

Original Article

FOXA1 positively regulates gene expression by changing gene methylation status in human breast cancer MCF-7 cells

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Received November 4, 2014; Accepted December 23, 2014; Epub January 1, 2015; Published January 15, 2015

Abstract: Objective: DNA methylation is an important epigenetic modification with tumor suppressor gene silencing in cancer. The mechanisms underlying DNA methylation patterns are still poorly understood. This study aims to evaluate the potential value of FOXA1 for controlling gene CpG island methylation in breast cancer. Methods: FOXA1 was down-regulated by transfection with siRNA and up-regulated by transfection with plasmid in MCF-7 cell lines. The DNA methylation and mRNA levels were examined by qMSP and qRT-PCR. The cell proliferation and apoptosis was detected by MTT and Flow cytometry. Results: Suppression of FOXA1 enhanced the methylation status of DAPK, MGMT, RASSF1A, p53, and depressed mRNA levels of these tumor suppressor genes, whereas over-expression of FOXA1 showed the opposite effects. DNMT1, DNMT3A and DNMT3B mRNA were up-regulated by siRNA knock-down of FOXA1. At the same time, FOXA1 suppression promoted cell growth and inhibited apoptosis. Conclusions: FOXA1 may be associated with methylation of the tumor suppressor genes promoter through changing DNMTs expression. FOXA1 could be a potential demethylation target for prevention and treatment of breast cancer.

Keywords: MCF-7, FOXA1, tumor suppressor gene expression, methylation

Introduction

Breast cancer is one of the most prevalent and well-studied forms of cancer. Despite abundant research, knowledge of the molecular basis of breast cancer subtypes is still incomplete, due in large part to the heterogeneous nature of the disease. At least five subtypes can be identified based on unique gene expression patterns; this subtype classification is distinct from the histopathological classification. The lack of somatic mutations in sporadic breast carcinomas suggests that gene inactivation might be achieved by mechanisms other than coding region mutations, such as epigenetic or regulatory changes [1]. At the same time, the transcription factor network required for the specific gene expression signature in each of these subtypes is currently being elucidated.

DNA methylation, one of the most studied epigenetic modifications, is involved in regulating gene expression [2], growth and development [3], and carcinogenesis [4, 5]. In particular,

hypermethylation of CpG islands may lead to aberrant silencing of tumor suppressor genes [6]. In breast cancer it had already found HIN-1, RASSF1A, p16, Cyclin D2, APC, BRCA and DAPK with CpG island methylation [7].

FOXA1 (fork-head box A1) is the founding member of the FOX family of transcription factors that is comprised of at least 40 members, which was originally identified for its transcriptional regulation of the genes liver-specific trans-thyretin (Ttr) and α 1-antitrypsin (SerpinA1). Since this seminal study, FOXAs have been found to regulate many genes involved in developmental specification of not just hepatic, but several other tissues, such as prostate cancer, breast cancer [8]. The unique ability of FOXA family transcription factors to bind to target sites in silent chromatin in a dominant manner and initiate regulatory events distinguishes them from the rest of the transcription factors (~2000 bp) in the mammalian genome; therefore, these transcription factors are dubbed "pioneer factors" [9].

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Table 1. Primers used in real-time PCR

| Genes | Sequence (5'-3') | Amplification size (bp) |
|---------|---|-------------------------|
| FOXA1 | F: CGCTTCGCACAGGGCTGGAT R: TGCTGACCGGGACGGAGGAG | 144 bp |
| p53 | F: GGCCCACTTCACCGTACTAA R: GTGGTTTCAAGGCCAGATGT | 156 bp |
| RASSF1A | F: GTTCTTGGTGGTGGATGACC R: CCTTCAGGACAAAGCTCAGG | 157 bp |
| MGMT | F: GCCGGCTCTTACCATCCCG R: GCTGCACACCACTCTGTGGCAGC | 210 bp |
| DAPK | F: GCCTGGAGACGGAGAAGAT R: AAGTCCCGTGGCTGGTAGA | 153 bp |
| DNMT1 | F: GTTCTTCTCCTGGAGAATGTC R: GTCTGGGCCACGCCGACTG | 146 bp |
| DNMT3A | F: TAAGCTGGAGCTGCAGGAGT R: GGAAACCAATACCCTTTCCA | 179 bp |
| DNMT3B | F: ACCACCTGCTGAATTACTCACGC R: GATGGCATCAATCATCACTGGATT | 146 bp |
| GAPDH | F: TCAACGGATTTGGTCGATTGGGC R: TCCTGGAAGATGGTGGTGGATT | 209 bp |

Table 2. Primers used in qMSP

| Genes | Sequence (5'-3') | Amplification size (bp) |
|------------|--|-------------------------|
| DAPK(M) | F: GGATAGTCGGATCGAGTTAACGTC R: CCCTCCAAACGCCGA | 98 |
| DAPK(U) | F: GGAGGATAGTTGGATTGAGTTAATGTT R: CAAATCCCTCCAAACACCAA | 106 |
| MGMT(M) | F: TTTTCGACGTTCTAGGTTTTTCGC R: GCACTCTTCGAAAACGAAAACG | 81 |
| MGMT(U) | F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT R: AACTCCACACTCTTCCAAAACAAAACA | 93 |
| RASSF1A(M) | F: GTGTAAACGCGTTGCGTATC R: AACCCCGCGAACTAAAAACGA | 93 |
| RASSF1A(U) | F: TTTGGTTGGAGTGTGTTAATGTG R: CAAACCCACAACTAAAAACAA | 105 |
| p53(M) | F: GTAGTTTGAACGTTTTTATTTTGGC R: CCTACTACGCCCTCTACAAACG | 115 |
| p53(U) | F: GTAGTTTGAATGTTTTTATTTTGGT R: CCTACTACACCCTCTACAAACA | 115 |
| ALU(M) | F: GGTTAGGTATAGTGGTTTATATTTGTAATTTAGTA R: ATTAACATAAATAATCTTAAACTCCTAACCTCA | 150 |
| ALU(M) | F: TTATTAGAGGGTGGGGTGGATTGT R: CAACCCCAACCAACCAACATAA | 151 |

Mammalian transcription factors (TFs) regulate gene expression via different classes of regulatory elements including promoters, enhancers, and silencers [10]. Interestingly, Aurelien found FOXA1 expression was associated with DNA

demethylation [11], but the mechanism is still uncertain. Using real-time PCR, contrast clinical specimens of breast cancer and normal tissue adjacent to cancer patients, we found that the level of FOXA1, tumor suppressor genes, and DNA methyltransferase expression exist certain differences, it is hypothesized that FOXA1 through regulating the expression of DNA methyltransferase expression to change gene expression. In order to test this hypothesis, we undertook to (i) Contrast FOXA1 expression changes before and after the tumor suppressor genes and DNMTs expression changes; (ii) FOXA1 expression compared before and after the change related to the change of tumor suppressor genes methylation level; (iii) Analyze whether FOXA1 can through regulating DNMTs expression changes, in turn, control genes; (iiii) Explore proliferation and apoptosis of MCF-7 cells with FOXA1 transfection.

Materials and methods

Cell lines and cell culture

Human breast cancer cell line MCF-7 was supplied by the cell center, China University of Science and Technology. MCF-7 cells were cul-

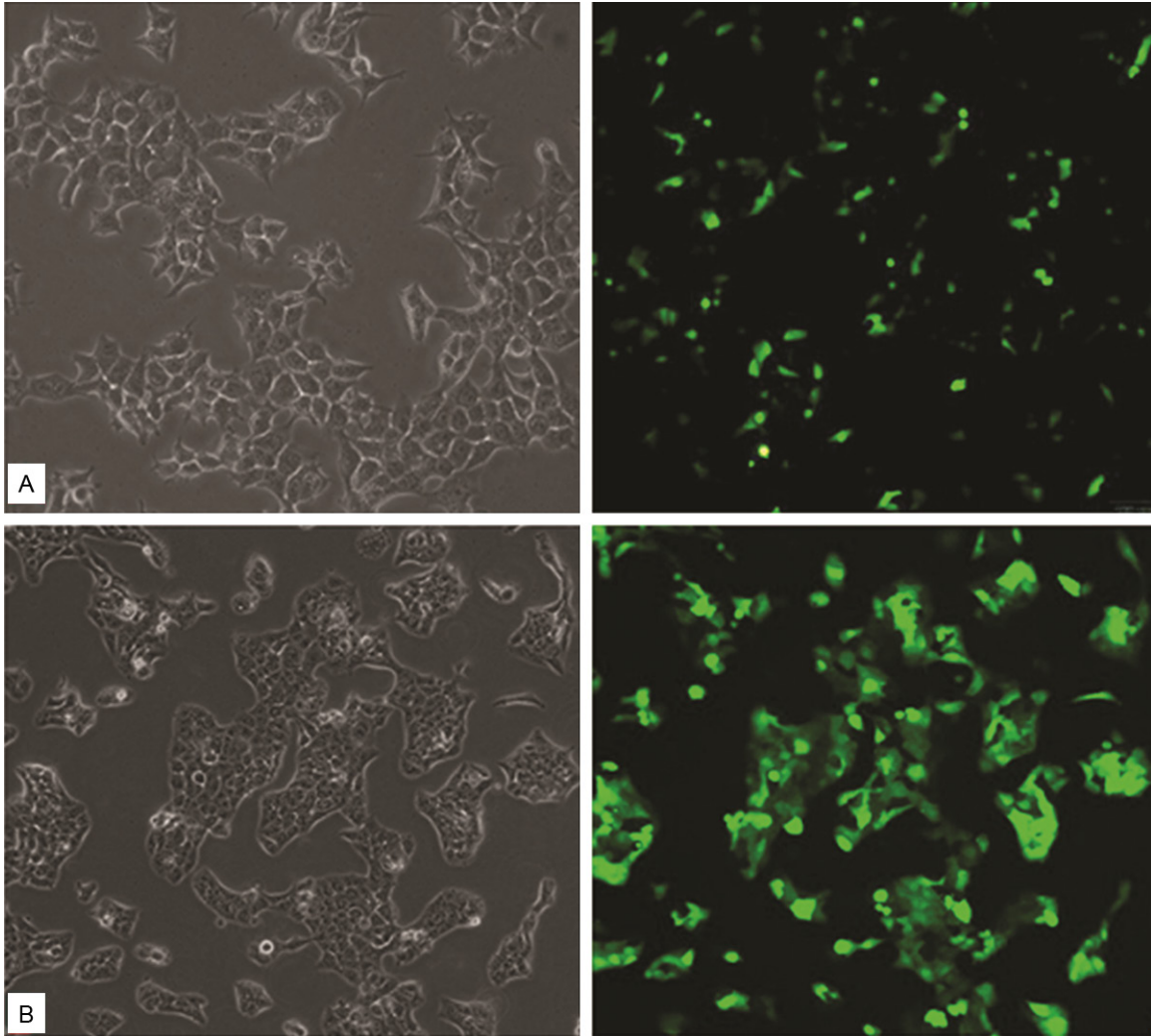


Figure 1. The transfection efficiency observed by Fluorescence microscope (A) siRNA-FOXA1 group; (B) FOXA1 over-expression group. Pictures were taken at $\times 200$ magnification.

tured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), and 0.02 mg/ml kanamycin (GIBCO) at 37°C in 5% CO₂.

RNA interference and plasmid transfection

SiRNA oligonucleotides corresponding to human FOXA1 (5'-CCATGAACACCTAC ATGACC-ATGAA-3') were used for transfection of MCF-7 cells using lipofectamine 2000 (Life Technologies). The following negative control siRNA was used: 5'-GTTG CATTGCGATTAATTACACG-3' (GenePharma, China).

RNA extraction and real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 1 μ g total RNA was subjected to reverse

transcription using reverse transcription system (Promega). Real-time PCR were performed on a Rotor-Gene 3000 (Qiagen), using SYBR Green PCR Master Mix (Qiagen) and 1/20 of RT reaction. At the end of each reaction, a melting curve analysis was performed to confirm the absence of primer dimers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantifications of target genes mRNA was performed using the 2^{- $\Delta\Delta$ Ct} method. The PCR products underwent electrophoresis on 2.5% agarose gel and were then visualized under UV illumination using ethidium bromide staining. Primers sequences were listed in **Table 1**.

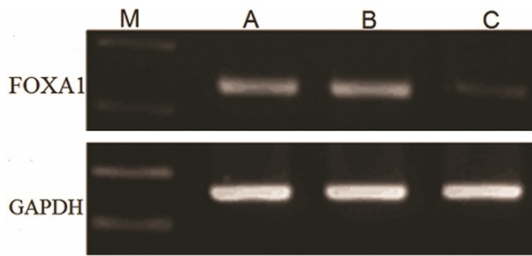


Figure 2. Different expression of FOXA1 and GAPDH mRNA in cells of each group by RT-PCR. A: MCF-7 group; B: siRNA Control group; C: siRNA-FOXA1 group; M: marker.

DNA extraction and quantitative methylation specific PCR (qMSP)

Total DNA was extracted using DNA Mini Kit (Qiagen) according to the manufacturer's protocol. 1 mg of genomic DNA from each sample was modified by sodium bisulfite with the BisulFlash DNA Modification Kit (Epegentek). qMSP was performed on a Rotor-Gene 3000 (Qiagen), using EpiTect[®] MethyLight PCR kit (Qiagen). Expression levels methylated and unmethylated DNA was normalized to the expression level of ALU gene, a region of ALU devoid of any CpG dinucleotide was amplified. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study and the annealing temperatures were listed in **Table 2**.

MTT assay

The tested cells were seeded at density 5000 cells/well in 96-well plates and measured in 1, 2, 3, 4 days after cultivating. Before measured the two kinds of cells were added 20 μ l MTT (5 mg/ml) and the cells were incubated for an additional 4h at 37°C. The culture medium was removed; 100 μ l of DMSO were added to each well. With shaking at low speed for 10 min, the MTT solution was aspirated and optical densities (OD) of the supernatant were read at 492 nm using a Microplate Reader (Thermo, Molecular Device Co). The experiments were repeated three times and the negative control was conducted using only cell-free culture medium (means \pm SEM).

Flow cytometry analysis

Fluorescein Annexin V-FITC/PI double labeling was performed with the Annexin V-FITC Apoptosis Detection Kit (Beckman) to detect

the apoptotic effects of FOXA1 expression on MCF-7 cells. The cells were stained with Annexin V-FITC and PI according to the instructions of the kit manufacturer. The apoptotic cells were determined with a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CELLQUEST software (BD Biosciences). The MCF-7 and control siRNA cells served as controls.

Tissue samples and immunohistochemistry

Tumor tissue and adjacent-tumor tissue specimens were collected from 50 patients with breast cancer resected between 2013 and 2014 at the second hospital of Anhui medical university. The sections were incubated with monoclonal mouse anti-human FOXA1 antibody (1:100, Abcam, USA). And the visualization signal was developed with 3'-diaminobenzidine (DAB) solution.

Statistical analyses

The results are expressed as mean \pm S.D. and the Mann-Whitney U test was used to evaluate the differences between groups. A value of $P < 0.05$ and $P < 0.01$ was taken to denote statistical significance.

Results

FOXA1 siRNA is effective knocking down FOXA1 expression in MCF-7 cells

After infected, the mRNA expression profiles of FOXA1 gene in each group were examined by RT-PCR with MCF-7 cells as the blank control group and control siRNA MCF-7 cells as a negative control group. The results suggested the expression profiles of FOXA1 of recombinant infection groups were under expression and over expression (**Figures 1, 2**).

Expression levels of FOXA1 was correlated to alterations of tumor suppressor genes and DNMTs expression

To determine the change in expression of tumor suppressor genes and DNMTs correlated to FOXA1 expression in the MCF-7 cell line, we analyzed expression levels of tumor suppressor genes (DAPK, MGMT, p53, RASSF1A) and DNMTs (DNMT1, DNMT3A, DNMT3B) in Control and siRNA-FOXA1 cells with RT-PCR and Real-time PCR. The expression of tumor suppressor genes mRNA were significantly decreased in siRNA-FOXA1 cells compared with Control cells,

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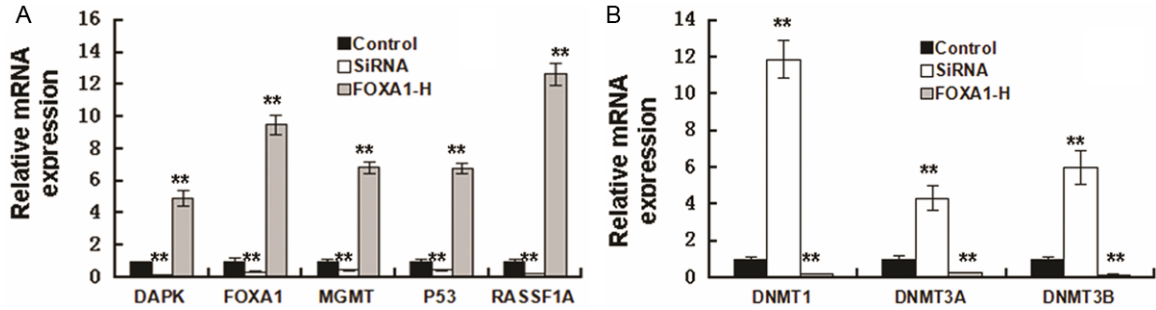


Figure 3. The mRNA levels of tumor suppressor genes. A: The mRNA levels of DAPK, FOXA1, MGMT, p53 and RASSF1A detected by RT-PCR; B: The mRNA levels of DNMTs. ** $P < 0.01$.

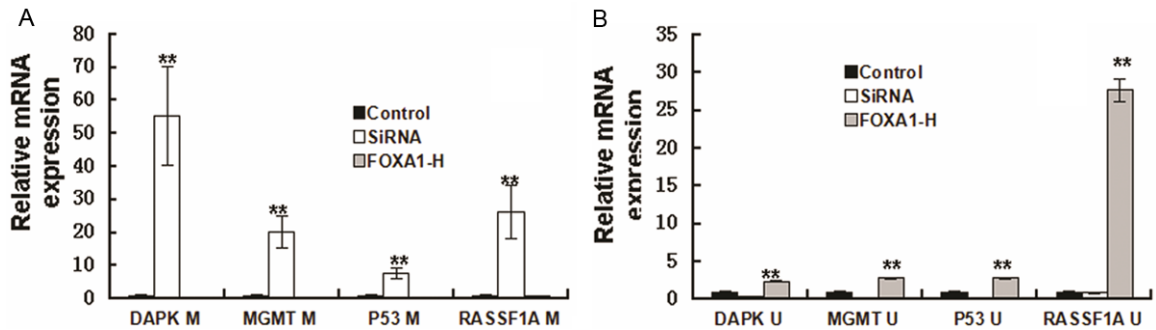


Figure 4. qMSP was used to detect the methylation status of tumor suppressor genes. A: The levels of DAPK, MGMT, p53 and RASSF1A methylation were up-regulated in siRNA-FOXA1 cells; B: The levels of DAPK, MGMT, p53 and RASSF1A unmethylation were dramatically up-regulated in FOXA1 over-expression cells. In this study, each experiment was repeated three times (** $P < 0.01$).

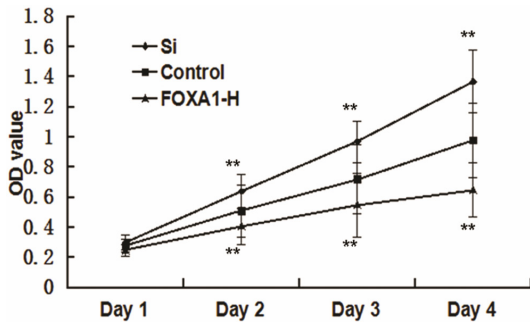


Figure 5. FOXA1 significantly promoted the proliferation of MCF-7 cells. An MTT assay was used to detect the proliferation of MCF-7 cells. The growth curve showed that cell proliferation was significantly promoted in siRNA-FOXA1 cells. Data represent mean \pm S.D. of three independent experiments, ** $P < 0.01$.

The expression of DNMT1 was not significantly changed whereas the DNMT3A and DNMT3B were increased, which was consistent with the alterations of genomic DNA methylations. These data indicated that the alterations of tumor suppressor genes expression induced by FOXA1 was specifically correlated to expression level of DNMT3A and DNMT3B (Figure 3).

Tumor suppressor genes DNA promotor methylation status was significantly altered with the change of FOXA1 expression

We analyzed the methylation and unmethylation levels of tumor suppressor genes (DAPK, MGMT, p53, RASSF1A) promotor in our established siRNA-FOXA1 and FOXA1 over-expression cells as well as control cell using quantitative methylation specific real-time PCR (qMSP). By qMSP methylation analysis, we found that the level of DAPK, MGMT, p53 and RASSF1A methylation was dramatically up-regulated in siRNA-FOXA1 cells compared with the control cells (Figure 4A). And the level of unmethylation was dramatically up-regulated in FOXA1 over-expression cells (Figure 4B). These results suggested that tumor suppressor genes methylation was significantly altered following FOXA1 expressing in breast cancer cells and FOXA1 might contribute to regulate DNA methylation.

FOXA1 expression and association with cell proliferation and apoptosis in MCF-7 cell lines

MCF-7, siRNA-FOXA1 and over-expression FOXA1 cells proliferation was detected using an

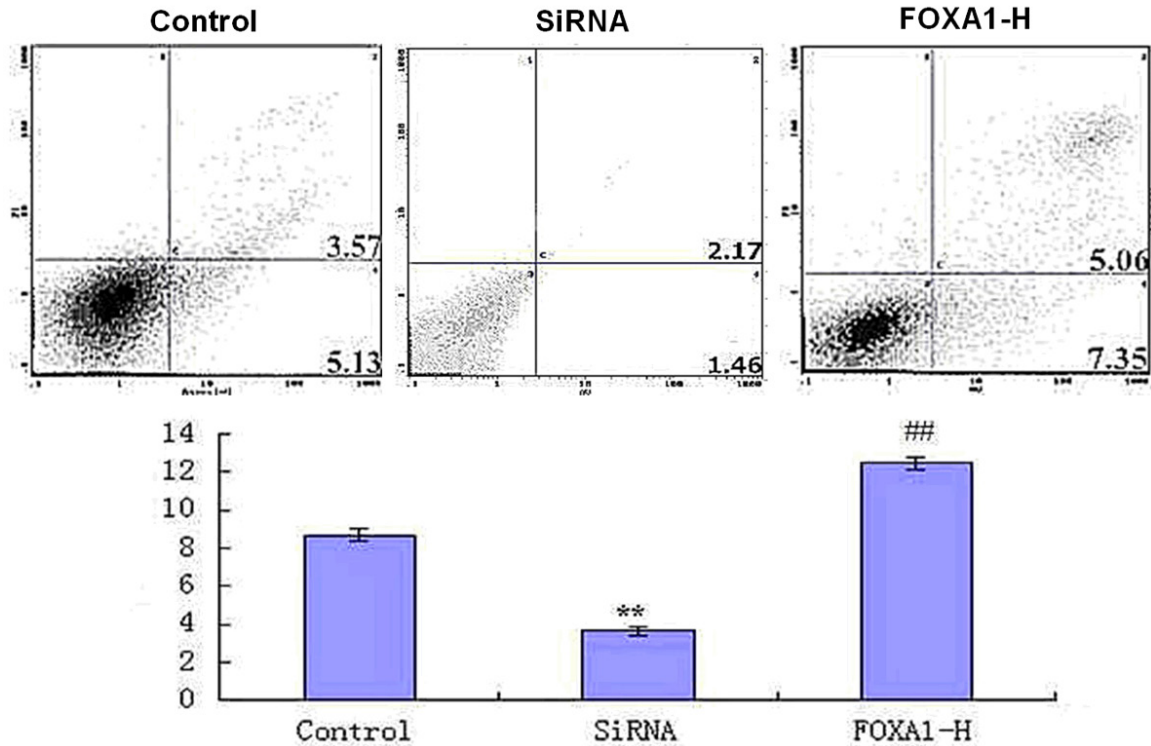


Figure 6. Scatter plots of bivariate flow cytometry, the cell apoptosis of siRNA FOXA1 transfected group was significantly decreased ($P < 0.05$).

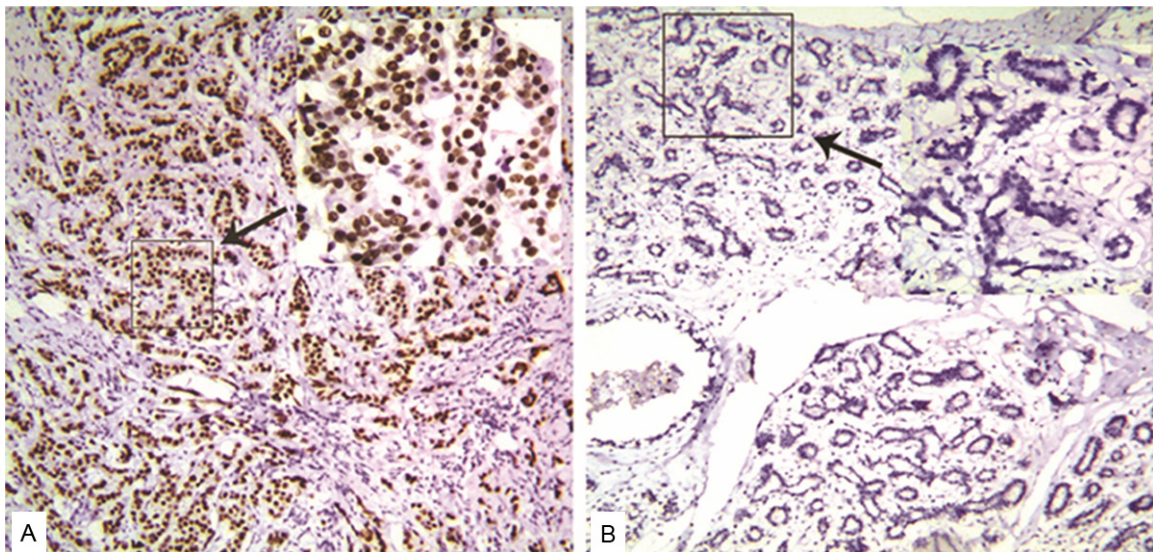


Figure 7. The FOXA1 expressing in tumor and adjacent-tumor tissue observed by immunohistochemistry. A. The positive expression in tumor tissue; B. The negative expression in adjacent tumor tissue. Pictures were taken at $\times 100$ and $\times 400$ magnifications.

MTT assay. Cell proliferation of the siRNA and over-expression FOXA1 was significantly promoted and suppression, compared to the

MCF-7 cells (Figure 5). Apoptotic analysis showed a pronounced induction decrease of apoptosis cell death by FOXA1 silencing in

MCF-7 cells (**Figure 6**). The apoptosis rates of MCF-7, siRNA Control group and siRNA FOXA1 group were $15.87\% \pm 1.24\%$, $18.49\% \pm 2.01\%$ and $11.32 \pm 3.03\%$, respectively. The apoptosis rates of MCF-7 cells with siRNA infection were lower than those of normal MCF-7 group and FOXA1 over-expression group. The differences were statistically significant ($P < 0.05$).

Different expression of FOXA1 in breast cancer tissue and adjacent-tumor tissue

There were both FOXA1 expressing in tumor and adjacent-tumor tissue. The FOXA1 level was significant greater (72.7%, 40/55) in tumor tissue than in adjacent-tumor tissue (54.5%, 30/55), $P < 0.01$ (**Figure 7**). Increased FOXA1 levels were not associated with age, Ki-67, or pathological. FOXA1 were positively correlates with the tumor subtypes (83.3% in luminal A and 58.3% in triple-negative tumors).

Discussions

The forkhead box (FOX) proteins represent a large family of evolutionary conserved transcriptional regulators that contain a highly conserved 100 amino acid DNA binding domain (DBD), known as a 'forkhead' or 'winged helix' domain [12]. Based on sequence conservation, FOX genes have been divided into 19 subfamilies (from FOXA to FOXS) [13, 14]. The mammalian genome contains more than 40 FOX genes, and mutations in many of these genes are linked to disease processes, including developmental defects, metabolic disorders, and cancer [15]. The FOXA subfamily contains three founding members of FOX proteins: FOXA1, FOXA2, and FOXA3, which were originally cloned from rat liver cells, and therefore, named as hepatocyte nuclear factor (HNF) 3 α , 3 β , and 3 γ [16, 17]. Extensive genetic, biological, and biochemical studies over the last two decades have established their essential roles in early development, organogenesis, metabolism and stem cell differentiation [18-21].

FOXA1 (forkhead box A1) is the founding member of the FOXA family. The FOXA1 gene at human chromosome 14q21.1 is amplified in lung cancer, esophageal cancer [22], estrogen receptor (ER)-positive breast cancer [23], anaplastic thyroid cancer [24], and metastatic prostate cancer [25]. In addition to gene amplifications, FOXA1 point mutations also

occur in prostate cancer [26]. FOXA1 upregulation is associated with good prognosis in breast cancer patients owing to its preferential upregulation in the ER-positive subtype [27]. FOXA1 has become a research hotspot.

Notably, in recent years epigenetics has attracted everybody's attention. Many genes methylation modification, these "chromatin marks" are thought to be involved in a functional dialogue with chromatin-binding factors in which one influences the presence of the other to finely tune the activity of cis-regulatory elements in space and time. Despite the recognized central regulatory role exerted by chromatin in control of TF functions, much remains to be learned regarding the chromatin structure of enhancers and how it is established. FOXA1, as "pioneer" has also been identified engage several nuclear receptors onto chromatin transcription factor [28].

We demonstrate for the first time that FOXA1 is associated with DNA methylation of the tumor suppressor genes promoter, the mechanism may be through the change of DNMTs. At the same time, decreasing the expression of FOXA1 promote cell growth, inhibit apoptosis. Wolf found breast cancer cells overexpressing FOXA1 will led to the decrease of the cell [29]. Our results found decrease the expression of FOXA1 will led to the increase grown of the cell, It would also be FOXA1 another potential research value. Williamson thought the mechanism was by inhibiting the activity of P27Kip1, the cyclin-dependent kinase inhibitor p27Kip [30].

Inactivation of tumor suppressor genes and reduced cell apoptosis have been reported to be essential mechanisms for breast tumorigenesis, contributing to deregulated tumor cell proliferation, invasion and metastasis. We selected the tumor suppressor genes related breast cancer. The role of these genes covers, apoptosis, cell growth cycle, blood vessel growth, and other aspects so as to comprehensive analysis FOXA1 in cancer development, growth, invasion and metastasis. According to Jing F results, the methylation rate of the gene in breast cancer specimen was RASSF1A 74%; DAPK 18%, while the methylation rate was 6% for RASSF1A in nontumor sera, there was no methylation of DAPK in nontumor sera [31]. Our study shows

that in MCF-7 cells, RASSF1A was methylated; MGMT, p53 and DAPK were unmethylated.

Death-associated protein kinase (DAPK) is a multidomain calcium/calmodulin-regulated and cytoskeletal-associated serine/threonine-kinase mandatory for interferon- γ , tumor necrosis factor α , and activated Fas-induced apoptotic cell death and detachment from the extracellular matrix, comprising modules such as ankyrin repeats mediating protein-to-protein interactions and a death domain.

The DNA repair protein O⁶-methylguanine DNA methyltransferase (MGMT) removes alkyl adducts from the O⁶ position of guanine. MGMT expression is decreased in some tumor tissues, and lack of activity has been observed in some cell lines. Loss of expression is rarely due to deletion, mutation, or rearrangement of the MGMT gene, but methylation of discrete regions of the CpG island of MGMT has been associated with the silencing of the gene in cell lines. Esteller [32] found all normal tissues and expressing cancer cell lines were unmethylated, whereas nonexpressing cancer cell lines were methylated. Among the more than 500 primary human tumors examined, MGMT hypermethylation was present in a subset of specific types of cancer. In gliomas and colorectal carcinomas, aberrant methylation was detected in 40% of the tumors, whereas in non-small cell lung carcinomas, lymphomas, and head and neck carcinomas, this alteration was found in 25% of the tumors. MGMT methylation was found rarely or not at all in other tumor types. It is worth noting, Alkam found in Basal-like breast cancer (BLBC) MGMT was methylated in 27% [33], whereas in our study, MGMT was unmethylated in MCF-7 cell lines. Changing MGMT methylation level may as a potential target of basal-like breast cancer.

The p53 tumor suppressor gene has a central role in cell cycle regulation, DNA repair and apoptosis, and a large number of reports have discussed the important role of p53 alterations in breast cancer. Also, a number of studies have shown that breast tumors with p53 mutations are strongly associated with poor prognosis and lacking methylation in a number of regulatory genes [34, 35].

RASSF1A functions as a tumor suppressor by inhibiting cell proliferation and migration and

promoting apoptosis [36, 37]. RASSF1A mediates the apoptotic effects of TNF- α through the activation of modulator of apoptosis protein 1 (MOAP1) and Bax leading to caspase 3 activation [38]. TNF- α is an inflammatory cytokine that regulates cell proliferation and cell death, and resistance to TNF- α -induced apoptosis may lead to tumor formation and growth. Klajic found that RASSF1A was hypermethylated in approximately 85% of all invasive tumors and DCIS, RASSF1A is a putative tumor-suppressor gene [39]. It belongs to an increasing list of tumor suppressor genes that are frequently inactivated by promoter methylation rather than by somatic mutations [40].

DNA methyltransferase 1, 3A, and 3B affect DNA methylation, and it is thought that they play an important role in the malignant transformation of various cancers [41-43]. DNMT1 is the "maintenance" methyl transfer that ensures faithful transmission of the methylation profile from maternal to daughter cells during cell division, whereas DNMT3A and DNMT3B are mainly involved in de novo establishment of methylation patterns during embryogenesis [44, 45]. Overexpression of DNMTs has been reported in various types of cancers. Riadh suggest that overexpression of various DNA methyltransferases might represent a critical event responsible for the epigenetic inactivation of multiple tumor suppressor genes [46].

Molecular classification models will dramatically impact clinical practice as individualized treatments are likely to become standard care in the near future. However, surrogate histopathological markers that mimic the molecular classification are being sought for clinical use. MapQuant DxTM (previously called genomic grade index) is a newly commercialized diagnostic tool that combines the expression levels of 97 genes to help discriminate between histological grades 1 to 3 [47]. It can be used if indeed reduction in FOXA1 expression in an experimental setting leads to lowered expression of the above genes, one could replace the expensive MapQuant DxTM with simple IHC for FOXA1 to determine additional benefits of chemotherapy for ER α -positive breast cancer. Our studies have shown the potential value of FOXA1 expression in assessment of tumor suppressor genes methylation level and the post-operative outcomes in breast cancer and have inspired further studies on breast cancer prognosis.

Acknowledgements

This study was supported by Anhui Natural Science Foundation (NO. 1308085QH152).

Disclosure of conflict of interest

None.

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