KDM3A is not associated with metastasis and prognosis of breast cancer

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Abstract. Lysine demethylase 3A (KDM3A), also known as JMJD1A, has been associated with metastasis and poor prognosis in several cancer types, including renal cell carcinoma, prostate cancer and Ewing sarcoma. However, little is known regarding the clinicopathological significance of KDM3A expression in breast cancer (BCa). To investigate the clinical relevance of KDM3A expression in the setting of BCa, immunohistochemistry was performed on a tissue microarray consisting of 150 commercially available BCa samples. No significant correlation was identified between KDM3A expression and various clinicopathological variables, including clinical stage, pathological grade, tumor size and the expression statuses of human epidermal growth factor receptor 2, estrogen receptor, and progesterone receptor. In addition, no significant association between KDM3A expression and overall prognosis was observed. Taken together, these findings suggest that there is no significant association between KDM3A expression and clinicopathological variables, indicating that KDM3A may not be associated with the malignant behavior of BCa.

Introduction

Breast cancer (BCa) is the most prevalent cancer among women worldwide and annually accounts for 25% (1.7 million) of new cases and 15% (more than 0.5 million) of cancer-related deaths (1). Despite therapeutic advances, including local interventions (mastectomy and radiotherapy) and systemic treatments (chemo/hormonal or targeted therapies) (2,3), thousands of women still die of BCa every year due to relapse and metastasis (4). It has been proposed that relapse and metastasis involve genetic and epigenetic alterations to BCa-related oncogenes or tumor suppressors (5). Therefore, understanding the phenotypic relevance of oncogenes reported to be associated with relapse and metastasis of BCa is crucial.

Some studies have suggested an association between lysine demethylase 3A (KDM3A) and BCa relapse and metastasis (6,7). KDM3A, also known as JMJD1, is an iron- and oxoglutarate-dependent dioxygenase that can specifically demethylate monomethyl- and dimethyl-H3K9 (8). Initially, it was reported that KDM3A was upregulated during hypoxia and is required for hypoxic-mediated gene expression (9). KDM3A has also been reported to be involved in the chemoresistance (7), proliferation (10,11), migration and invasion (7,12,13), angiogenesis (14) and recurrence (13) of several different cancer types, which suggests the potential of KDM3A as a druggable target for cancer therapy. Most studies linking KDM3A with cancer are mechanistic and primarily use *in vitro* cancer cell culture systems, and there is a paucity of data concerning KDM3A expression in terms of clinicopathological relevance in cancer tissue.

Considering the limited information regarding KDM3A expression in clinical BCa tissues, we explored the clinicopathological significance of KDM3A expression via immunohistochemistry of a BCa tissue microarray (TMA). No significant association between KDM3A expression and any clinicopathological variables, including demographic parameters, clinical stage, tumor grade, lymph node metastases, and the expression status of human epidermal growth factor receptor 2 (Her2), ER or PR was observed. Furthermore, KDM3A expression was not significantly associated with overall prognosis. These results suggest that KDM3A expression may not be associated with metastasis and prognosis of BCa as previously reported.

Materials and methods

Clinical tissues. The present study was approved by the Medical Ethics Committee at the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China). The TMA used for the immunostaining analysis of KDM3A was commercially purchased from Shanghai Outdo Biotech. Co. Ltd. (Shanghai, China). The array consisted of 150 individual BCa tissues. Staging and grading of the samples was assessed in

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accordance with the World Health Organization classification and grading system. None of the samples were collected from patients who underwent chemoradiotherapy prior to resection. Informed consent was obtained from all the subjects involved. The corresponding clinicopathological information, including age, clinical stage, tumor grade, estrogen receptor (ER) status, progesterone receptor (PR) status, Her2 status, lymph node metastasis and overall 5-year and 10-year prognoses, was available for each BCa tissue sample.

Immunohistochemical staining. Briefly, the BCa TMA was deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using citrate buffer (pH=6) and a microwave histoprocessor (Haier, Qingdao, China), after which the tissue sections were incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Tissue sections were then incubated with an antibody targeting KDM3A (dilution, 1:100; TA332173; Origene, Technologies, Inc., Rockville, MD, USA) overnight in a humidified chamber at 4˚C. Immunostaining was visualized using a labeled horseradish peroxidase (HRP)-conjugated AffiniPure mouse Anti-rabbit IgG antibody with 3,3'-diaminobenzidine as a chromogen (Dako Canada Inc., Mississauga, Ontario, Canada), and the tissues were counterstained with hematoxylin.

Immunoscoring. The sections were evaluated under a light microscope, and cellular localization of the protein and the immunostaining intensity of each section were assessed by two pathologists. The staining patterns were scored based on the signal intensity as follows: Negative (no positive staining), weak (<15% of cells with positive staining), medium (>15% but <30%) and strong (>30% of cells with positive staining). For the clinicopathological analysis, the negative and weak samples were recategorized as low expression, whereas medium and strong samples were recategorized as high expression. Additionally, the use of a general rabbit anti-human IgG in place of the primary antibody served as a negative control as recommended by Hewitt *et al* (15).

Statistical analysis. Statistical analysis was conducted using SPSS 17.0 version (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0. Data are expressed as the mean ± SD and were analyzed using Student's t-test and the χ^2 test as appropriate. Kaplan-Meier survival curves were plotted, and log-rank tests were performed. $P<0.05$ was considered to indicate as statistically significant difference, and all listed P-values ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$) were calculated vs. the respective control groups.

Results

KDM3A staining. The primary aim of our study was to investigate the clinicopathological significance of KDM3A expression in BCa. To measure the expression of KDM3A in BCa tissues, IHC was performed using a TMA consisting of 150 unique BCa samples. The specificity of the primary antibody against KDM3A used for IHC was first evaluated using the antigen preabsorption approach as previously recommended (16); the results of this trial suggested that the specificity of the primary antibody against KDM3A was adequate for detection in our TMA (Fig. 1). KDM3A staining was primarily nuclear, and KDM3A was heterogeneously expressed in BCa tissues, with negative, weak, moderate or strong staining, as shown in Fig. 2.

Association between KDM3A and clinicopathological characteristics of BCa. We next analyzed the clinicopathological significance of KDM3A expression. Interestingly, no significant correlation was observed between KDM3A expression in the BCa tissues and the clinicopathological parameters, including age, clinical stage, tumor grade, ER status, PR status, Her2 status and lymph node metastasis (Table I). Furthermore, it was shown that there was no significant association regarding prognosis among patients with high KDM3A expression vs. patients with low KDM3A expression (Fig. 3). The statistical analysis suggests that KDM3A expression did not correlate with any of the clinicopathological variables available.

Association between KDM3A, and Her2, ER and PR expression. To analyze whether KDM3A expression is correlated with Her2, ER or PR expression, the Spearman correlation was used. The results indicated that there was no significant correlation between KDM3A and Her2, ER or PR expression (Table II), suggesting that there is no causal relationship between KDM3A and the status of Her2, ER or PR.

Discussion

In the present study, to the best of our knowledge, we showed for the first time that there was no significant association between KDM3A expression and metastasis and prognosis of BCa, which suggests that KDM3A might not be involved with metastasis and prognosis in BCa.

Originally, KDM3A was reported to be involved in H3K9 demethylation and transcriptional activation of the androgen receptor (8) as well as in spermatogenesis (17) and hypoxia (18,19). In the context of cancer, KDM3A has been implicated in lung cancer carcinogenesis (20) and has been shown to be required for growth of tumor xenografts (21,22); furthermore, KDM3A can induce migration and invasion in neuroblastoma (12), hepatocarcinoma (13) and BCa (7). The literature therefore supports a potential role for KDM3A in cancer growth and metastasis. To assess the role of KDM3A on tumor progression and metastasis in the context of BCa, we investigated KDM3A expression using a BCa TMA and focused on the clinicopathological significance of its expression.

Unexpectedly, we observed no significant associations between KDM3A expression in BCa tissues and clinicopathological variables, including demographic parameters, TNM stage, tumor size, and Her2, ER and PR expression. Additionally, no significant association between KDM3A expression and overall prognosis was found after statistical analysis. However, in other types of cancer such as gastric cancer, elevated JMJD1A expression (an analog of KDM3A) was associated with its prognosis and metastasis (23). In our study of BCa, we did not observe this type of correlation. In addition, increased JMJD1A levels have been purported to be associated with the progression of renal cell carcinoma. Nevertheless, we did not find a similar association in our own study compared to the results by Guo *et al* (14). One

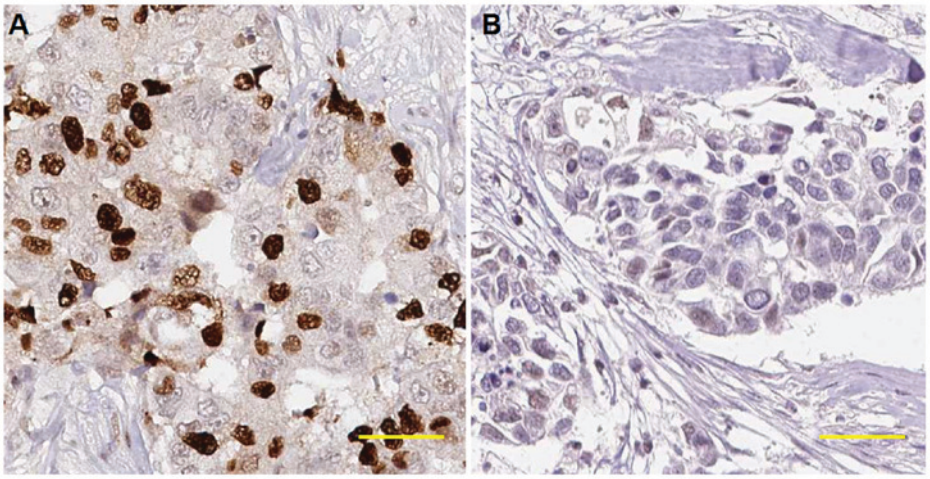

Figure 1. Preliminary testing and evaluation of primary antibody specificity against KDM3A using the antigen preadsorption approach. Immunostaining after direct incubation with the antibody against KDM3A at a dilution of 1:100 (A) on a BCa sample; immunostaining after the antibody was preincubated with recombinant KDM3A (20 μg/ml) before antibody treatment on the tissue sample (B). Magnification, x400. KDM3A, lysine demethylase 3A; BCa, breast cancer.

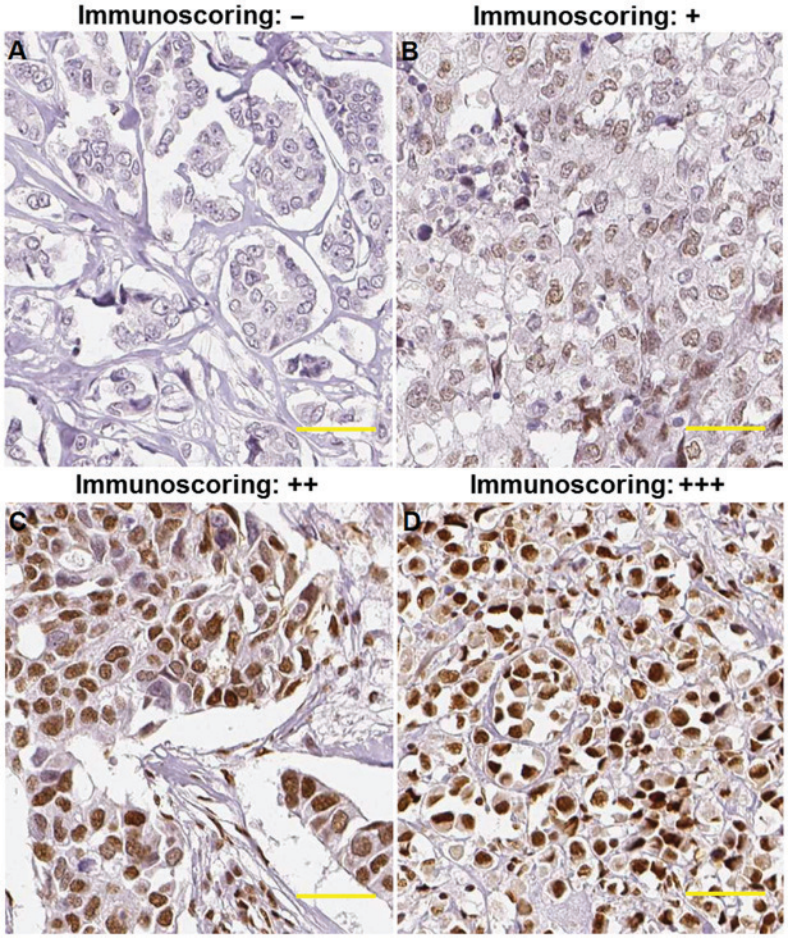

Figure 2. Heterogeneous expression of KDM3A in BCa tissues. Immunostaining of KDM3A was negative (A), weakly positive (B), moderately positive (C) or strongly positive (D) in BCa tissues. Magnification, x400. KDM3A, lysine demethylase 3A; BCa, breast cancer.

study (13) of hepatocellular carcinoma did not identify an association between elevated JMJD1A expression and any clinicopathological characteristics using multivariate Cox regression analysis (similar to the results of our study) but found that JMJD1A was an independent predictor of recurrence. However, another study by Suikki *et al* (24) detected the mRNA levels of JHDM2A (another name of KDM3A) in prostate cancer tissues and found that despite significant increases in JHDM2A mRNA expression in prostate cancer than in benign prostate hyperplasia, the authors claimed that JMJD2 was unlikely contributing a major role in the progression of prostate cancer after statistical analysis.

Table I. Analysis of the association between KDM3A expression and clinicopathological variables in BCa (n=150).

Clinicopathological variables	No.	KDM3A expression		χ^2	P-value
		Low	High		
Age, years					
≤50	72	43	29	1.079	0.299
>50	78	40	38		
Clinical stage					
I	11	6	5	0.03	0.985
II	86	49	37		
III	50	28	22		
Pathological grade					
I	38	25	13	2.102	0.147
II	111	58	53		
Diameter, cm					
<2	14	7	7	0.388	0.824
2-5	111	61	50		
>5	25	15	10		
Lymph nodes metastases					
0	54	31	23	0.103	0.950
1-3	47	27	20		
≥4	44	24	20		
ER					
-	38	21	17	0.117	0.733
+	63	37	26		
PR					
-	46	25	21	0.327	0.567
+	55	33	22		
HER2					
-	76	45	31	0.400	0.527
+	25	13	12		

The cases involved, totaling 150, did not mean that all cases whose clinicopathological variables were available. In our analysis, of these 150 cases, there were 3 cases whose clinical stage information were unavailable, 1 case without pathologic grade, and 5 cases whose lymph nodes metastases were unavailable. In terms of positive staining of ER, PR and HER2, there were only 101 cases have had whereas the remainder were unavailable. KDM3A, lysine demethylase 3A; BCa, breast cancer; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor.

Consequently, the abovementioned studies together with our own results support the suggestion that the role of KDM3A and its expression levels appear to vary in different types of cancer.

In a recent functional study performed in BCa (25), KDM3A expression was observed to gradually increase during BCa transformation and was elevated in BCa tissues compared to paired control tissues. Consequently, the authors deemed increased KDM3A expression levels as an important event during BCa transformation. In consideration of this, it would be difficult to compare the data from our study with previous studies of KDM3A in BCa because most of the data are derived from *in vitro* models of BCa, whereas our study focused on the relationship between KDM3A expression in BCa tissue and clinicopathological variables. Another recent study (26) of note showed a significant association between elevated JMJD1A expression and poor overall prognosis of patients with BCa. This result appears

Table II. Analysis of the association between KDM3A and Her2, ER and PR expression.

Protein	KDM3A	ER	PR	HER2
KDM3A	1.000	-	-	-
ER	-0.034	1.000	-	-
PR	-0.057	0.767	1.000	-
HER2	0.063	-0.218	-0.305	1.000

KDM3A, lysine demethylase 3A; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor.

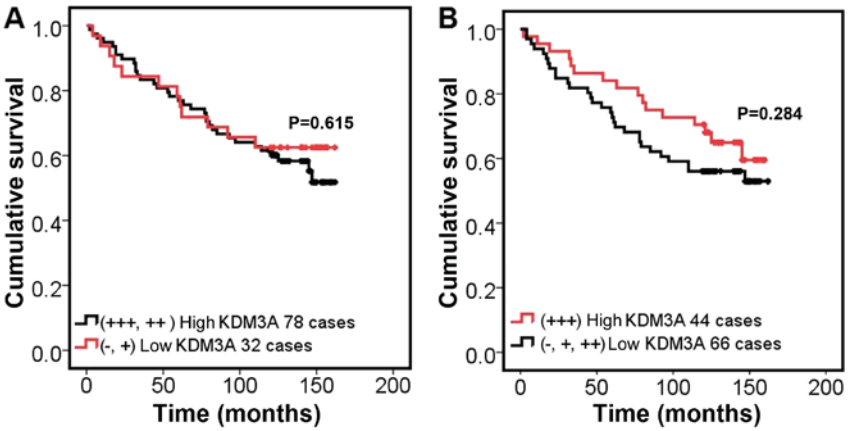

Figure 3. Prognostic significance of KDM3A expression using Kaplan-Meier survival curves. Among the 150 samples in the TMA, only 110 samples had survival information available for the corresponding patients; the remaining data either were missing or unavailable. All the patients involved were subdivided based on the expression status of KDM3A. Patients with either negative (-) or weakly positive (+) KDM3A expression were defined as low expression, whereas patients with moderately (++) or strongly positive (+++) KDM3A expression were defined as high expression (A). Alternatively, the negative (-), weak (+) and moderate (++) groups were reclassified as low expression, and patients with strong (+++) KDM3A expression were redefined as high expression (B). In both scenarios, no significant differences were observed. Log-rank tests were used for statistical analysis. KDM3A, lysine demethylase 3A; TMA, tissue microarray.

to contradict the observation in our setting that despite no significant association, there exists a small trend toward a better overall prognosis of patients with BCa and elevated JMJD1A expression.

Because there were no paired normal control tissues available with the BCa TMA, we were unable to assess the relative expression of KDM3A in BCa tissues compared with corresponding normal control tissues. In addition, we only explored the correlation between KDM3A expression and the status of Her2, ER and PR expression. It was determined that in our experimental setting, there was no significant correlation between KDM3A expression and the Her2, ER or PR status. This result leads to the suggestion that there might be no explicitly causal relation between KDM3A and Her2, ER or PR; however, this appears to be inconsistent with the observation made by Wade *et al*, who, using mechanistic investigations, reported that KDM3A was required for ER signaling in BCa (6). Thus, more studies are necessary to determine whether KDM3A plays any causal role in BCa with a different Her2, ER or PR status. BCa can be classified as either triple-negative or non-triple-negative based on the expression of Her2, ER and PR. In our analysis, we were unable to further stratify the results based on the Her2, ER and PR status. Consequently, whether there exists an association between KDM3A and Her2, ER or PR in triple-negative and non-triple-negative BCa remains unknown and should be investigated in future studies. However, Ramadoss *et al* (7) discovered that KDM3A played a dual role in the invasion and apoptosis of triple-negative BCa by demethylating histones and the non-histone protein p53, respectively. Whether KDM3A plays a dual role in the invasion and apoptosis of non-triple-negative BCas remains unknown.

Several technical factors could potentially account for the discrepancy between our findings and other relevant reports. First and foremost, given the importance of the accuracy and specificity of primary antibodies (27), the primary antibody against KDM3A used in our study was distinctly different from that employed by previous studies (14,28). Second, considering the inherent limitations of TMAs (29,30), the BCa TMA we used could contribute underlying bias. Third, the influence of slide aging (31) as well as the immunoscoring criteria adopted (32) also cannot be neglected in the analysis. Finally, in the absence of a functional analysis of KDM3A *in vitro* cell culture systems, we cannot measure the basic biological roles it mediates regarding the proliferation, migration and invasion of BCa cells.

Taking our findings together, we showed, for the first time to our knowledge, that KDM3A expression was not associated with metastasis and prognosis in BCa as assessed using a TMA, suggesting that KDM3A may not play a key role in the progression of BCa.

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Availability of data and materials

All data generated or analyzed during this study has been included in this published article.

Authors' contributions

JY was involved in data collection. SZ interpreted the data and performed statistical analysis. BL and XL provided the biochemical reagents and performed immunohistochemistry. WL conceived the whole study design and provided funding.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee at the First Affiliated Hospital of Xinjiang Medical University. Informed consent was obtained from all the subjects involved.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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