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Acetylation Enhances the Promoting Role of AIB1 in Breast Cancer Cell Proliferation

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The oncogene nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a transcriptional coactivator, which is overexpressed in various types of human cancers, including breast cancer. However, the molecular mechanisms regulating AIB1 function remain largely unknown. In this study, we present evidence demonstrating that AIB1 is acetylated by MOF in human breast cancer cells. Moreover, we also found that the acetylation of AIB1 enhances its function in promoting breast cancer cell proliferation. We further showed that the acetylation of AIB1 is required for its recruitment to E2F1 target genes by E2F1. More importantly, we found that the acetylation levels of AIB1 are greatly elevated in human breast cancer cells compared with that in non-cancerous cells. Collectively, our results shed light on the molecular mechanisms that regulate AIB1 function in breast cancer.

INTRODUCTION

Breast cancer is one of the main causes for cancer-related death in women in the past a few decades, and is the most common malignant cancer in women, posting a grave threat to the women health (McPherson et al., 2000; Parkin et al., 2005). There are about one million women diagnosed of breast cancer each year. Most of the women died of breast cancer underwent the metastatic spread of this malignant tumor to other organs, including lung, liver and bone (Hortobagyi, 1998; Weigelt et al., 2005). The current treatment for women with breast cancer includes surgical therapies, chemotherapeutic and biological therapies. Surgical therapies are still the best option for women with breast cancer in early stage. However, surgery is not a good option for women with breast caner in advanced stages (Kranzfelder et al., 2011). Although scientists have made great efforts trying to understand the mechanism of breast cancer and made significant advances in understanding breast cancer pathogenesis, we still don't fully understand why this disease occurs and have very limited knowledge of its regulating mechanisms.

Thankful to the efforts made by many scientists around the globe, several important genes, which play crucial roles in the tumorigenesis of breast cancer, have been identified so far. In the mid-1990s, BRCA1 and BRCA2 were first identified as two breast cancer susceptibility genes (Miki et al., 1994; Wooster et al., 1995). The life-long breast cancer risk for BRCA mutation carriers is 55-85%, and these women have a high risk of suffering breast cancer at a very young age (Easton et al., 1993). Therefore, the genetic testing has been developing rapidly in recent years. However, BRCA mutation can only explain about 20% of the heritable risk of breast cancer (Mavaddat et al., 2010; Thompson and Easton, 2004).

AIB1 gene is another breast cancer-related gene, which was found to be amplified in breast cancer cell lines and in about 5-10% of primary breast cancers (Anzick et al., 1997; Bautista et al., 1998). The product of AIB1 gene was shown to promote breast cancer cell proliferation (Louie et al., 2004). However, the mechanism by which AIB1 regulates breast cancer cell proliferation is not full understood. In this study, we investigated how acetylation regulates the role of AIB1 in promoting breast cancer cell proliferation.

MATERIALS AND METHODS

Cell culture and cell transfection

All cells were obtained from the ATCC (American Type Culture Collection) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics in a 5% CO_2 incubator. siRNAs or constructs were transfected into cells using Lipofectamine 2000 (Invitrogen) following the company's specification. MOF siRNAs used in MOF knockdown experiments were purchased from Santa Cruz Biotechnology Company (sc-37129). For the expression of FLAG tagged MOF, we transfected cells with the construct pCMV-MOF-FLAG. For the control experiments, empty vector pCMV-FLAG was used for transfection.

Co-immunoprecipitation assay and Western blotting assay

Cells were washed in ice-cold PBS buffer and lysed in buffer containing 50mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 2mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), and protease inhibitor cocktail (Roche). The cell debris was removed from cell lysate by centrifugation. The whole-cell lysates were incubated with antibodies for 4 h at 4°C, and then

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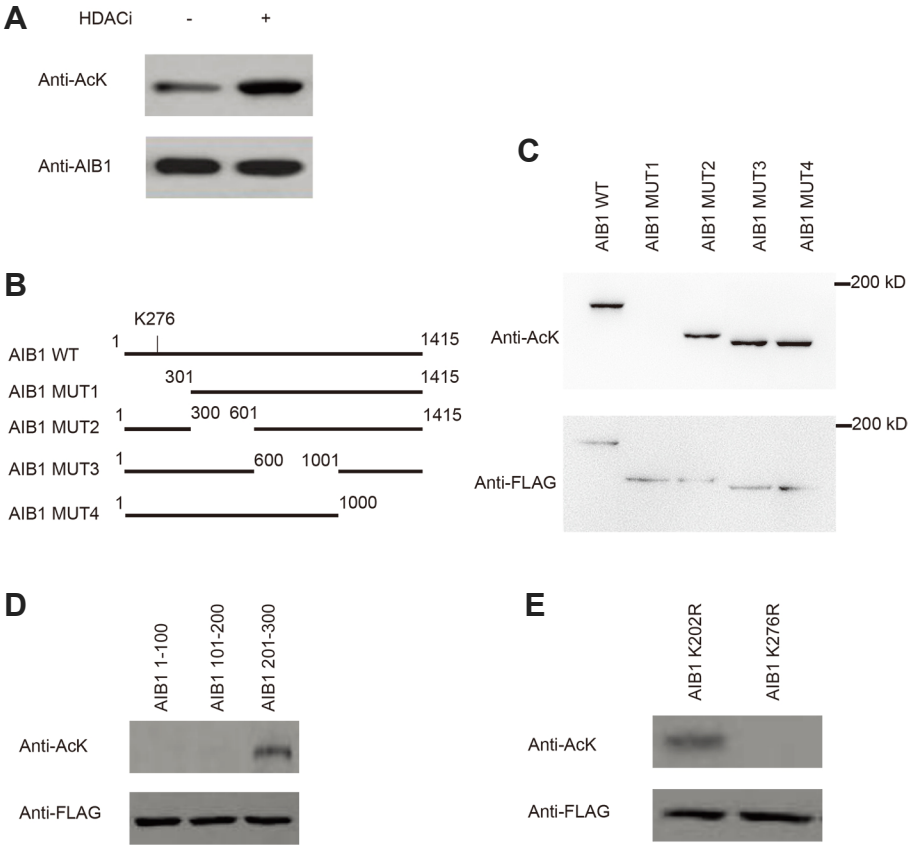

Fig. 1. AIB1 is acetylated at Lysine 276 in breast cancer cells. (A) AIB1 is acetylated in MCF-7 cells. Endogenous AIB1 was purified by immunoprecipitation from MCF-7 cells treated with or without HDAC inhibitors. Western blotting assay was performed to detect AIB1 acetylation with anti-acetyl lysine antibody. (B) Schematic representation of AIB1 truncation mutants used in this study. (C) The acetylation of AIB1 is lost in AIB1 truncation mutant 1. These truncation mutants were individually expressed in MCF-7 cells. The acetylation of AIB1 truncation mutants was examined using anti-acetylated lysine antibody. (D) The acetylated lysine residue is located in 201-300 aa of AIB1. Three AIB1 mutants containing 1-100 aa, 101-200 aa, 201-300 aa of AIB1 were individually expressed in MCF-7 cells. The acetylation levels were examined by Western blotting assay using anti-acetylated lysine antibody. (E) Lysine 276 is the acetylated residue of AIB1. AIB1 K202R mutant and AIB1 K276R mutant was individually expressed in MCF-7 cells. The acetylation levels of two mutants were examined by Western blotting assay using anti-acetylated lysine antibody.

with protein A/G beads for 4 h at 4°C. The protein precipitates were then washed and analyzed by Western blotting assay using antibodies indicated. The following antibodies were used in Co-immunoprecipitation assay and Western Blotting assay: Anti-acetylated lysine (Abcam, ab21623); anti-FLAG (Sigma, F3165); anti-AIB1 (Abcam, ab2831); GAPDH (Santa cruz, sc-48166); anti-E2F1 (Santa cruz, sc-56661); anti-MOF (Santa cruz, sc-13677)

Cell proliferation

The effect of AIB1 WT, AIB1 K276R or AIB1 K276Q overexpression on cell proliferation was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For MTT assay, MTT was dissolved in 0.8 % NaCl solution, and the final concentration of MTT is 5 mg/ml. Cells were incubated with MTT solution at 37°C for 3 h. The absorbance at 490 nm was measured to determine the number of viable cells.

Real-time quantitative PCR

The mRNA levels of cyclin A and cyclin E were measured by quantitative real-time PCR. Total RNA samples were prepared from cells transfected with AIB1 WT, AIB1 K276R, or AIB1 K276Q. The 2 ug of total RNA was used in reverse transcription reaction. The resultant cDNA samples were diluted and used in real-time PCR reactions as templates. IQ5 Real-Time PCR System (Bio-rad) was used to determine relative mRNA levels of cyclin A and cyclin E using SYBR green dye. Beta-actin was used as internal control for normalization using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001)

RESULTS AND DISCUSSION

AIB1 is acetylated at Lysine 276 in breast cancer cells

Post-translational modifications play crucial roles in regulating protein functions. In this study, we tried to investigate the role of post-translational modifications in regulating the function of AIB1. We were particularly interested in investigating the role of the acetylation of AIB1 in promoting breast cancer cell proliferation. We first tried to determine whether AIB1 is acetylated in human breast cancer cells. In order to do so, we performed immunoprecipitation to pull down endogenous AIB1 in MCF-7 cells. The protein precipitate was analyzed by Western blot assay using anti-acetylated lysine antibody and anti-AIB1 antibody. As shown in Fig. 1A, the endogenous AIB1 is acetylated in breast cancer cells. Moreover, we detected a marked increase in the acetylation levels of AIB1 in cells treated with HDAC inhibitors. This result suggests that AIB1 is highly acetylated in breast cancer cells.

We next tried to determine the acetylated lysine residue or residues of AIB1. In order to do so, we constructed four AIB1 mutants. In each of these AIB1 mutants, a certain fragment was deleted (Fig. 1B). These constructs were transfected into MCF-7 cells and the cell lysates were collected for immunoprecipitation assay. As shown in Fig. 1C, we were unable to detect any signal in AIB1 MUT1 samples using anti-acetylated lysine anti

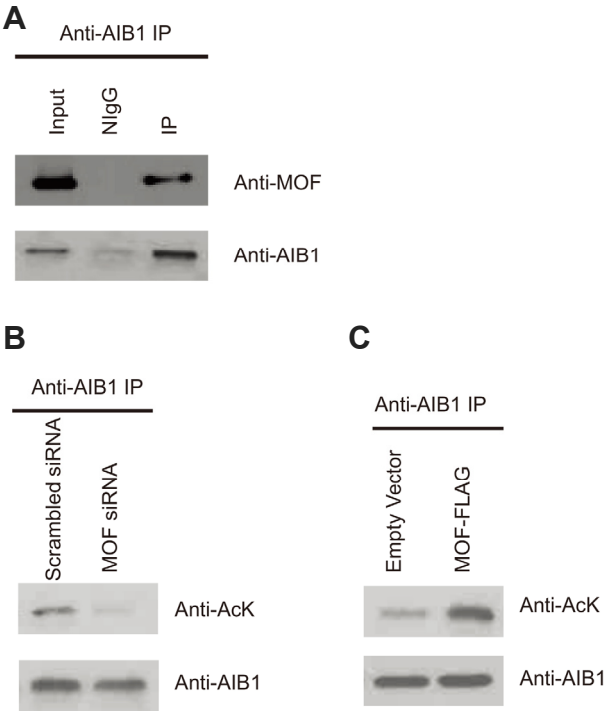

Fig. 2. MOF is the acetyl transferase responsible for AIB1 acetylation. (A) AIB1 co-precipitates with MOF. Co-precipitation experiment was performed to examine the interaction between AIB1 and MOF. (B) MOF knockdown dramatically reduced AIB1 acetylation levels. MCF-7 cells were treated with MOF siRNA to knock down MOF expression. MCF-7 cells treated with scrambled siRNA were used as negative control. (C) MOF overexpression greatly elevated AIB1 acetylation levels. FLAG tagged MOF was overexpressed in MCF-7 cells to examine the effect of MOF overexpression on AIB1 acetylation. MCF-7 cells transfected with empty vector expressing FLAG alone were used as negative control.

bodies, while other AIB1 mutants showed comparable acetylation levels. This data indicated that the acetylated lysine resides in the first 300 aa of AIB1. We next individually expressed the fragment of 1-100, 101-200 and 201-300 aa of AIB1. The results suggested that the acetylated lysine resides in 201-300 aa of AIB1 (Fig. 1D). There are two lysine residues in fragment of 201-300 of AIB1, lysine 202 and lysine 276. To further determine which residue is acetylated, we generated AIB1 K202R and AIB1 K276R mutants separately. The two mutants were transfected into MCF-7 cells, and the cell lysates were analyzed by immunoprecipitation assay and Western Blot assay (Fig. 1E). The result showed that the lysine 276 of AIB1 is acetylated. Thus, we concluded that AIB1 is acetylated in human breast cancer cells and the acetylated lysine residue is lysine 276.

AIB1 is acetylated by MOF in breast cancer cells

We next tried to identify the acetyl transferase that is responsible for AIB1 acetylation. In order to do so, we performed co-immunoprecipitation assay and Western Blot assay to determine which acetyl transferase co-precipitate with AIB1. We used antibodies against various acetyl transferases in the Western Blot assay, and found that MOF co-precipitates with AIB1, indicating that MOF might catalyze the acetylation of AIB1 in breast cancer cells (Fig. 2A and Supplementary Fig. S1).

To further confirm that MOF is responsible for the acetylation of endogenous AIB1 in breast cancer cells, we used RNAi technique to knock down MOF expression in MCF-7 cells, and analyzed the acetylation of AIB1. The data demonstrated that the depletion of MOF dramatically reduced the acetylation of AIB1 in MCF-7 cells (Fig. 2B). We also overexpressed MOF in MCF-7 cells and found that the overexpression of MOF greatly increased the acetylation levels of AIB1 in MCF-7 cells (Fig. 2C and Supplementary Fig. S2). Collectively, these results demonstrate that MOF interacts AIB1 and is responsible for the acetylation of AIB1 in breast cancer cells.

The acetylation of AIB1 by MOF enhances its effect on promoting breast cancer cell proliferation

The above data demonstrate that AIB1 is acetylated at Lysine 276 by MOF in human breast cancer cells. We next tried to investigate the function of the acetylation of AIB1. AIB1 is known to be able to promote breast cancer cell proliferation. Therefore, we first examined the effect of AIB1 acetylation in promoting breast cancer cell proliferation. In order to do so, we transfected T-47D cells and MDA-MB-361 cells with constructs expressing AIB1 K276R, which cannot be acetylated, and AIB1 K276Q, which mimics the acetylation of AIB1. The empty vector and construct expressing wild-type AIB1 were used as negative control and positive control. Western Blot results showed that AIB1 K276R, AIB1 K276Q, and AIB1 WT were expressed at comparable levels in both T-47D cells and MDA-MB-361 cells (Fig. 3A). We also examined the acetylation status of these mutants along with AIB1 WT to confirm the proper function of each vector (Supplementary Fig. S3).

MTT assays were performed to examine the ability of AIB1 K276R, AIB1 K276Q, and AIB1 WT in promoting breast cancer cell proliferation. The results showed that the overexpression of AIB1 WT greatly promoted breast cancer cell proliferation, consistent with previous report. I also observed even faster growth rate when AIB1 K276Q was overexpressed (Fig. 3B). However, We did not observe too much difference between cells overexpressing AIB1 K276R and negative control cells (Fig. 3B). These results demonstrate that the acetylation of AIB1 is required in its function in promoting breast cancer cell proliferation.

The acetylation of AIB1 by MOF is required for its interaction with E2F1

It was previously reported that AIB1 functions as a coactivator of transcription factor E2F1 to promote cell proliferation (13). Having found that the acetylation of AIB1 is required for its positive role in promoting breast cancer cell proliferation, we next investigated whether AIB1 acetylation regulates its interaction with E2F1. We expressed FLAG-tagged wild-type AIB1, AIB1 K276R and AIB1 K276Q in MCF-7 cells. We then performed co-immunoprecipitation using anti-FLAG antibodies followed by Western blot assay using anti-FLAG antibody and anti-E2F1 antibody. As shown in Figure 4A and Supplementary Fig. S4, wild-type AIB1 coprecipitated with E2F1, and AIB1 K276Q showed even stronger interaction with E2F1. However, the interaction between AIB1 and E2F1 was nearly completely abolished by K276R mutation. This result indicates that the acetylation of AIB1 is required for its interaction with E2F1. We also performed ChIP assay to examine the effect of AIB1 acetylation on its recruitment to E2F1 target gene promoter. Consistent with co-immunoprecipitation results,

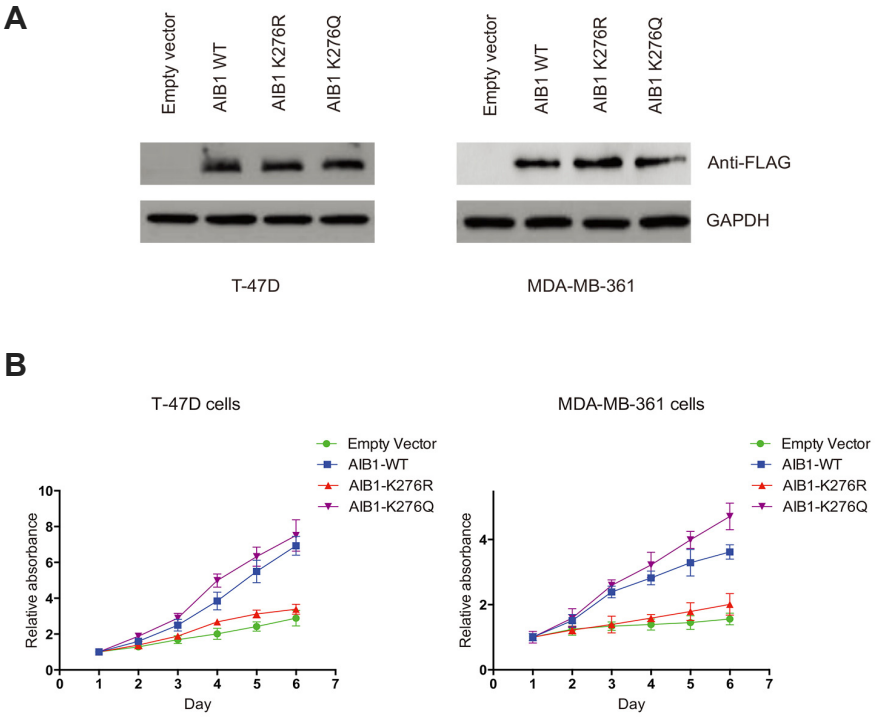

Fig. 3. The acetylation of AIB1 by MOF enhances its effect on breast cancer cell proliferation. (A) AIB1 K276R, AIB1 K276Q, and AIB1 WT were expressed at comparable levels in transfected T-47D cells and MDA-MB-361 cells. Western blotting assay was performed to examine the expression level of FLAG-tagged proteins using anti-FLAG antibody. (B) The acetylation of AIB1 by MOF enhances its effect on breast cancer cell proliferation. MTT assays were performed to examine the ability of AIB1 K276R, AIB1 K276Q, and AIB1 WT in promoting breast cancer cell proliferation.

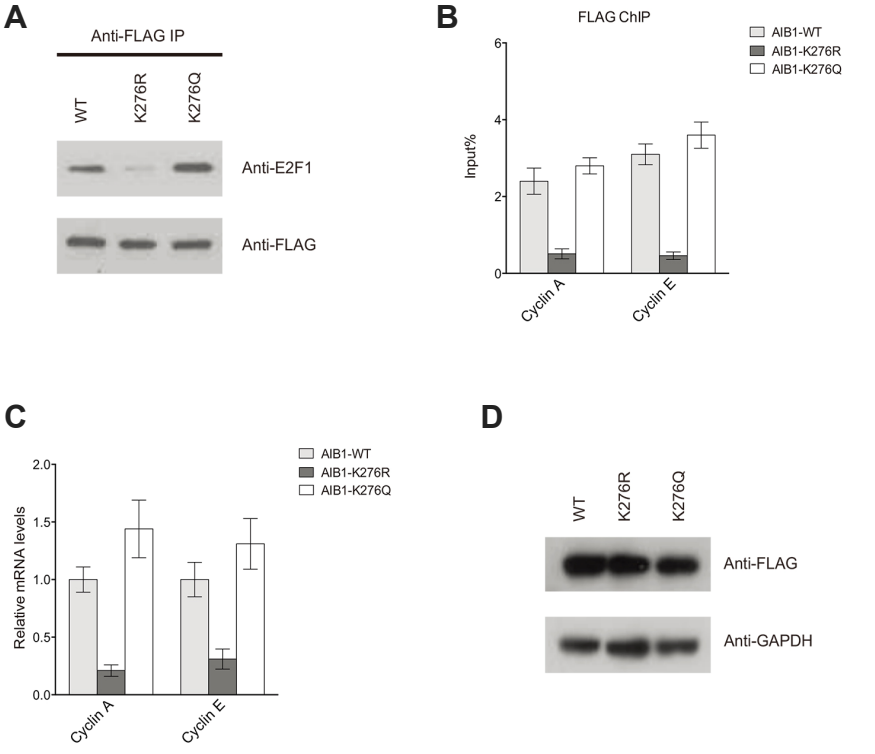

Fig. 4. The acetylation of AIB1 by MOF is required for its interaction with E2F1. (A) E2F1 co-precipitates with AIB1 K276Q, but not AIB1 K276R. Cells expressing FLAG-tagged AIB1 K276R or AIB1 K276Q were harvested for co-immunoprecipitation assay using anti-FLAG antibody. (B) Acetylated AIB1 binds to E2F1 target genes. ChIP experiments were performed to detect the occupancy of AIB1 on E2F1 target genes. Cells expressing FLAG-tagged wild-type AIB1, AIB1 K276R or AIB1 K276Q were harvested for ChIP assay using anti-FLAG antibody. (C) The acetylation of AIB1 is required for its positive regulatory role in E2F1 target gene expression. Total RNAs were extracted from Cells expressing FLAG-tagged wild-type AIB1, AIB1 K276R or AIB1 K276Q. Quantitative real time PCR experiments were performed to measure E2F1 target gene expression. (D) AIB1 K276R, AIB1 K276Q, and AIB1 WT were expressed at comparable levels in transfected cells. Western blotting assay was performed to examine the expression level of FLAG-tagged proteins using anti-FLAG antibody.

we observed a dramatic decrease in AIB1 binding levels on the promoter of E2F1 target gene cyclin A and cyclin E when K276R mutation was introduced (Fig. 4B). Accordingly, the mRNA levels of cyclin A and cyclin E were significantly reduced in cells expressing AIB1 K276R compared with that in cells expressing wild-type AIB1.

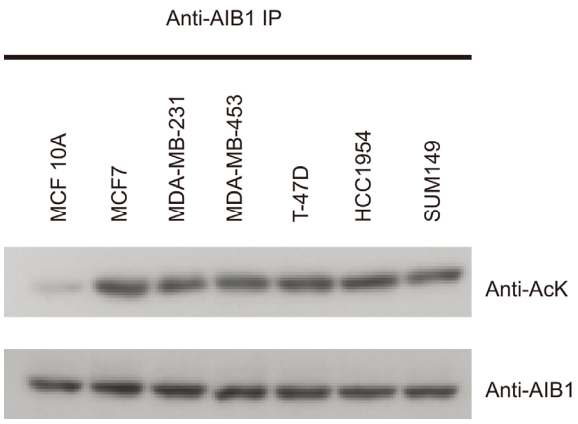

Fig. 5. AIB1 is highly acetylated in breast cancer cells. Western blotting assay was performed to examine AIB1 acetylation in various breast cancer cell lines using anti-acetylated lysine antibody. Non-cancerous cell line MCF-10A was used as control.

We observed even high mRNA levels of cyclin A and cyclin E in cells expressing AIB1 K276Q (Fig. 4C). Wild type AIB1, AIB1 K276R and AIB1 K276Q were expressed at comparable levels in cells used in above analyses (Fig. 4D).

AIB1 is highly acetylated in breast cancer cells

Having found that AIB1 is highly acetylated in MCF-7 cells, and that the acetylation of AIB1 plays a crucial role in its function in promoting breast cancer cell proliferation, we next aimed to investigate whether AIB1 is also highly acetylated in other breast cancer cell lines. In order to do so, we performed immunoprecipitation assay using anti-AIB1 antibody using various breast cancer cell lines, including MDA-MB-231, MDA-MB-453, T-47D, HCC1954, and SUM149 cells. MCF-7 was also included in this analysis as positive control. MCF-10A cells were included as non-cancerous control. As shown in Fig. 5, AIB1 was highly acetylated in all the breast cancer cell lines examined compared with that in non-cancerous cells MCF-10A. This result confirmed that the acetylation of AIB1 is common in breast cancer cells, not specific to MCF-7 cells. We also examined the protein levels of AIB1 in these breast cancer cell lines (Supplementary Fig. S5). The results showed that AIB1 is overexpressed in most breast cancer cell lines we examined, in line with the reports that AIB1 is frequently amplified in breast cancer.

Gene amplification is one of the most common genetic events in human cancers (Albertson, 2006; Santarius et al., 2010), which results in the overexpression of the oncogene in tumorigenesis. The oncogene AIB1 was previously reported to be amplified and overexpressed in many human cancers, including breast cancer (Anzick et al., 1997; Sakaguchi et al., 2007; Zhou et al., 2005). AIB1 has been shown to interact with a broad range of nuclear receptors and transcription factors and enhance their function in gene expression regulation. These nuclear receptors and transcription factors include estrogen receptor, androgen receptor, activator protein-1 (AP-1), E2F1, and nuclear factor-kB (NF-kB) (Louie et al., 2004; Werbajh et al., 2000; Yan et al., 2006).

In this study, we investigated the effect of the acetylation on the regulatory function of AIB1. We found that AIB1 is acetylated in breast cancer cells, and that Lysine 276 is the only acetylation site. We further demonstrated that MOF is the acetyl transferase responsible for AIB1 acetylation. We also found that the acetylation of AIB1 is required for its promoting role in breast cancer cell proliferation. More importantly, we found that the acetylation levels of AIB1 are greatly elevated in breast cancer cells compared with that in noncancerous cells. It's been nearly two decades since it was first observed that AIB1 was overexpressed in human breast cancer. Since then the promoting role of AIB1 in tumorigenesis has also been revealed in many other human cancers. However, the mechanism regulating the role of AIB1 in tumorigenesis remains unclear. Our findings revealed one of the mechanisms regulating AIB1 function, and also provide a potential therapeutic target for human breast cancer.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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