Expression of JMJD2A in infiltrating duct carcinoma was markedly higher than fibroadenoma, and associated with expression of ARHI, p53 and ER in infiltrating duct carcinoma

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Received 25 June 2012; revised 12 October 2012

Jumonji Domain Containing 2A (JMJD2A) may be a cancer-associated gene involved in human breast cancer. With a view to investigating expression of JMJD2A in human breast cancer and benign lesion tissues as well as relationship between JMJD2A and tumor related proteins, histological and immunohistochemical analysis, Western blot and quantitative real-time PCR in infiltrating duct carcinoma and fibroadenoma for JMJD2A and immunohistochemical analysis and quantitative real-time PCR in infiltrating duct carcinoma for tumor related proteins (ARHI, p53, ER, PR and CerbB-2) were performed. Histological examination validated the clinical diagnosis. The JMJD2A positive rate of infiltrating duct carcinoma was significantly higher than fibroadenoma by immunohistochemical analysis. The mean optical density of JMJD2A in infiltrating duct carcinoma was higher than fibroadenoma by western blot. JMJD2A mRNA level in infiltrating duct carcinoma was higher than fibroadenoma by quantitative real-time PCR. Spearman correlation analysis revealed that the expression of JMJD2A was associated with ARHI, p53 and ER from immunohistochemical results respectively. Pearson correlation analysis revealed that the expression of JMJD2A was associated with ARHI, p53 and ER from quantitative real-time PCR results respectively. Expression of JMJD2A in infiltrating duct carcinoma was higher, and associated with ARHI, p53 and ER. The results may take JMJD2A as a potential diagnostic and therapeutic target in human breast cancer.

Keywords: Correlation, Fibroadenoma, Infiltrating duct carcinoma, JMJD2A, Tumor related protein

Human breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females according to the GLOBOCAN 2008 estimates, accounting for 23% of the total cancer cases and 14% of the cancer deaths. With the incidence rate increasing year by year, prognosis of patients with advanced stage breast cancer is poor because of the progression and metastasis of the tumor, even if various therapies were employed for most cases. Better understanding the molecular mechanisms underlying the progression of breast cancer is required for prevention and treatment.

Jumonji Domain Containing 2A (JMJD2A, also known as JHDM3 or KDM4A) identified and characterized in 2004 is widely expressed in human tissues and cell lines, and high expression of endogenous JMJD2A mRNA was found in several cell types, including human T-cell lymphotropic virus 1-infected cell lines, HT1376 bladder carcinoma cell line, U2OS osteosarcoma cell line and prostate cancer cell line. JMJD2A belongs to the cancer-associated genes family JMJD2 proteins containing JmjC domain are lysine trimethyl–specific histone demethylases with the capability of catalyzing the demethylation of trimethylated H3K9 (H3K9me3) and H3K36 (H3K36me3).

Li et al. have verified that knock down of JMJD2A in human breast cancer cell line MCF-7 and MDA-MB-231 could result in inhibition of proliferation, migration and invasion. The lowly metastatic MCF-7 cells and highly invasive and aggressive MDA-MB-231 cells would mimic the expression patterns of their in vivo counterparts. It can be inferred that JMJD2A may take part in breast carcinogenesis and cancer progress.

However, there have been no information on the expression of JMJD2A in both malignant breast tumor and benign lesion so far. This present study has been undertaken to examine the expression of JMJD2A

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in human breast cancer and benign lesion tissues. Further, investigation the relationship between JMJD2A and other tumor related protein such as ARHI, p53, ER, PR and CerbB-2 has also been carried out in the study.

**Materials and Methods**

Tissue samples of human breast cancer (infiltrating duct carcinoma, 104) and benign lesion (fibroadenoma, 60) were obtained from Fudan University Shanghai Cancer Center. Among the 104 infiltrating duct carcinoma tissue samples, 24 samples were randomly selected to additional immunohistochemical analysis and Quantitative real-time PCR for ARHI, p53, ER, PR and CerbB-2. Trizol, RT Kit and SYBR Premix Ex Taq were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd, China. Monoclonal rabbit anti-JMJD2A, ARHI, p53, ER, PR and CerbB-2 antibodies were purchased from Cell Signaling Technology, USA. S-P immunohistochemical detection kit and DAB reaction kit were purchased from Beijing Zhongshan Biotechnology Co., Ltd, China. All primers used in the study were synthesized by Shanghai Daweike Biotechnology Co., Ltd, China.

**Histological and immunohistochemical analysis—** All the samples were paraffin-embedded and cut into 4 μm thick sections. These were mounted on glass slides and stained with Hematoxylin and Eosin (H&E) for histological examination. The slides were deparaffinized and treated with 1% hydrogen dioxide to block endogenous peroxidase. Heat induced epitope retrieval was performed. After pre-incubating in 0.2% Tween-20/PBS buffer containing 5% dry milk, slides were incubated with anti-JMJD2A, ARHI, p53, ER, PR and CerbB-2 monoclonal antibody respectively at 4 °C overnight. After rinsing, biotinylated secondary antibody and horseradish peroxidase labeled streptavidin were added. The signal was developed with DAB-H₂O₂ solution, and slides were counterstained with 5% hematoxylin. These slides were observed under microscope. The brown signals represent positive staining for JMJD2A, ARHI, p53, ER, PR and CerbB-2 respectively. Results were defined in 4 groups by the average percentage of positive cell in 5 fields [negative <10% (-), weakly positive 11% ~50% (+), moderately positive 51% ~75% (++)], strongly positive >76% (+++)]

**Western blot—** Different samples were homogenized in Western blot analysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 0.28 kU/L aprotinin, 50 mg/L leupeptin, 1 mM benzamidine and 7 mg/L pepstatin A. The homogenate was then centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was retained and preserved at -80 °C for later use. Protein concentration was determined using a BCA kit (Pierce). Protein (20 μg) from each group samples was subject to electrophoresis on 10% SDS-PAGE gel using a constant current. Proteins were transferred to nitrocellulose membranes on a semidy electrotransferring unit and incubated with monoclonal rabbit anti-human JMJD2A antibody (Cell Signaling Technology, USA, 1:1000) in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk overnight at 4 °C. After the overnight incubation with the primary antibodies, membranes were washed and incubated with HRP-labelled goat anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., USA) in TBST for 2 h. Immunoreactivity was detected with enhanced chemoluminescent autoradiography (ECL kit, Amersham), according to the manufacturer’s instructions. The membranes were reprobed with GAPDH (Cell Signaling Technology, USA, 1:1000) after stripping. The signal intensity of primary antibody binding was quantitatively analyzed with Sigma Scan Pro 5 and was normalized to a loading control, GAPDH³³.

**Quantitative real-time PCR—** Total RNA of these samples was extracted respectively with the RNAiso Reagent kit (TaKaRa, Dalian, China) at 48 h after transfection. cDNA was generated by reverse transcription of 2 µg of total RNA using oligonucleotide primers and PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Dalian, China) in a total reaction volume of 40 µL according to the manufacturer’s instructions. The sequences of forward and reverse oligonucleotide primers, specific to JMJD2A, ARHI, p53, ER, PR, CerbB-2 and housekeeping genes, used were as follows:

- **JMJD2A:** F 5'-TGTCCTCGTGGCTCGTCTAG-3' R 5'-GTCTCCTCGTCTGTCCATCC-3'
- **GAPDH:** F 5'-TGACGGTGGGCTGCTGGAC-3' R 5'-GTCCTTCCTCTGTCGTCG-3'
- **ARHI:** F 5'-AGAAAGGGGTCTCCTGCTG-3' R 5'-GCAGCTTCTGTTCCTTGGAG-3'
- **p53:** F 5'-CACGTACTCTCCTCCCAG-3' R 5'-ATTTCCTCACCACCGATAC-3'
- **CerbB-2:** F 5'-TGTCCTCGTGGCTCGTCTAG-3' R 5'-GTCTCCTCGTCTGTCCATCC-3'
ER: F 5’-CCACCAACCAGTGCACCATT-3’
R 5’-GGTCTTTTCGTATCCCACCTTTC-3’
PR: F 5’-GAACCAGATGTGATCTATGCAGGA-3’
R 5’-CGAAAACCTGGCAATGATTTAGAC-3’
CerbB-2: F 5’-TCACCTACAACACAGACCGTTTG-3’
R 5’-ATCCCACGTCCGTAGAAGGTA-3’

Primers were synthesised by Shanghai Daweike Biotechnology Co. Ltd (Shanghai, China).

Real-time quantitative PCR was performed in an ABI PRISM 7500 Real-Time System. A 10-fold dilution of each cDNA was amplified in a 20 µL volume, using the SYBR Premix Ex TaqTM Perfect Real Time (TaKaRa, Dalian, China), with 0.2 µM final concentrations of each primer. PCR cycle conditions were 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The amplification specificity was evaluated with melting curve analysis. Threshold cycle Ct, which correlates inversely with the target mRNA levels, was calculated using the second derivative maximum algorithm provided by the iCycler software. For JMJD2A, the mRNA levels were normalized to GAPDH mRNA levels.

Statistics analysis—The data were presented as means±SE for human breast cancer and benign lesion tissue samples. Statistical analysis was carried out by one-way ANOVA followed by Dunnett t-test or Student’s t-test (two means comparison), Spearman correlation analysis and Pearson correlation analysis. Statistical analysis was given using the related programs in SPSS 12.0. Differences were considered significant when P<0.05.

Results
Validation of the hospital clinical diagnosis—All the clinical diagnosis from Fudan University Shanghai Cancer Center was proved to be correct by histological examination (Fig. 1).

Expression of JMJD2A in infiltrating duct carcinoma tissue was markedly higher than that in fibroadenoma—In the immunohistochemical analysis, the positive products were mainly located in the cell nuclei and a few located in the cytoplasm. According to immunohistochemical analysis and statistic analysis (Fig. 2), it was shown that the positive rate of infiltrating duct carcinoma tissue (82.7%, 86/104) is significantly higher than benign lesion tissue (21.7%, 13/60). The 104 infiltrating duct carcinoma tissue samples were made up of 22 strongly positive results (21.2%), 34 moderately positive results (32.7%), 30 weakly positive results (28.8%) and 18 negative results (16.7%). While 2 strongly positive results (3.3%), 5 moderately positive results (8.3%), 6 weakly positive results (10.0%) and 47 negative results (78.3%) composed the 60 benign lesion tissue samples.

Western blot analysis (Fig. 3A) showed that, the expression of JMJD2A protein in infiltrating duct carcinoma tissue was notably higher than benign lesion tissue (P<0.05). The mean optical density of infiltrating duct carcinoma tissue was 1.094±0.151, while that of benign lesion tissue was 0.563±0.105. These data indicated that in protein level expression of JMJD2A infiltrating duct carcinoma tissue was higher.

Based on the results of quantitative real-time PCR (Fig. 3B), there was significant difference (P<0.05) in the levels of JMJD2A mRNA between...
Fig. 2—Immunohistochemical analysis results of expression of JMJD2A in infiltrating duct carcinoma and fibroadenoma. [A: JMJD2A positive cells in infiltrating duct carcinoma in 4 groups (+++, ++, +, -). B: JMJD2A positive cells in fibroadenoma in 4 groups (+++, ++, +, -). C: Pie chart analysis for 4 groups’ percentages of infiltrating duct carcinoma and fibroadenoma samples respectively. The positive rate of infiltrating duct carcinoma tissue (82.7%, 86/104) is significantly higher than fibroadenoma (21.7%, 13/60), (×20)].
infiltrating duct carcinoma tissue (0.621±0.117) and fibroadenoma tissue (0.286±0.105). These data suggested that the expression of JMJD2A mRNA in infiltrating duct carcinoma tissue was higher than benign lesion tissue.

All the immunohistochemical, western blot and quantitative real-time PCR results has proved that expression of JMJD2A in infiltrating duct carcinoma tissue was markedly higher than in benign lesion tissue from three aspects including in situ, protein level and mRNA level.

Expression of JMJD2A was associated with that of ARHI in infiltrating duct carcinoma tissues—The positive products of ARHI were mainly located in the cell membrane (Fig. 4A). And the outcome of the SPSS software’s Spearman correlation analysis revealed that the expression of JMJD2A and ARHI protein was related ($P=0.026$, $r_s=0.456$).

Additionally, the outcome of the SPSS software’s Pearson correlation analysis revealed that the expression of JMJD2A and ARHI was related ($P<0.05$, $r_p=0.522$) in the mRNA level (Fig. 4B).
Expression of JMJD2A was associated with that of p53 in infiltrating duct carcinoma tissues—The positive products of p53 were mainly located in the cell nuclei (Fig. 5A). Spearman correlation analysis suggested that the expression of JMJD2A and p53 protein was related ($P=0.026$, $r_s=0.218$).

Meanwhile, the outcome of Pearson correlation analysis revealed that the expression of JMJD2A and p53 was related ($P<0.05$, $r_p=0.336$) in the mRNA level (Fig. 5B).

Expression of JMJD2A was associated with that of ER in infiltrating duct carcinoma tissues—The positive products of ER were mainly located in the cell nuclei (Fig. 6A). It was similar to ARHI and p53 that JMJD2A was associated with ER by the Spearman correlation analysis in immunohistochemical assay ($P=0.031$, $r_s=0.296$).

The RT-PCR results were analyzed by the SPSS software’s Pearson correlation analysis. And it was proved that the expression of JMJD2A and ER was related ($P<0.05$, $r_p=0.167$) in the mRNA level. The outcome of Pearson correlation analysis is shown in Fig. 6B.

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**Fig. 5**—Expression of JMJD2A was associated with that of p53 in infiltrating duct carcinoma tissues. [A: p53 positive cells in infiltrating duct carcinoma. The positive products of p53 were mainly located in the cell nuclei. B: Pearson correlation analysis result. Expression of JMJD2A and p53 were positively correlated ($\times20$)].

**Fig. 6**—Expression of JMJD2A was associated with that of ER in infiltrating duct carcinoma tissues. [A: ER positive cells in infiltrating duct carcinoma. The positive products of ER were mainly located in the cell nuclei. B: Pearson correlation analysis result. Expression of JMJD2A and ER were positively correlated ($\times20$)].
Expression of JMJD2A was not associated with that of PR in infiltrating duct carcinoma tissues—The positive products of PR were mainly located in the cell nuclei (Fig. 7A). Spearman correlation analysis suggested that there was no correlation between JMJD2A and PR protein \((P=0.227)\).

Simultaneously, Pearson correlation analysis pointed out that there was no correlation between JMJD2A and PR in the mRNA level \((P>0.05)\) (Fig. 7B).

Expression of JMJD2A was not associated with that of CerbB-2 in infiltrating duct carcinoma tissues—The positive products of CerbB-2 were mainly located in the cell membrane (Fig. 8A). JMJD2A was not associated with CerbB-2 in protein level by Spearman correlation analysis \((P=0.300)\).

Similarly, there was no correlation between JMJD2A and CerbB-2 in the mRNA level \((P>0.05)\) by Pearson correlation analysis (Fig. 8B).
Discussion

In the present study, it was proved that expression of JMJD2A in infiltrating duct carcinoma tissue was markedly higher than that in benign lesion tissue from three aspects including in situ, protein level and mRNA level. Further, it was revealed that expression of JMJD2A was associated with that of ARHI, p53 and ER in infiltrating duct carcinoma by statistics analysis.

Human breast cancer is the leading cause of cancer death among females with the features of powerful invasive ability and early metastatic property. It is meaningful to diagnose correctly, understand the pathologic mechanism of breast cancer and find treatment target site. The present results may provide a new perspective in understanding the molecular mechanisms underlying the progression of breast cancer and a potential therapeutic target in breast cancer.

Previous studies showed that knock down JMJD2A by siRNA could suppress proliferation, migration and invasion of breast cancer MCF-7 and MDA-MB-231 cell line in vitro. The results of the present study showed expression differences between malignant breast tumor and benign lesion and correlation with some tumor makers like ARHI, p53 and ER as complement to previous studies.

Combined with the inhibition from knock down of JMJD2A in MCF-7 and MDA-MB-231 cell line, the higher expression of JMJD2A in infiltrating duct carcinoma suggested that JMJD2A might hint the tumor malignant and play a role as a tumor marker.

Further investigation brought JMJD2A’s correlation with ARHI, p53 and ER to light, but the bathypelagic meaning and relation of the correlation is still not exposed. However, based on the present results and JMJD2A’s correlation with ARHI and ER, there may be two possible pathways involved in the mechanism of JMJD2A in breast cancer.

One of the possible pathways is that JMJD2A may be involved in the estrogen signaling pathway. A recent research focused on JMJD2 family proteins, especially JMJD2B which is considered to have the similar function as JMJD2A in breast cancer. The research demonstrated that JMJD2B constitutes a key component of the estrogen signaling pathway and the establishment of local epigenetic state and chromatin structure required for proper induction of ER responsive genes. JMJD2B which interacts with ERα and components of the SWI/SNF-B chromatin remodeling complex was recruited to ERα target sites, demethylated H3K9me3 and facilitated transcription of ER responsive oncogenes including MYB, MYC and CCND1, and knock down of JMJD2B severely impaired estrogen induced cell proliferation and the tumor formation capacity of breast cancer cells as a consequence. Knock down of JMJD2A also damage cell proliferation, migration and invasion capacity of breast cancer cells. The positive correlation between JMJD2A and ER found in the present study may support this pathway from another aspect.

The other pathway is the pRB-E2F complex pathway. Depletion of JMJD2A caused only a marginal defect in ER target gene induction in contrast to JMJD2B, which implies another pathway JMJD2A may participate. JMJD2A has molecular characterization in binding both retinoblastoma protein (pRB) and histone deacetylases (HDACs). Associating with pRB, JMJD2A may recruit HDACs to the pRB-E2F complex, change the chromatin structure at the E2F-responsive promoters and induce repression transcription from E2F-dependent promoters. E2F1, 4 and their complexes with HDAC play an important role in down-regulating the expression of the maternally imprinted tumor suppressor gene ARHI in breast cancer cells. Expression of ARHI is markedly down-regulated in breast cancer. The negative correlation between JMJD2A and ARHI is consisted with this assumption.

 Participating either estrogen signaling pathway or pRB-E2F complex pathway or both, JMJD2A may play a diverse role in human breast cancer. Further researches to understand the pleiotropic functions of JMJD2A and its contribution to human breast cancer both in vitro and in vivo are required. Though the exact mechanism is not very clear, the results of the present study focused on differences of JMJD2A expression in between human breast cancer and benign lesion tissues and the correlation with other tumor related proteins early. The results shed light on the novel role of JMJD2A as a neo-hopeful diagnosed and therapeutic target in human breast cancer.
Acknowledgement
The work was supported by the National Science Foundation of China (No. 81172897 and No. 81072512).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
BX-L and J-L carried out experiments and drafted the manuscript. CL-L and MC-Z participated in study design and helped to draft the manuscript. H-L, LL-L, HF-X, YW-S and AM-X participated in study design, performed experiments and ZQ-Z participated in study design and revised manuscript. All authors approved the final manuscript.

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