-6192, 2013. PMID: 24045185. DOI: 10.1158/1078-0432.CCR-12-3904

15 Shu S, Lin CY, He HH, Witwicki RM, Tabassum DP, Roberts JM, Janiszewska M, Huh SJ, Liang Y, Ryan J, Doherty E, Mohammed H, Guo H, Stover DG, Ekram MB, Brown J, D'Santos C, Krop IE, Dillon D, McKeown M, Ott C, Qi J, Ni M, Rao PK, Duarte M, Wu SY, Chiang CM, Anders L, Young RA, Winer E, Letai A, Barry WT, Carroll JS, Long H, Brown M, Liu XS, Meyer CA, Bradner JE and Polyak K: Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. Nature *529(7586)*: 413-417, 2016. PMID: 26735014. DOI: 10.1038/nature16508

16 Shi J, Wang Y, Zeng L, Wu Y, Deng J, Zhang Q, Lin Y, Li J, Kang T, Tao M, Rusinova E, Zhang G, Wang C, Zhu H, Yao J, Zeng YX, Evers BM, Zhou MM and Zhou BP: Disrupting the interaction of BRD4 with diacetylated Twist suppresses tumorigenesis in basal-like breast cancer. Cancer Cell *25(2)*: 210-225, 2014. PMID: 24525235. DOI: 10.1016/j.ccr.2014.01.028

17 Yamada A, Ishikawa T, Ota I, Kimura M, Shimizu D, Tanabe M, Chishima T, Sasaki T, Ichikawa Y, Morita S, Yoshiura K, Takabe K and Endo I: High expression of ATP-binding cassette transporter ABCC11 in breast tumors is associated with aggressive subtypes and low disease-free survival. Breast Cancer Res Treat *137(3)*: 773-782, 2013. PMID: 23288347. DOI: 10.1007/s10549-012-2398-5

18 Allred DC, Harvey JM, Berardo M and Clark GM: Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol *11(2)*: 155-168, 1998. PMID: 9504686.

19 Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF, American Society of Clinical Oncology and College of American Pathologists: Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol *31(31)*: 3997-4013, 2013. PMID: 24101045. DOI: 10.1200/JCO.2013.50.9984

20 Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal *6(269)*: pl1, 2013. PMID: 23550210. DOI: 10.1126/scisignal.2004088

21 Liu J, Lichtenberg T, Hoadley KA, Poisson LM, Lazar AJ, Cherniack AD, Kovatich AJ, Benz CC, Levine DA, Lee AV, Omberg L, Wolf DM, Shriver CD, Thorsson V, Cancer Genome

Atlas Research Network and Hu H: An integrated TCGA pan-cancer clinical data resource to drive high-quality survival outcome analytics. Cell *173(2)*: 400-416.e11, 2018. PMID: 29625055. DOI: 10.1016/j.cell.2018.02.052

22 Lovén J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, Bradner JE, Lee TI and Young RA: Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell *153(2)*: 320-334, 2013. PMID: 23582323. DOI: 10.1016/j.cell.2013.03.036

23 Du Z, Song X, Yan F, Wang J, Zhao Y and Liu S: Genome-wide transcriptional analysis of BRD4-regulated genes and pathways in human glioma U251 cells. Int J Oncol *52(5)*: 1415-1426, 2018. PMID: 29568956. DOI: 10.3892/ijo.2018.4324

24 Akıncılar SC, Khattar E, Boon PL, Unal B, Fullwood MJ and Tergaonkar V: Long-Range chromatin interactions drive mutant TERT promoter activation. Cancer Discov *6(11)*: 1276-1291, 2016. PMID: 27650951. DOI: 10.1158/2159-8290.CD-16-0177

25 Lu L, Chen Z, Lin X, Tian L, Su Q, An P, Li W, Wu Y, Du J, Shan H, Chiang CM and Wang H: Inhibition of BRD4 suppresses the malignancy of breast cancer cells via regulation of Snail. Cell Death Differ *27(1)*: 255-268, 2020. PMID: 31114028. DOI: 10.1038/s41418-019-0353-2

26 Lee M, Tayyari F, Pinnaduwage D, Bayani J, Bartlett JMS, Mulligan AM, Bull SB and Andrulis IL: Tumoral BRD4 expression in lymph node-negative breast cancer: association with T-bet+ tumor-infiltrating lymphocytes and disease-free survival. BMC Cancer *18(1)*: 750, 2018. PMID: 30029633. DOI: 10.1186/s12885-018-4653-6

27 Zhong L, Yang Z, Lei D, Li L, Song S, Cao D and Liu Y: Bromodomain 4 is a potent prognostic marker associated with immune cell infiltration in breast cancer. Basic Clin Pharmacol Toxicol *128(1)*: 169-182, 2021. PMID: 32799413. DOI: 10.1111/bcpt.13481

28 Crawford NP, Alsarraj J, Lukes L, Walker RC, Officewala JS, Yang HH, Lee MP, Ozato K and Hunter KW: Bromodomain 4 activation predicts breast cancer survival. Proc Natl Acad Sci USA *105(17)*: 6380-6385, 2008. PMID: 18427120. DOI: 10.1073/pnas.0710331105

29 Fernandez P, Scaffidi P, Markert E, Lee JH, Rane S and Misteli T: Transformation resistance in a premature aging disorder identifies a tumor-protective function of BRD4. Cell Rep *9(1)*: 248-260, 2014. PMID: 25284786. DOI: 10.1016/j.celrep.2014.08.069

30 Wu SY, Lee CF, Lai HT, Yu CT, Lee JE, Zuo H, Tsai SY, Tsai MJ, Ge K, Wan Y and Chiang CM: Opposing functions of BRD4 isoforms in breast cancer. Mol Cell *78(6)*: 1114-1132.e10, 2020. PMID: 32446320. DOI: 10.1016/j.molcel.2020.04.034

31 Zhang S and Roeder RG: The long and the short of BRD4: Two tales in breast cancer. Mol Cell *78(6)*: 993-995, 2020. PMID: 32559427. DOI: 10.1016/j.molcel.2020.05.033

32 Bai L, Zhou B, Yang CY, Ji J, McEachern D, Przybranowski S, Jiang H, Hu J, Xu F, Zhao Y, Liu L, Fernandez-Salas E, Xu J, Dou Y, Wen B, Sun D, Meagher J, Stuckey J, Hayes DF, Li S, Ellis MJ and Wang S: Targeted degradation of BET proteins in triple-negative breast cancer. Cancer Res *77(9)*: 2476-2487, 2017. PMID: 28209615. DOI: 10.1158/0008-5472.CAN-16-2622

33 Doroshow DB, Eder JP and LoRusso PM: BET inhibitors: a novel epigenetic approach. Ann Oncol *28(8)*: 1776-1787, 2017. PMID: 28838216. DOI: 10.1093/annonc/mdx157

34 Lewin J, Soria JC, Stathis A, Delord JP, Peters S, Awada A, Aftimos PG, Bekradda M, Rezai K, Zeng Z, Hussain A, Perez S, Siu LL and Massard C: Phase Ib trial with birabresib, a small-molecule inhibitor of bromodomain and extraterminal proteins, in patients with selected advanced solid tumors. J Clin Oncol *36(30)*: 3007-3014, 2018. PMID: 29733771. DOI: 10.1200/JCO.2018.78.2292

35 Filippakopoulos P: What is the BET on solid tumors? J Clin Oncol *36(30)*: 3040-3042, 2018. PMID: 29847297. DOI: 10.1200/JCO.2018.78.8695

Received May 11, 2021
Revised June 6, 2021
Accepted June 7, 2021