Original Article
Low PBRM1 identifies tumor progression and poor prognosis in breast cancer

Dan Mo[1*], Chunhong Li[2*], Junrong Liang[1], Qunfeng Shi[1], Naiwei Su[1], Shuyou Luo[1], Tian Zeng[1], Xinning Li[1]

[1]Department of Surgery, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning, China; [2]Public Health College of Guangxi Medical University, Nanning, China. *Equal contributors.

Abstract: Background: To investigate the expression and role of PBRM1 in breast cancer, and to evaluate the clinical and prognostic significance of PBRM1 protein in patients with breast cancer. Methods: The expression of PBRM1 was examined in breast cancer tissue and paired non-cancerous tissues by real-time PCR. Moreover, PBRM1 protein expression was evaluated by immunohistochemistry in 150 paraffin-embedded breast cancer specimens. The correlation between PBRM1 expression and clinicopathological features were statistically analyzed. Results: The status of PBRM1 protein in breast cancer tissues is much lower than that in paracarcinoma tissues. Low PBRM1 expression was positively correlated with tumor stage (P =0.003) and lymph node metastasis (P =0.013). The overall (P =0.003) and recurrent-free survival (P =0.001) of the patients with high PBRM1 expression was significantly lower than the low PBRM1 expression group. Multivariate analysis showed that the expression of PBRM1 was an independent factor of overall survival for the patients with breast cancer (P =0.030). Conclusions: PBRM1 might involve in the development and progression of breast cancer as a tumor suppressor, and thereby may be a valuable prognostic marker for breast cancer patients.

Keywords: PBRM1, prognosis, breast cancer

Introduction

Breast cancer is one of the most frequently occurring malignancies and the fourth leading cause of cancer death in females worldwide, with about 230,000 new cases in women per year [1]. For the advanced breast cancer, its prognosis is still poor, with the median survival time less than 2 years [2]. Current the molecular mechanisms in the development and progression of breast cancer remain largely unknown. Methods used to predict the outcome of breast cancer patients mainly depend on the clinicopathological factors, such as clinical stage, tumor differentiation, and lymph node metastasis [3]. Therefore, other parameters such as molecular markers are needed in predicting the clinical outcome of this disease. It is of great value in further understanding the molecular mechanisms of breast cancer and find valuable molecular prognostic factors with high specificity and sensitivity and novel therapeutic strategies.

PBRM1, a component of the SWI/SNF chromatin-remodeling complex found in mammalian cells, is located in chromosome 3p21 and encodes the BRG1-associated factor 180 protein [4]. The gene is composed of multiple domains, including 6 bromodomains involved in binding acetylated lysine residues on histone tails, 2 bromo-adjacent homology domains important in protein-protein interaction and an HMG DNA binding domain [5]. The protein encoded by this gene participates in DNA replication, transcription, DNA repair, and regulation of cell proliferation and differentiation [5, 6]. Recent exome sequencing studies led to the unexpected finding that mutations in PBRM1 were frequently identified, including in over 40% of renal cell carcinoma samples [7]. PBRM1 mutations were uniformly correlated with the loss of heterozygosity (LOH). The majority of PBRM1 mutations result in the loss of this protein. Most often, PBRM1 mutations occurred together with VHL mutations and Varela et al. reported that nearly all of the PBRM1-mutant

Table 1. Correlation between PBRM1 expression with clinicopathologic parameters of breast cancer

Variable	PBRM1		P
	Low	High	
Age			0.106
< 50 y	29	21	
≥ 50 y	44	56	
Side			0.636
Left	37	42	
Right	36	35	
Tumor stage			0.003
I	25	45	
II-IV	48	32	
Tumor size			0.136
≤ 2 cm	30	41	
> 2 cm	43	36	
Tumor grade		0.353	0.353
G1, G2	19	26	
G3	54	53	
Histological type			0.550
Ductal carcinoma	61	67	
Others	12	10	
Lymph nodes status			0.013
Negative	27	44	
Positive	46	33	

tumors exhibited a hypoxia signature, including some cases without a detectable VHL mutation [8]. PBRM1 mutation in renal cancers was associated with advanced stage, high tumor grade, and poor overall survival [9]. These studies indicate that PBRM1 plays a critical role in preventing tumorigenesis. Recently, Xia et al. demonstrated that PBRM1 binds to the p21 promoter and regulates baseline and signal-dependent p21 transcription to suppress tumorigenesis in breast cancer cells, indicating that PBRM1 is a tumor suppressor mutated in breast cancer [10]. However, the clinical significance of PBRM1 in breast cancer remains unclear.

In the present study, we aimed to investigate the expression of PBRM1 in breast cancer and the clinical significance of PBRM1 in breast cancer. We first examined the expression level of PBRM1 in breast cancer tissues by using Real-time PCR. Then we analyzed its association with clinicopathological characters in order to determine the clinical significance of PBRM1

in breast cancer. Taken together, our research revealed a novel biomarker involved in the development and progression of breast cancer.

Materials and methods

Patients and tissue specimens

Fresh tumor tissue with paired non-cancerous tissue samples of 10 breast cancer patients were obtained in operation from the Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region. A total of 150 paraffin-embedded breast cancer samples, which were histologically and clinically diagnosed in patients with radical surgery in Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, between 1998 and 2004, were also included in this study. The clinical and pathologic parameters were reviewed from impatient medical records and presented in **Table 1**. None of these patients had received radiotherapy or chemotherapy prior to surgical treatment. Clinical and pathological data of the 150 patients with breast cancer were collected, such as age, tumor stage, differentiation grade, lymph node metastases, pathologic characteristics and recurrence. The tumor stages were classified according to the American Joint Committee on Cancer guidelines. Clinical follow-up information was obtained by telephone or from the outpatient records. Written Ethics Approval and Patient Consent from the Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region Ethics Committee and written informed consent from all participants were obtained.

RNA extraction and real-time PCR

Total RNA from human cancer tissues and non-cancerous tissues was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 mg of total RNA by use of the SuperScriptH III First-Strand Synthesis System (Invitrogen).

Real-time PCR was carried out using an CFX96 Real-Time System (BIO-RAD). SYBR green 2X master mixture (Invitrogen) was used in a total volume of 10 mL. The primer sequences were as follows: PBRM1 sense, 5'-AAGAAGAAAGAGC-TTGCCAG-3'; antisense, 5'-TCTCGAGCTTCAAGA-
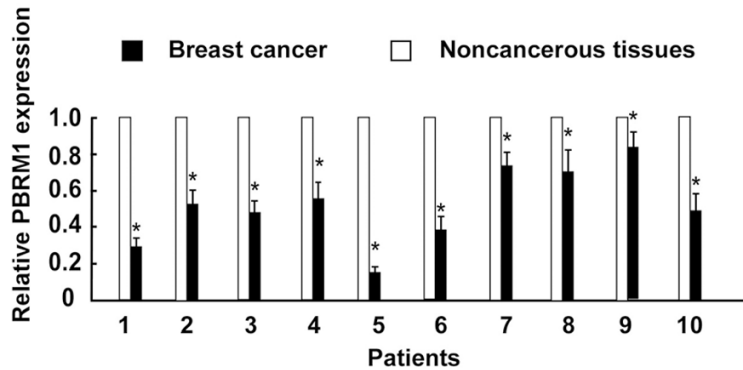
Figure 1. The PBRM1 expression in breast cancer specimens was detected by Real-time PCR compared with paired non-cancerous tissues. asterisks, *P* < 0.05.

ACAAC-3'; GAPDH sense, 5'-GAAGGTGAAGGTC-GGAGTC-3', antisense, 5'-GAAGATGGTGATGGG-ATTTC-3'. GAPDH was used as an internal control. All reactions were run in triplicate in three independent experiments.

Immunohistochemical analysis

The paraffin-embedded specimens were cut into 4m m sections and baked 1 h at 65u C. All sections were deparaffined with xylenes and rehydrated through graded ethanol series to distilled water. Then, the sections were submerged into EDTA antigenic retrieval buffer (pH 8.0) and microwaved for antigenic retrieval. The sections were treated with 0.3% H_2O_2 for 15 min to block the endogenous peroxidase at RT, and then were treated with normal goat serum for 30 min to reduce the nonspecific binding. Consequently, the sections were incubated with rabbit polyclonal anti-PBRM1 antibody (1:200; Sigma, USA) overnight at 4°C. After washing, the sections were incubated with bio-tinylated anti-rabbit secondary antibody (Zymed) followed by further incubation with streptavidin-horse-radish peroxidase (Zymed) at 37°C for 30 min. Diaminobenzidine (DAB) was used for color reaction, and the antibody was replaced by normal goat serum for negative controls.

The immunohistochemically stained tissue sections were scored independently by two pathologists blinded to the clinical parameters, and the final score was the average of the scores by two observers. We used the intensity and extent of the staining to evaluate the expression of PBRM1. The staining intensity was scored as 0 (no staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), 3 (strong staining exhibited as brown). Extent of staining was scored as: 1 (< 10% positive tumor cells); 2 (10-50% positive tumor cells); 3 (51-80% positive tumor cells), and 4 (> 80% positive tumor cells), according to the percentages of the positive staining areas relative to the whole cancer area or entire section for the normal samples. The sum of intensity and extent score was used as the final staining scores (0 to 7) for PBRM1. For the purpose of statistical evaluation, tumors having a final staining score of < 4 classified tumors with low PBRM1 expression and score ≥ 4 classified as high PBRM1 expression.

Statistical analysis

Statistical analyses were performed using a statistical software package (SPSS13.0, Chicago, IL). The significance of PBRM1 levels was determined by t-test. The chi-square test was used to analyze the relationship between PBRM1 expression and clinicopathological characteristics. Survival times were evaluated using the Kaplan-Meier survival curves, and compared by the log-rank test. The significance of various variables for survival was analyzed by multivariate survival analysis using Cox's regression model. *P* value less than or equal to 0.05 were considered to be statistically significant.

Results

Expression of PBRM1 in breast cancer issues

To determine the expression of PBRM1 protein in breast cancer tissues, real-time RT-PCR was performed in 10 breast cancer tissues with paired non-cancerous mucosa. In all the 10 breast cancer tissues with paired normal mucosa, clearly decreased levels of PBRM1 expression was detected in all the tumors tissues in comparison to the paired non-cancerous muco-
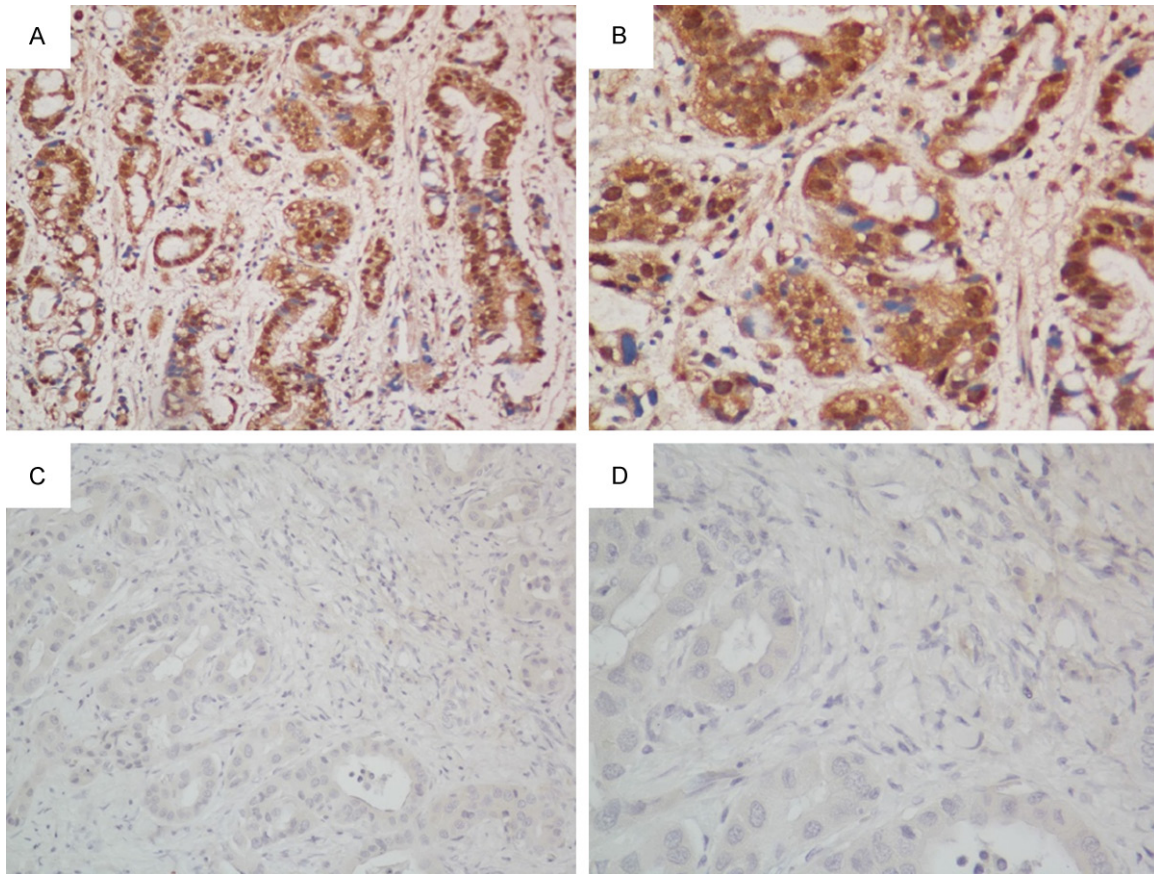
Figure 2. High (A. 200 ×; B. 400 × magnification) and low (C. 200 ×; D. 400 × magnification) expression of PBRM1 in breast cancer tissues by immunohistochemistry.

sal tissues (**Figure 1**). The immunohistochemical data showed that PBRM1 protein was predominantly localized in the cytoplasm. The representative immunostaining of PBRM1 in breast cancer tissues was shown in **Figure 2**. These data suggested that PBRM1 might serve as a tumor suppressor gene in breast cancer.

Correlation of PBRM1 expression with clinico-pathological features

The median follow-up time for overall survival was 67 months for all patients. The association between PBRM1 expression and the clinico-pathological outcomes is shown in **Table 1**. PBRM1 expression was significantly correlated with tumor stage clinical stage (P =0.003) and lymph nodes status (P =0.013) of patients with breast cancer. There was no significant correlation between PBRM1 expression and age, side, tumor size, histological type, or tumor grade (**Table 1**).

Correlation of PBRM1 expression with clinical survival

The prognostic impact of PBRM1 in breast cancer was evaluated using the Kaplan-Meier survival curve analysis. Among these patients, the overall and recurrent-free survival of the patients with high PBRM1 expression was significantly lower than the low PBRM1 expression group (**Figure 3**, P =0.003, and P =0.001, respectively). Multivariate Cox regression analysis showed that PBRM1 is an independent prognostic factor for these patients (**Table 2**). Our results demonstrated that PBRM1 could be used as a potential prognostic marker for breast cancer patients.

Discussion

In the present study, we investigated the clinical value of PBRM1 and found that it is down-regulated in breast cancer tissues in comparison with that in normal breast tissues by
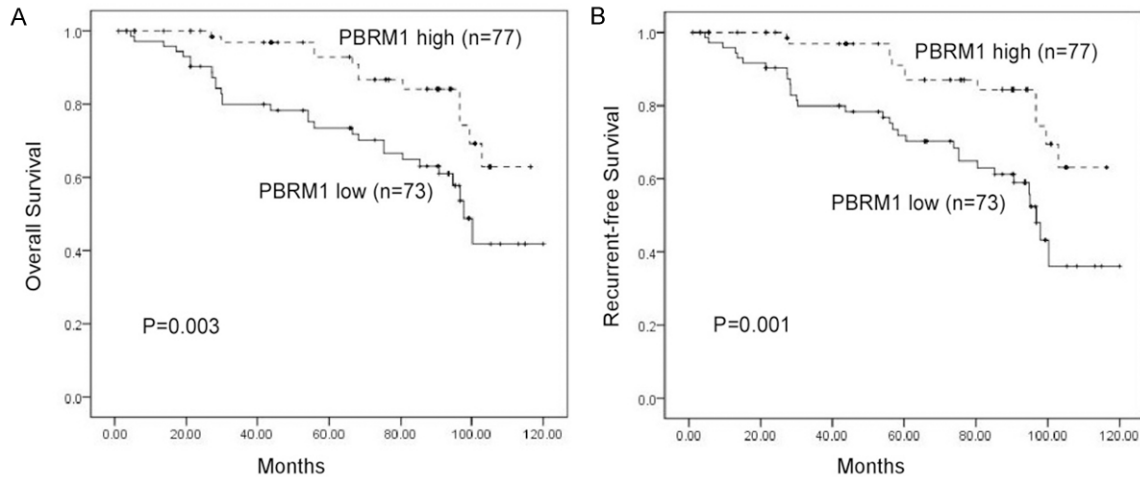
Figure 3. Survival analysis of PBRM1. Patients with lower PBRM1 expression in breast cancer were closely correlated with poorer overall and recurrent free survival than patients with tumor with higher PBRM1 expression.

Table 2. Multivariate Cox regression analyses of clinicopathological variables and breast cancer survival

Variables	OS		RFS	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Age (< 50 vs. ≥50)	0.676 (0.541-2.419)	0.594	0.724 (0.528-2.530)	0.578
Side (Left vs. Right)	0.953 (0.462-1.814)	0.891	1.035 (0.617-1.193)	0.848
Tumor stage (II-IV vs. I)	2.235 (0.756-7.982)	0.031	2.293 (0.503-9.628)	0.045
Tumor grade (G3 vs. G1-2)	1.505 (0.310-4.825)	0.161	1.085 (0.291-4.634)	0.165
Tumor size (> vs. ≤ 2 cm)	1.717 (0.638-2.148)	0.061	2.139 (0.368-3.803)	0.097
Histological type (> vs. ≤ 5 μg/ml)	1.623 (0.519-5.342)	0.413	1.467 (0.424-4.078)	0.544
Lymph nodes status (+ vs. -)	2.581 (0.928-2.695)	0.047	2.296 (0.754-3.876)	0.063
PBRM1 expression (Low vs. High)	2.195 (0.335-3.604)	0.030	1.930 (0.658-3.172)	0.051

Real-time PCR. The expression of PBRM1 was significantly correlated with clinical stage and lymph nodes status of patients with breast cancer. Moreover, PBRM1 expression was a significant indicator of both overall survival and recurrent-free survival for breast cancer patients. Multivariate Cox regression analysis showed that PBRM1 is an independent prognostic factor for breast cancer patients. Our study may suggest a critical role of PBRM1 in the development and progression of breast cancer.

SWI/SNF is a multisubunit chromatin-remodeling complex that performs fundamental roles in a variety of cellular process including gene regulation, cell lineage specification, and organism development [11]. The SWI/SNF family was originally discovered and characterized genetically in yeast. Mutations in genes encoding SWI2/SNF2 or any of several other subunits of the 11 subunit yeast SWI/SNF complex result in transcriptional defects at specific promoters [12]. Existing evidence indicated that mutations that inactivate SWI/SNF subunits are found in nearly 20% of human cancers, which indicates that the SWI/SNF complex is involved in human cancer pathogenesis. Genetic alterations of two SWI/SNF subunits, hSNF5/INI1/BAF47 (hSNF5) and BRG1, are associated with the suppression of tumor development, which likely occurs through their ability to regulate cell cycle factors [10]. Loss of SWI/SNF subunits has been reported in a number of malignant cell lines and tumors, and a large number of experimental observations suggest that this complex functions as a tumor suppressor [13]. Recently, the SWI/SNF complex subunit PBRM1 has been suggested to exert its tumor suppressive functions in renal cell cancer [8]. Moreover,

PBRM1 has been showed to be a mutated tumor suppressor in breast cancer [10]. However, the prognostic role of PBRM1 in breast cancer remains unknown.

In this study, we for the first time reported significantly lower PBRM1 expression in breast cancer tissues compared with matched adjacent noncancerous tissues. In addition, as determined by clinicopathological data, PBRM1 expression was significantly correlated with clinical stage and lymph nodes status. The results indicate that PBRM1 might play an important role in the development and progression of breast cancer. In agreement with our results, Huang et al. demonstrated that PBRM1 expression was significantly reduced in bladder cancer cells and tissues compared to their normal counterparts, and that PBRM1 suppresses bladder cancer by cyclin B1 induced cell cycle arrest [14]. Pawłowski et al. found that a significant number of ccRCC cancer cell lines lack detectable PBRM1 expression, and that functional inactivation of PBRM1 in the context of pVHL loss-of-function may represent a key event in facilitating the development of key aspects of an aggressive tumor behavior [9]. In line with their study, Xiao et al. revealed that MicroRNA-590-5p could enhance proliferation and invasion of clear cell renal carcinoma cells via targeting PBRM1 [15]. Jiao et al. discovered frequent inactivating mutations in BAP1, ARID1A and PBRM1 in intrahepatic cholangiocarcinomas, which highlight the key role of dysregulated chromatin remodeling in intrahepatic cholangiocarcinomas [16]. These studies indicate important roles of PBRM1 in the development of breast cancer. Further studies are required to explore the underlying molecular mechanism of the process.

Importantly, we found that PBRM1 expression was associated with poor clinical outcomes of patients with breast cancer, further indicate the tumor-suppressing role of PBRM1 in cancers. Nam et al. found that decreased expression of PBRM1 predicts unfavorable clinical outcome in patients with clear cell renal cell carcinoma [17]. In supporting with them, Kim et al. reported that PBRM1 expression level is a potential prognostic marker for advanced renal cell carcinoma [18]. Our findings underline that PBRM1 could be used as a prognostic marker in cancers other than renal cancer.

In conclusion, this study showed the first evidences of the expression and clinical significance of PBRM1 in breast cancer, suggesting that PBRM1 might involve in the development and progression of breast cancer as a tumor suppressor, and thereby may serve as a valuable prognostic indicator for patients with breast cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xinning Li, Department of Surgery, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning 530003, China. Tel: +86-1345-7977-273; Fax: +86-771-580-2114. E-mail: xinningli530003@sina.com

References

[1] Stuckey A. Breast cancer: epidemiology and risk factors. Clin Obstet Gynecol 2011; 54: 96-102.

[2] Saad ED, Katz A and Buyse M. Overall survival and post-progression survival in advanced breast cancer: a review of recent randomized clinical trials. J Clin Oncol 2010; 28: 1958-1962.

[3] Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL and Liu ET. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A 2003; 100: 10393-10398.

[4] Reisman D, Glaros S and Thompson EA. The SWI/SNF complex and cancer. Oncogene 2009; 28: 1653-1668.

[5] Thompson M. Polybromo-1: the chromatin targeting subunit of the PBAF complex. Biochimie 2009; 91: 309-319.

[6] Ye K, Schulz MH, Long Q, Apweiler R and Ning Z. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. Bioinformatics 2009; 25: 2865-2871.

[7] Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA and Swanton C. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012; 366: 883-892.

[8]   Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, Davies H, Jones D, Lin ML, Teague J, Bignell G, Butler A, Cho J, Dalgliesh GL, Galappaththige D, Greenman C, Hardy C, Jia M, Latimer C, Lau KW, Marshall J, McLaren S, Menzies A, Mudie L, Stebbings L, Largaespada DA, Wessels LF, Richard S, Kahnoski RJ, Anema J, Tuveson DA, Perez-Mancera PA, Mustonen V, Fischer A, Adams DJ, Rust A, Chan-on W, Subimerb C, Dykema K, Furge K, Campbell PJ, Teh BT, Stratton MR and Futreal PA. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 2011; 469: 539-542.

[9]   Pawlowski R, Muhl SM, Sulser T, Krek W, Moch H and Schraml P. Loss of PBRM1 expression is associated with renal cell carcinoma progression. Int J Cancer 2013; 132: E11-17.

[10]   Xia W, Nagase S, Montia AG, Kalachikov SM, Keniry M, Su T, Memeo L, Hibshoosh H and Parsons R. BAF180 is a critical regulator of p21 induction and a tumor suppressor mutated in breast cancer. Cancer Res 2008; 68: 1667-1674.

[11]   Hohmann AF and Vakoc CR. A rationale to target the SWI/SNF complex for cancer therapy. Trends Genet 2014; 30: 356-363.

[12]   Phelan ML, Sif S, Narlikar GJ and Kingston RE. Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. Mol Cell 1999; 3: 247-253.

[13]   Lu P and Roberts CW. The SWI/SNF tumor suppressor complex: Regulation of promoter nucleosomes and beyond. Nucleus 2013; 4: 374-378.

[14]   Huang L, Peng Y, Zhong G, Xie W, Dong W, Wang B, Chen X, Gu P, He W, Wu S, Lin T and Huang J. PBRM1 suppresses bladder cancer by cyclin B1 induced cell cycle arrest. Oncotarget 2015; 6: 16366-78.

[15]   Xiao X, Tang C, Xiao S, Fu C and Yu P. Enhancement of proliferation and invasion by MicroRNA-590-5p via targeting PBRM1 in clear cell renal carcinoma cells. Oncol Res 2013; 20: 537-544.

[16]   Jiao Y, Pawlik TM, Anders RA, Selaru FM, Streppel MM, Lucas DJ, Niknafs N, Guthrie VB, Maitra A, Argani P, Offerhaus GJ, Roa JC, Roberts LR, Gores GJ, Popescu I, Alexandrescu ST, Dima S, Fassan M, Simbolo M, Mafficini A, Capelli P, Lawlor RT, Ruzzenente A, Guglielmi A, Tortora G, de Braud F, Scarpa A, Jarnagin W, Klimstra D, Karchin R, Velculescu VE, Hruban RH, Vogelstein B, Kinzler KW, Papadopoulos N and Wood LD. Exome sequencing identifies frequent inactivating mutations in BAP1, ARID1A and PBRM1 in intrahepatic cholangiocarcinomas. Nat Genet 2013; 45: 1470-1473.

[17]   Nam SJ, Lee C, Park JH and Moon KC. Decreased PBRM1 expression predicts unfavorable prognosis in patients with clear cell renal cell carcinoma. Urol Oncol 2015; 33: 340.e9-340.e16.

[18]   Kim JY, Lee SH, Moon KC, Kwak C, Kim HH, Keam B, Kim TM and Heo DS. The impact of PBRM1 expression on prognostic and predictive marker in metastatic renal cell carcinoma. J Urol 2015; [Epub ahead of print].