Histone Acetylation Modification H4K16ac Involved in Notch1 Signaling in Breast Cancer

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Abstract
The Notch signaling pathway is a fundamental intercellular signaling system that regulates cell fate decisions in terms of proliferation and differentiation in multiple tissues. Abnormal activation of Notch1 signaling pathway leads to a variety of diseases, including the malignant tumors. Dysregulated Notch receptor activity has been implicated in breast cancer but the epigenetic mechanisms remains not clear. Compared to adjacent tissues, high expression of NIC1 and low expression of H4K16ac were found in cancer tissues. Notch1 knocked down in the breast cancer cell line MCF-7 resulted in increased H4K16ac expression and a parallel reduction in their proliferation and migration capacity. Our study prompting H4K16ac to participate in the signal transduction of Notch1 in breast cancer cells. These results confirm Notch1 as a signal triggering epigenetic mechanisms in breast cancer cells, which may have implications in tumor dissemination, metastasis and proliferation. The identification of specific factors interacting with NOTCH signaling could thus be relevant to fully understanding the role of NOTCH in breast neoplasia.

Keywords: breast cancer, notch1 signaling in tumorigenesis, epigenetic modification, H4K16ac

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1. Introduction

Breast cancer is the most diagnosed cancer in American women except for skin cancer, reaching nearly a third [1]. Breast cancer is also the second leading cause of death in women after lung cancer [1]. In China, breast cancer has also become the most common cancer in women [2]. The number of new breast cancer cases and the number of deaths each year account for 12.2 percent and 9.6 percent, respectively, in the world [2]. The occurrence and development of breast cancer is associated with abnormal regulation of multiple genes. Notch signaling pathway plays an important role in regulating the growth and development of normal mammary gland [3,4,5]. Notch1 is the focus of current research, its role is to suppress cancer or promote cancer is still under debate [4,5]. Histone acetylation modification is an important apparent modification [6]. A large number of studies have shown that the decrease of acetylation caused by the overexpression of histone deacetylase or the decrease of acetylase expression in cancer cells is closely related to the occurrence of tumor [7,8,9,10,11]. There are reports that acetylation H4 the 16th lysine (H4K16ac) residue of histone can promote the transcription of corresponding genes by changing the spatial conformation of chromatin [12,13]. In contrast, reduced acetylation of chromatin in the transcriptional regulatory region of these genes can inhibit the transcription of these genes and thus silence gene expression [12,14]. Also some studies have found that there is a decrease in H4K16ac level in many types of human malignant tumors [15], and speculate that this change can be used as a common marker of human malignant tumors [14,15,16,17]. The expression of Notch1 and H4K16ac in human breast cancer tissues and adjacent tissues (<2 cm away from the tumor area) was detected by immunohistochemical technique, and the relationship between them and clinicopathological characteristics was preliminarily analyzed. The specific mechanism of the two in the development of breast cancer was discussed, and the expression regulation of genes involved in cell proliferation and migration ability was analyzed.

2. Materials and Methods

2.1. Tissue Collection

One hundred tissue samples including 50 primary diagnosed breast cancer, paired 50 adjacent tissues from
the same patients were collected. All patients underwent modified radical mastectomy surgery between September 2015 and July 2017 at Tangshan People's Hospital, (Hebei, China) and did not receive any adjuvant therapy before the surgical operation. The median age of the patients was 54 years (range, 44-70 years). Written informed consent was obtained from all participants, and the study was approved by the Institutional Ethics Board of Tangshan People's Hospital. Patient medical records including patient age and gender, tumor staging, pathological diagnosis, and surgical records were reviewed. All specimens were fixed by 10% formaldehyde and embedded in conventional paraffin. All specimens were continuously sliced 4μm and applied to 1% polylysine treated slide.

2.2. Cell Lines

Human MCF-7 cells were obtained from the Cell Culture Center of Peking Union Medical College. The cells were maintained in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% Penicillin/Streptomycin (Solarbio, China) at 37°C with 5% CO2.

2.3. Antibodies

Antibodies against Notch1 (ab8925), H4 (acetyl K16) (acetyl K16) were purchased from Abcam (Shanghai, China), PV-9000 Polymer Detection System For Immuno-Histological Staining was purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China).

2.4. Immunohistochemistry (IHC)

Breast tissue samples from 50 patients were collected in accordance with the Declaration of Helsinki and rules of Tangshan People's Hospital of Science and Technology Ethics Committee. Tissue samples were scored as previously reported [15,18]. Two identical samples derived from consecutive sections of same lesion were placed on each glass slide, one of which did not add primary antibody as a negative control. IHC was performed with a 2-step plus Poly-HRP Detection System (PV-9000, Zhongshan Golden Bridge, Beijing, China).

2.5. Transfection of Gene Expression Plasmids and siRNAs

The siRNAs against human Notch1 (si-Notch1) and the negative control siRNAs (NC), were all purchased from Shanghai Gene Pharma Company (Gene Pharma, China). The sequences were used as Table 1.

Table 1. Si-RNA sequence

| Sequence | Dilution |
|----------|----------|
| Sense | 5'-CACGGAGACGCAUGUGUAACACUTT-3' |
| Antisense | 5'-AUGUUAACAGCUCCUGTT-3' |
| NC | 5'-UUUCGCGAACGGUCAGCAGUdTdT-3' |
| Anti-sense | 5'-ACGUGACCGUUCGGAGAAdTdT-3' |

MCF-7 cells were seeded in 6-well plates (2×10^5/well) and incubated overnight. The cells at 50% confluence were transfected with siRNA using the Lipofectamine 2000 transfection reagent (Invitrogen, USA) and incubated. We set MOCK groups (using the Lipofectamine 2000 transfection reagent only) as blank controls. Forty-eight hours post-transfection, the cells were collected. Overexpression and silencing efficacy were tested by western blot and/or real time PCR. The experiments were repeated 3 times.

2.6. Reverse Transcription and Quantitative Real-time PCR (qRT-PCR)

Total RNA from cultured cells (include si-Notch1, NC and Mock cell lines) was isolated using TRIzol® LS Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) from each sample was used as a template to produce cDNA with PrimeScript First-strand cDNA Synthesis kit (Takara). Notch1 mRNA levels were analyzed by quantitative real-time PCR (qPCR) with an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). All PCR reactions were performed as follows: initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 62°C for 40 sec, annealing at 94°C for 1 min and extension at 60°C for 3 min. Primer sets used for PCR were as follows: GAPDH, 5'-GCCGTGAACAAAGTTGGAAGT-3' (forward) and 5'-CACCTTGCCGGTCCTCCTGA-3' (reverse); Notch1, 5'-GCCGTGAAACATTGTTGATG-3' (forward) and 5'-CACCTTGCCGGTCCTCCTGA-3' (reverse).

2.7. Western Blot

Total cell lysates were obtained from MCF-7 cells RIPA buffer (Beyotime Institute of Biotechnology P0013B, Haimen, Jiangsu, China). Protein concentrations in the samples were determined by the BCA protein assay kit (Pierce, Rockford, IL USA). Cell lysate was loaded and run on a 10% SDS-PAGE, and the protein was transferred to a PVDF membrane (Millipore, Billerica, MA, USA) using the BioRad Semi-dry transfer system (BioRad, Hercules, CA, USA). The membrane was incubated with the primary antibody followed by the secondary alkaline phosphatase-conjugated goat anti-rabbit (ZB-2301 dilution 1:5000, Zhongshan Golden Bridge, China) and goat anti-mouse antibodies (ZB-2301 dilution 1:5000, Zhongshan Golden Bridge, China). Primary antibodies were used as follows: Anti-activated Notch1 (Abcam, USA, ab8925, 1:1000), Anti-Histone H4 (acetyl K16) (Abcam, USA, ab109463, 1:1000), Anti-Histone H4 (Abcam, USA, ab61255, 1:500), anti-β-actin (#βA09, Zhongshan Golden Bridge, China), anti-Flag (#βA09, Zhongshan Golden Bridge, China), anti-MyC (Zhongshan Golden Bridge, China, #βA01), anti-AKT antibody (#α272, Cell Signaling Technology, USA), anti-pidermal growth factor (EGF) (E5036, Sigma, USA). Dilution was performed 1000-fold for all of the antibodies. Protein expression levels in tumor samples were normalized to those of paired normal tissues (P < 0.01, Pearson’s chisquare test).

2.8. Cell proliferation Assays

A cell Counting Kit-8 (CCK-8; APEX BIO, USA) was
used for cell proliferation assays. Cells were seeded into a 96-well plate at a density of $1 \times 10^3$ cells/well in quintuplicate wells. At 1, 2, 3, 4, 5 and 6 day after culture, 100μl of CCK-8 solution was added to each well, and after another 2h of incubation at 37°C, the absorbance of cells was measured at a wavelength of 450nm for calculation of the optical density (OD) values.

2.9. Transwell Migration Assay

Cell migration capabilities were detected through Transwell assays (3422; Corning USA). In brief, cells were collected and suspended in 200 μl of serum-free medium. For the migration assay, $1 \times 10^5$ cells in serum-free medium were added to the upper chambers. After incubation for 24 h at 37°C and 5% CO₂, the invaded cells were fixed with methanol for 25 min and were then stained with 0.1% crystal violet for 30min. Ten, the invaded cells were counted in at least five random fields under a light microscope (200×).

3. Results

3.1. The NIC1 of Breast Cancer is Higher than that of Adjacent Tissues, but the H4K16ac Level is Lower than That of Adjacent Tissues

The expression of NIC1 (Notch1 active fragments) and H4K16ac in breast cancer and adjacent tissues was detected by immunohistochemistry. Among 50 cases of breast cancer and corresponding adjacent tissues, 48 cases NIC1 in breast cancer expressed higher than the corresponding adjacent tissues ($P<0.05$). Expression in breast cancer tissues were found to be lower than H4K16ac corresponding adjacent tissues in all 48 cases.

The results showed that the NIC1 of breast cancer is higher than that of adjacent tissues, but the H4K16ac level is lower than that of adjacent tissues ($P<0.05$) (Figure 1).

3.2. Inhibition of Notch1 Pathway Activity Leads to H4K16ac Upregulation

The MCF-7 cells were transfected with siRNAs against human Notch1 (si-Notch1) and the negative control siRNAs (NC). We set MOCK groups (using the Lipofectamine transfection reagent only) as blank controls. Forty-eight hours post-transfection, the cells were collected. The expression of NIC1 genes and internal reference GAPGH genes in each group were measured by quantitative real-time PCR (qRT-PCR). The experimental results were analyzed by $2^{\Delta\DeltaCT}$ method. The results showed that the mRNA level of Notch1 in si-Notch1 group was significantly lower than that in the NC group and the Mock group ($P_{\text{Mock,si-Notch1}}<0.001; P_{\text{NC,si-Notch1}}<0.001$). The difference between Mock and NC groups was not statistically significant ($P_{\text{Mock, NC}}=0.336$) (Figure 2A). These results suggest that the knock-down Notch1 in the MCF-7 cell line was successful and successfully simulated the state of inhibition of the Notch1 pathway. The levels of NIC1 and H4K16ac proteins in each group were detected by Western blot. The expression of NIC1 protein in si-Notch1 group was lower than that in Mock group and NC group ($P_{\text{Mock,notch1}}<0.05; P_{\text{NC,notch1}}<0.001$) (Figure 2B). The difference between Mock and NC groups was not statistically significant ($P_{\text{Mock, NC}}=0.511$). The expression level of H4K16ac protein in si-Notch1 group was significantly higher than that in the other two groups ($P_{\text{Mock,notch1}}<0.05; P_{\text{NC,notch1}}<0.001$) (Figure 2C), there was no significant difference between the other two groups ($P_{\text{Mock, NC}}=0.967$). These results suggest that knock-down Notch1 expression leads to upregulation of H4K16ac levels.

Figure 1. The expression level of NIC1 and H4K16ac in breast cancer and corresponding adjacent tissues. A: Immunohistochemical analysis of NIC1 protein expression in breast cancer and corresponding adjacent tissues. B: NIC1 protein expression level in 48 samples of breast cancer and corresponding adjacent tissues. C: Immunohistochemical analysis of H4K16ac protein expression in breast cancer and corresponding adjacent tissues. D: H4K16ac protein in 48 samples of breast cancer and corresponding adjacent tissues.
3.3. Inhibition of Notch1 Pathway Activity Leads to a Decrease in Breast Cancer Cell Proliferation

According to the procedure described above, U138 and U373 cells transfected with Notch1 specific si-RNA, si-RNA negative control or LipofectamineTM2000 reagent without any si-RNA. Cell proliferation was detected by CCK8 experiment on day 1, day 2, day 3, day 4, day 5 and day 6, and the growth curve was drawn. The results showed that the value-added ability of MCF-7 cells decreased significantly after knocking low Notch1, but the difference of cell proliferation ability between Mock group and NC group was not statistically significant (Figure 3A).

3.4. Inhibition of Notch1 Pathway Activity Leads to a Decline in Breast Cancer Cell Migration

Transwell experimental results, compared with the Mock group and the NC group, the cell migration ability of the si-Notch1 group was the weakest ($P_{\text{Mock, si-Notch1}}<0.001$; $P_{\text{NC, si-Notch1}}<0.001$), while the difference between the two groups was not statistically significant ($P_{\text{Mock, NC}}=0.736$) (Figure 3B).
4. Discussion

Notch was first found in Drosophila melanogaster, a specific form of gene mutation in Drosophila, which is named after the partial loss of function in the wings of Drosophila melanogaster [19]. Notch signaling pathway has the function of regulating [20], initiating embryonic or fetal cell differentiation [21], and once Notch is activated by oncogenes, it can induce a variety of cancers [22,23]. Notch belongs to the Notch receptor family and is a transmembrane protein [24]. NIC1 is the active form of Notch1 receptor proteins [25]. When the Notch signaling pathway is activated Notch the receptor protein hydrolyzes the NIC fragment into the nucleus to regulate the transcription of the downstream target gene [25]. The expression level of NIC can represent the degree of activation of the Notch signaling pathway [25,26].

Recent studies have shown that Notch1 may be a potential therapeutic target for many kinds of tumor therapy and can be used as a biomarker for early detection of tumor because of the basic biological activities that regulate the occurrence and development of many human tumors, including cell proliferation, apoptosis, migration and invasion [27,28]. For example, colorectal cancer studies have found that inhibition of Notch1 signaling pathway activity negatively regulates stem cell characteristics in colorectal cancer cells [29]. In breast cancer, previous studies have shown that Notch1 may be a potential driving force for breast tumorigenesis, development and metastasis [30]. High expression in human breast cancer tissues has been verified again in 50 cases Notch1 human breast invasive ductal carcinoma, but the specific mechanism is not clear. We found that the NIC1 of breast cancer was higher than that of adjacent tissues in 50 human breast cancer invasive ductal carcinoma samples, but the H4K16ac level was lower than that of adjacent tissues, and the H4K16ac level was up-regulated after cell experiment interfered with Notch1 signaling pathway activity.

H4K16ac loss in skin cancer and gastric cancer may be involved in malignant tumorigenesis and malignancy [31]. Our group found that the increase of H4K16ac level in human breast cancer MCF-7 cell line will lead to the decrease of tumor cell proliferation and migration ability. The exact mechanism of H4K16ac loss to promote tumorigenesis and development is unclear. Studies have shown that histone lysine acetylation can activate or inhibit gene transcription, maintain the spatial structure of chromosomes, and participate in DNA damage repair. These effects may be involved in tumorigenesis and development. Our group will continue to explore the specific mechanism that H4K16ac affect the occurrence and development of breast tumors by using ChIP-seq and other experimental techniques.

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Conflict of Interest

All authors declare no conflicts of interest in this study.

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