



Immunohistochemical detection and clinicopathological significance of JARID1B/KDM5B and P16 expression in invasive ductal carcinoma of the breast

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ABSTRACT. The aims of this study were to investigate the expression of the H3K4 demethylase, jumonji AT-rich interactive domain 1B (JARID1B/KDM5B) and of p16 (multiple tumor suppressor gene *MTS1*) in breast cancer tissue and determine its clinicopathological significance. JARID1B/KDM5B and P16 protein expression in 176 resected breast cancer specimens and adjacent normal breast tissue was detected by the streptavidin-peroxidase (S-P) immunohistochemical method. The TNM staging grade was assigned according to the World Health Organization (2012) breast classification system. The positive staining rate of JARID1B/KDM5B and p16 protein in cancer tissue was 74.43 and 35.8%, respectively. JARID1B/KDM5B protein expression was positively associated with T grade, Bloom and Richardson (B&R) score and axillary lymph node metastasis ($P < 0.05$). p16 protein expression was negatively associated with T grade, B&R score, and axillary lymph node metastasis ($P < 0.05$). JARID1B/KDM5B and p16 protein expression in breast cancer and adjacent normal breast tissue were negatively correlated ($r = -0.303$, $P < 0.001$). The data demonstrated

that protein expression of p16 and JARID1B/KDM5B is negatively correlated in invasive ductal carcinoma of the breast.

Key words: p16; Invasive ductal carcinoma of the breast; JARID1B/KDM5B; Immunohistochemistry

INTRODUCTION

Carcinoma of the breast is the most common malignancy in women and the number of women with breast cancer is increasing. Among several histologic types of breast cancer, ductal carcinoma generally referred to as adenocarcinoma without other designation comprises the majority (79%) of breast cancer (Robbins et al., 2009). Abnormal cell cycle regulation in breast cancer is closely related to the incidence of breast cancer, as the loss of cell cycle regulation is an early event. Abnormalities in p16, which impact the G1 phase of the cell cycle, have been observed in a majority of breast cancer patients (Geradts and Ingram, 2000).

The *p16/INK4a* gene, also known as *MTS1*, is located on chromosome 9p21. p16 plays a key role in regulating senescence induction, which is the reverse effect on cell cycle regulation than that seen in cancers; namely, inhibition of cell division and proliferation. p16 acts through the retinoblastoma pathway to inhibit cyclin-dependent kinases, leading to G1 cell cycle arrest and senescence (Rayess et al., 2012). Loss of the inhibition provided by the p16 protein in a cell leads to dysplasia, cell division, and eventually complete loss of control over cell proliferation, leading to cancer (Bazarov et al., 2010).

JARID1B (KDM5B/PLU-1/RBP2-H1) is a member of the AT-rich DNA interaction domain (ARID) containing the JmjC family of demethylases that specifically target histone H3 lysine 4 (H3K4; ref. Yamane et al., 2007). *JARID1B/KDM5B* was originally isolated as a gene that was overexpressed in breast carcinomas (Li et al., 2011) and is strongly associated with estrogen receptor-positive (ER+) cancers. Its role in breast cancer is believed to be inhibition of the tumor suppressor gene *BRCAl*, which plays an important role in the proliferative ability of breast cancer cells (Yamane et al., 2007). JARID1B/KDM5B is an important part of the Rb-E2f pathway, and works in combination with E2f target genes in the process of cellular senescence, inhibiting the anti-cell-aging role of E2f (Nijwening et al., 2011). Quantitative reverse transcription polymerase chain reaction analysis has confirmed that expression levels of *JARID1B/KDM5B* are significantly higher in carcinogenesis through the E2F/RB pathway. Reduction of *KDM5B* expression resulted in suppression of cell growth of cancer cells, through co-regulation of the E2F/RB1 cell cycle regulation pathway, and possibly the promotion of apoptosis of cells remaining in sub-G₁ phase (Hayami et al., 2010).

Since JARID1B/KDM5B and the p16 protein both utilize the mechanism of the pRb pathway in regulating the cell cycle, we designed this study to examine JARID1B/KDM5B and p16 expression in invasive ductal breast cancer, and define its clinicopathological significance.

MATERIAL AND METHODS

Case selection

Patients and tumor samples

This study included tumor tissues surgically resected from 176 patients who visited

Beijing TongRen Hospital between 2011 and 2014, and were diagnosed with invasive ductal carcinoma of the breast. Patients' ages ranged between 29 and 77 (mean 49.5) years. Study qualifiers included: diagnosis of invasive ductal carcinoma; no preoperative chemotherapy, radiation therapy, or non-steroidal anti-inflammatory drug therapy; diagnosis confirmed by pathology; and integrity of clinical data. Demographic, clinical, and pathologic data were computed from patient files, surgical reports, and pathology reports.

The World Health Organization classification system was applied for histological tumor typing and grading. All pathology slides and gross photographic images were reviewed by two pathologists; regional lymph node metastasis, histological grade and tumor size were evaluated. Clinical data, including gender and age were obtained by chart review.

Microscopic grading of breast carcinoma

The Nottingham modification of the Bloom-Richardson system was applied as follows:

Tubule formation

1 point: Tubular formations in >75% of the tumor

2 points: Tubular formations in 10-75% of the tumor

3 points: Tubular formations in <10% of the tumor

Note: For scoring tubule formations, the overall appearance of the tumor has to be taken into consideration.

Nuclear pleomorphism

1 point: Nuclei with minimal variation in size and shape

2 points: Nuclei with moderate variation in size and shape

3 points: Nuclei with marked variation in size and shape

Note: The tumor areas having cells with greatest atypia should be evaluated.

Mitotic count

1 point: 0-5

2 points: 6-10

3 points: >11

Objective 40X

Field diameter (mm) 0.44

Field area (mm²) 0.152

Note: Mitotic figures are to be counted only at the periphery of the tumor. Counting should begin in the most mitotically active area; 10 high-power fields are to be counted in the same area (but not necessarily contiguous). The fields should be filled with as much tumor as possible; poorly preserved areas are to be avoided. Cells in the prophase should be ignored.

Final grading score

SUM OF POINTS: FINAL GRADE

3-5: I; 6-7: II; 8-9: III

Immunohistochemistry

Immunohistochemical staining was performed on 4- μ m sections of formalin-fixed, paraffin-embedded surgical tumor samples. The sections were mounted, deparaffinized in xylene three times, and rehydrated through 100, 90, 80, and 70% ethanol and Tris-buffered saline, pH 7.4. Antigen retrieval was performed using 10 mM citrate buffer, pH 6.0, heated

in a pressure cooker for 5 min. The blocking of endogenous peroxidases was accomplished by incubating the sections in 3% hydrogen peroxide (H_2O_2 ; Maixin Biotechnology; Fuzhou, China) for 5 min. The sections were incubated with rabbit anti-JARID1B/KDM5B antibody (1:100; DAKO, Hamburg, Germany) and rabbit anti-P16 antibody (Maixin Biotechnology) overnight at 4°C. Immunostaining for JARID1B/KDM5B was performed using the S-P immunohistochemistry kit according to manufacturer instructions (Maixin Biotechnology). The sections were counterstained with hematoxylin for nuclear counterstaining and examined for the extent and intensity of nuclear and non-nuclear staining in tumor cells and for background staining by two independent observers in a blinded manner. Discordant scores were resolved by review and consensus agreement or use of a third observer.

For p16 immunohistochemical (IHS) scoring, nuclear and cytoplasmic staining was identified as positive staining (Figure 1). For JARID1B/KDM5B IHS scoring, nuclear staining was identified as positive staining (Figure 2). The extent of positively stained cells was estimated and classified on a five-point scale as follows: grade 0, $\leq 10\%$; grade 1, $\geq 0\%$ and $\leq 25\%$; grade 2, $\geq 25\%$ and $\leq 50\%$; grade 3, $\geq 50\%$ and $\leq 75\%$; grade 4, $\geq 75\%$. The intensity of the positive staining was categorized into three groups: weak (1), moderate (2) and strong (3). A final IHS score was obtained by multiplying the score for the extent and the score for intensity as follows: 0, negative (-); 1 - 4, weakly positive (+); 5 - 8, moderately positive (++); 9 - 12, strongly positive (+++).

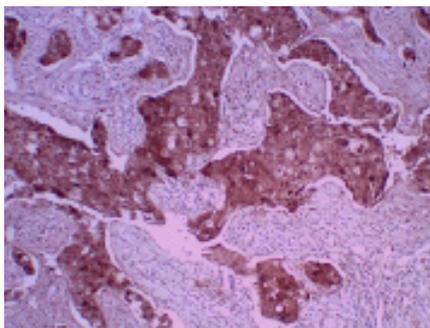


Figure 1. p16 protein-positive expression in invasive ductal carcinoma of the breast by immunohistochemistry (cytoplasmic staining); 200X.

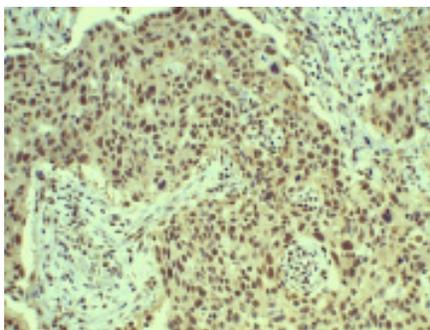


Figure 2. JARID1B/KDM5B protein-positive expression in invasive ductal carcinoma of the breast by immunohistochemistry (nuclear staining); 200X.

Statistical analyses

All statistical analyses were performed using the SPSS statistical software package, version 19 (SPSS Inc., Chicago, IL, USA) for Windows. The χ^2 test and Spearman's correlation coefficient were used to test for any associations between the levels of JARID1B/KDM5B and p16 proteins. A P value of <0.05 was considered to be statistically significant for all tests.

RESULTS

Patient characteristics

The patient tumors studied consisted of grade I (46), grade II (92), and grade III (38). The most frequent T stage was T1 (85), followed by T2 (59) and T3 (32) stages. Nodal stages were N0 (88), N1 (43), N2 (26), and N3 (19).

JARID1B/KDM5B and p16 expression in breast cancer tissue and adjacent normal tissues

In 176 patients, the breast cancer expression rate of JARID1B/KDM5B was 74.43% (131/176); in the adjacent normal tissues, the positive expression rate was 7.39% (13/176); the difference was statistically significant ($\chi^2 = 163.637$, $P < 0.05$). The breast cancer p16 expression rate was 35.80% (63/176); in the adjacent normal tissues, the positive expression rate was 82.95% (146/176), and the difference was statistically significant ($\chi^2 = 81.137$, $P < 0.05$) (Table1).

Table 1. Comparison of KDM5B and P16 protein expression in breast cancer and precancer tissue.

	N	KDM5B		P16	
		-	+ (%)	-	+ (%)
Precancer tissue	176	163	13 (7.39)	30	146 (82.95)
Breast cancer	176	45	131 (74.43)	113	63 (35.80)
χ^2			163.637		81.137
P			0.000		0.000

Relationship between JARID1B/KDM5B expression and the clinical pathological features of breast cancers

In 176 patients with breast cancer aged ≤ 45 years or ≥ 45 years, the JARID1B/KDM5B-positive expression rate determined using the Bloom and Richardson (B&R) scoring system, was 82.9 or 71.9% for each respective group, with no significant difference between groups ($\chi^2 = 2.027$, $P > 0.05$). The JARID1B/KDM5B-positive expression rate in T1 stage tumors was 65.9%; T2 positive expression was 79.7%, and T3 was 87.5%. There was a statistically significant difference between T1 and T3 rates ($\chi^2 = 5.365$, $P < 0.05$), but no statistically significant difference between T1 and T2 ($\chi^2 = 3.247$, $P > 0.05$) or T2 and T3 ($\chi^2 = 0.880$, $P > 0.05$) rates. The JARID1B/KDM5B-positive expression rate in grade I tumors was 63.0%, grade II was 75.0%, and grade III was 86.8%. There was a statistically significant difference

between grade I and grade III tumors ($\chi^2 = 6.097$, $P < 0.05$), but no statistically significant difference between grade I and grade II ($\chi^2 = 2.130$, $P > 0.05$) or grade II and grade III ($\chi^2 = 2.232$, $P > 0.05$) groups (Table 2).

Table 2. KDM5B and P16 expression in breast cancer tissue and clinicopathological significance.

Clinicopathology	N	KDM5B			χ^2	P	P16			χ^2	P
		-	+	%			-	+	%		
Age					2.027	0.155				0.389	0.533
≤45 years old	41	7	34	82.9			28	13	31.71		
>45 years old	135	38	97	71.9			85	50	37.04		
Tumor diameter (cm)					6.984	0.030				7.313	0.026
≤2 (T1)	85	29	56	65.9			47	38	44.71		
2-5 (T2)	59	12	47	79.7			40	19	32.20		
≥5 (T3)	32	4	28	87.5			26	6	18.75		
R&B score					6.226	0.044				7.138	0.028
Grade I	46	17	29	63.0			25	21	45.65		
Grade II	92	23	69	75.0			57	35	38.04		
Grade III	38	5	33	86.8			31	7	18.42		
Lymphnode metastasis					12.034	0.007				8.351	0.039
N0 (0)	88	32	56	63.6			48	40	45.5		
N1 (1-3)	43	8	35	81.4			30	13	30.2		
N2 (4-9)	26	3	23	88.5			19	7	26.9		
N3 (≥10)	19	2	17	89.4			16	3	15.8		

JARID1B/KDM5B protein expression rates in lymph node metastasis groups (N1, N2, N3) were significantly higher than that in the group without lymph node involvement (N0) ($\chi^2 = 4.295$, $\chi^2 = 5.814$, and $\chi^2 = 4.812$, respectively; $P < 0.05$); there was no statistically significant difference among the N1, N2, and N3 groups ($P > 0.05$) (Table 2).

In 176 patients with breast cancer aged ≤45 years or ≥45 years, the p16-positive expression rate was 31.71 or 37.04% respectively, with no significant difference between age groups ($P > 0.05$). The p16-positive expression rate in T1 was 44.71%, positive expression rate in T2 was 32.2% and that in T3 was 18.75%. There was a statistically significant difference between groups T1 and T3 ($\chi^2 = 6.675$, $P < 0.05$), but no significant difference between the T1 and T2 or the T2 and T3 groups ($\chi^2 = 2.276$, $\chi^2 = 1.885$, respectively; $P > 0.05$) (Table 2).

p16-positive expression in B&R grade I tumors was 45.65%; positive expression in grade II was 38.04% and that in grade III was 18.42%, with statistically significant differences between grade III and grades I and II ($\chi^2 = 6.944$, $\chi^2 = 4.735$, respectively; $P < 0.05$), but no significant difference between grade I and II tumors ($\chi^2 = 0.736$, $P > 0.05$). p16 protein expression rates between lymph node metastasis groups N3 and N0 had a statistically significant difference ($\chi^2 = 5.721$, $P < 0.05$), whereas no statistically significant difference was found among groups N1, N2, and N3 ($P > 0.05$), between the N0 and N1 and N2 groups ($P > 0.05$) (Table 2).

Correlation analysis of JARID1B/KDM5B and p16 protein expression

Statistical analyses demonstrated that JARID1B/KDM5B and p16 protein expression in breast cancer and adjacent normal breast tissue were negatively correlated ($r = -0.303$, $P < 0.001$) (Table 3).

Table 3. KDM5B and P16 protein expression in breast cancer tissue and correlation.

	N	P16			
		-	+	++	+++
KDM5B					
-	45	14	9	15	7
+	41	32	2	2	5
++	62	47	7	5	3
+++	28	20	4	2	2

$r = -0.303$, $P < 0.001$.

DISCUSSION

JARID1B is a lysine-specific demethylase 5B (KDM5B or PLU-1), and a member of the family of JmjC domain-containing proteins. The JARID1B/KDM5B protein sequence contains several well-conserved domains known to be involved in protein interactions, gene regulation, and chromatin remodeling including three PHD domains, the ARID domain and two potential hormone-binding motifs. The novel Trp/Tyr/Cys domain (overlapping the JmjC domain and renamed the PLU domain 9) interacts with a conserved motif found in two unrelated transcription factors (BF1 and PAX9). Thus, the JARID1B/KDM5B protein could be recruited to DNA directly through the ARID DNA-binding domain, or through other transcription factors (Barrett et al., 2007). JARID1B/KDM5B specifically removes methyl groups from tri-, di-, and mono-methylated lysine 4 residues on histone H3, facilitating access to DNA by RNA polymerase II (Radberger et al., 2012).

Using immunohistochemistry and western blot analysis, Catchpole et al. (2011) have demonstrated expression of the JARID1B/KDM5B protein in breast cancers and breast cancer cell lines. The limited expression of JARID1B/KDM5B in normal adult tissues, which was predicted from studies on expression of *PLU-1* mRNA, has also been confirmed. The JARID1B/KDM5B nuclear protein plays important roles in the development, differentiation, transcriptional regulation and chromatin remodeling. The only normal tissues showing high expression of JARID1B/KDM5B were the testis and bone marrow (Roesch et al., 2010), with weak expression observed in the ovary, whereas varying levels of expression were seen in a series of immortalized cell lines. In this study, we demonstrated that JARID1B/KDM5B expression in paracancer breast tissue was lower (7.39%) than that in breast cancer tissue 74.43% ($P < 0.05$).

In cancer, JARID1B/KDM5B functions as a transcriptional regulator of oncogenes (e.g., *BRCAl* in breast cancer) via direct interaction with promoter sites. In addition to its over-expression in breast cancers, JARID1B/KDM5B dysregulation has been reported in several types of solid tumors, including prostate cancer, melanoma, and bladder cancer (Li et al., 2011).

Therefore, the mechanistic role of JARID1B/KDM5B in tumor progression remains an intriguing question. It has become evident that the expression of JARID1B/KDM5B is dynamically regulated and might be associated with tumor grades; for example, the expression of JARID1B/KDM5B has been associated with the invasive and *in situ* components in primary breast cancers and is expressed only weakly or not at all in benign tumors. Thus JARID1B/KDM5B expression was found to be closely associated with the malignant phenotype in breast cancer (Lu et al., 1999). In this study, we demonstrated that JARID1B/KDM5B expression was statistically significantly different between T1 and T3 ($\chi^2 = 5.365$, $P < 0.05$) and grade

I and grade II ($\chi^2 = 6.097$, $P < 0.05$) tumors; that JARID1B/KDM5B protein expression in lymph node metastasis groups (N1, N2, N3) was significantly higher than in those tumors without lymph node involvement (N0) ($\chi^2 = 4.295$, $\chi^2 = 5.814$, and $\chi^2 = 4.812$, respectively; $P < 0.05$).

Surprisingly, however, mechanistic studies on the role of JARID1B/KDM5B in different types of cancer have yielded inconsistent and even confusing results (Roesch et al., 2008, 2010). In melanocytic tumors, JARID1B/KDM5B was found to be highly expressed in benign nevi, which typically are characterized by oncogene-induced senescence. However, in aggressive primary melanomas and melanoma metastases, there were only single cells with high JARID1B/KDM5B expression (~5-10% of the total population) (Roesch et al., 2005). In this study, we demonstrated that JARID1B/KDM5B expression had no statistically significant difference between T1 and T2 ($\chi^2 = 3.247$, $P > 0.05$) or T2 and T3 ($\chi^2 = 0.880$, $P > 0.05$) groups, and no statistically significant difference was observed between grade I and II ($\chi^2 = 2.130$, $P > 0.05$) or between grade II and III ($\chi^2 = 2.232$, $P > 0.05$) tumors. JARID1B/KDM5B protein expression in lymph node metastasis was also not significantly different among the N1, N2, and N3 groups ($P > 0.05$).

The tumor suppressor gene p16, the product of the gene encoding the relative molecular mass 16,000-kDa protein, is one of the cyclin-dependent kinase inhibitors (CDKIs) (Gröbe et al., 2013). *p16INK4a* is commonly inactivated in many human cancers, including breast, head and neck, and lung cancers, as well as malignant melanoma. The many mechanisms involved in p16 inactivation include promoter hypermethylation and hemizygous deletion, as noted by Silva et al. (2003) in primary breast cancers.

A number of investigators have examined p16 gene and protein expression in primary human breast cancers and endometrial carcinomas (Singh et al., 2004). High levels of p16 expression have been associated with loss of pRb expression in both primary cancers and cell lines of various kinds, presumably due to loss of a feedback loop and the fact that pRb expression is transcriptionally repressed by ectopic p16 expression. In other human cancers, loss of p16 expression, regardless of the mechanism, appears to confer a grave prognosis, presumably due to more rapid cell growth and increased mutation rate in *p16*-null cells (Robinson et al., 2003). Geradts and Ingram (2000) found p16 to be the most common target of cell cycle deregulation in invasive breast carcinomas. In recent years, a separate study revealed that the p16 protein was overexpressed in primary breast cancer, and this high level of expression was an indicator of poor prognosis (Di Vinci et al., 2005).

In our study, rather than decreased expression of p16 correlating with poor prognostic factors, there was a statistically significant difference between T1 and T3 ($\chi^2 = 6.675$, $P < 0.05$) tumors, and statistically significant differences among grade III and grades I and II tumors ($\chi^2 = 6.944$ and $\chi^2 = 4.735$, respectively; $P < 0.05$). There was also a significant difference in p16 protein expression between tumors with lymph node metastasis (N3) and without (N0) ($\chi^2 = 5.721$, $P < 0.05$).

However, mechanistic studies on the role of p16 in breast cancer have yielded inconsistent findings. Dublin et al. (1998) examined p16 expression by immunohistochemistry in 192 primary breast cancers and found that high expression was associated with poor outcome. Han et al. (2001) reported that p16 protein expression was associated with poor survival of breast cancer patients after chemotherapy.

In this study, we found that p16 expression showed no significant difference between T1 and T2, or T2 and T3 tumor groups ($\chi^2 = 2.276$, $\chi^2 = 1.885$, respectively; $P > 0.05$); no

significant difference between grades I and II tumors ($\chi^2 = 0.736$, $P > 0.05$); and no statistically significant difference between N1, N2, and N3 groups ($\chi^2 = 0.086$, $\chi^2 = 0.780$, $\chi^2 = 0.275$, respectively; $P > 0.05$).

Ohta et al. (2013) demonstrated that JARID1B/KDM5B may play a role in active chromatin compaction of the *p16* promoter, contributing to gene silencing. Therefore, JARID1B/KDM5B depletion may lead to p16 activation. It has also been suggested that JARID1B/KDM5B itself has tumor-suppressive activity, partly due to its binding and ability to stabilize hypophosphorylated pRb, leading to maintenance of pRb-mediated cell-cycle control (Roesch et al., 2006).

In this study, we demonstrated that JARID1B/KDM5B and p16 protein expression were negatively correlated ($r = -0.303$, $P < 0.001$) in 176 breast cancer tissues. The fact that JARID1B/KDM5B is overexpressed in breast cancer raised the question of whether it may also determine a population in epithelial malignancies with increased stemness capacity (Lu et al., 1999). Therefore, aberrant overexpression of JARID1B/KDM5B in any tumor, compared to corresponding non-neoplastic tissues, makes it an ideal molecular candidate with potential for use in cancer detection and as a therapeutic target. In particular, combined *p16* gene therapy and anti-JARID1B/KDM5B treatment may prove to be effective anti-breast cancer mechanisms.

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