



Original Research

The PI3K and AIB1 interaction is involved in estrogen treated breast cancer cells

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Received October 1, 2017; Accepted January 6, 2018; Published May 15, 2018

Doi: <http://dx.doi.org/10.14715/cmb/2018.64.6.12>

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Abstract: AIB1 was involved in the development and progression of breast cancer. Although it was found that AIB1 could be phosphorylated by some kinases including PI3K, the function of AIB1 and AKT interaction in breast cancer is not well defined. MCF-7 cells were transfected with pERE-Luc AKT and/or AIB1 plasmids, and then ERE luciferase activity in presence or absence of estrogen (E2) were measured. Plasmids containing PTEN and an PI3K inhibitor LY294002 were transfected into or treated cells to identify the interaction of PI3K/AKT and activation of AIB1, and examine their roles in cell cycle regulation. The AKT phosphorylation activity was evaluated by kinase assay using H2B as a substrate. The association between AIB1 and pS2 promoter was detected by the Chromatin Immunoprecipitation (ChIP) assay. AIB1 and AKT in the same complex were detected by Pull-down assay. IGF-1 can increase AIB1 recruitment to PS2 and enhance the ER-dependent transcription activity through the PI3K/AKT pathway. AIB1 associate with AKT to regulate cell cycle. The special relations concerning the AIB1 and AKT may arouse some new viewpoints for potential therapeutic targets in breast cancer.

Key words: AIB1; AKT; PI3K; IGF-1; Breast cancer.

Introduction

The phosphoinositide 3-kinase (PI3K)/AKT pathway, which is a pivotal signal transduction system that links multiple receptor classes with many essential cell functions including cell proliferation, cell survival and cell mobility (1, 2). However, its aberrant activation by constitutively activated receptor tyrosine kinases (IGF, IGFR, ErbB, FGF/FGFR systems) leading to loss of PTEN function, PI3K mutations, and aberrant activation of AKT, eIF4E, 4E-BP1 and p70S6K are found in numerous human cancers including breast cancer.

It was reported that the AKT1 was usually overexpressed and occurred mutations early in breast carcinoma development (3). Overexpression of AKT2 can lead to up-regulation of β 1 integrins, increase in invasion, and metastasis of human breast and ovarian cancer cells (4). AKT has been phosphorylated by the activated upstream proteins, thus to enforce various cell processes (1, 5-9). Particularly, the expression levels of AKT3 were significantly higher in the estrogen receptor (ER)-negative tumor in comparison to the ER-positive tumor, indicating that AKT3 may contribute to the more aggressive clinical phenotype of the ER-negative breast cancer (10).

As a pivotal coactivator, p160 steroid receptor (SRC) family can promote transcriptional activity of numerous nuclear receptors such as ER (11, 12). In steroid hormone-induced gene expression, the SRCs interact with CBP, p300, p/CAF, CARM1 and PRMT1 (13). SRC family consists of three members including SRC-1, SRC-2 and SRC-3 (AIB1). Overexpression of SRC-1 and SRC-2 can promote cell proliferation and invasion through inducing SDF-1 α expressions; meanwhile reduction of them in MCF-7 cells decreases estrogen dependent DNA synthesis and the estrogen-responsive pS2 gene expression (14, 15). The overexpression of AIB1 have been confirmed to promote cell metastasis and invasiveness and is correlated with poor clinical prognosis in many cancers (16-18).

Actually, many pathways or factors are relative to SRC family responsible for the cell functions (19-21). Many researches have elaborated the AIB1 could activate many proteins in PI3K/AKT/mTOR pathway in breast cancer. Serine 967 of AIB1 can be phosphorylated specifically by AKT both in vitro and in vivo and may be an essential factor required for Tam resistance and tumor growth independent of ER (22). AKT signaling can promote AIB1 stability independently of GSK3 phosphorylation (23). In the AIB1-tg mice, AIB1

overexpression increases mammary IGF-I mRNA and serum IGF-I protein levels activating many proteins such as the AKT in the IGF-R/PI3K/AKT/mTOR pathway (24), although the new standpoint supports that AIB1 maintains circulating IGF-1 just by controlling IGF-Binding protein 3 (IGFBP-3) expression (25). Meanwhile, reducing AIB1/ SRC-3 levels or activities in the mammary epithelium could potentiate therapies aimed at inhibiting HER2/neu signaling (26). Herein, we present the parallel questions that whether the PI3K pathway acts the similar role in aiding the AIB1 recruitment to estrogen-responsive promoters and whether there are some special relationships between AKT and AIB1. In this study, we mainly investigate the mechanisms related to relationship between PI3K pathway and AIB1 in the breast cancer cells. The compelling findings demonstrated that the AIB1 plays an important role in cell cycle regulation through the PI3K/AKT pathway in the breast cancer.

Materials and Methods

Cell culture

MCF-7, T47D and 911 cells were all obtained from ATCC, USA. Human breast cancer MCF-7 cells were maintained in DMEM/F-12 (Gibco, USA) containing 5% fetal bovine serum (FBS). T47D cells were maintained in DMEM (Gibco) containing 5% FBS and 911 cells were cultured in DMEM (Gibco) containing 10% FBS.

Cell transfection and Dual luciferase assay

0.1×10^6 cells were plated onto 12-well plates in Phenol red-free DMEM/F-12 containing 5% DCC-FBS. Cells were transfected in the next day with plasmids of 0.25 μg /well pAd1020sfidA-CMV-GFP-hAIB1 and/or 0.25 μg /well pLNCX-HA-AKT with 0.5 μg /well pERE-Luc reporter gene using Calcium phosphate precipitation method. Six hours after transfection, cells were shocked by 10% Glycerol for 3 min, followed by three washes with $1 \times \text{PBS}$, and treated with 1×10^{-9} M 17- β estradiol (estrogen, E2) for 48 hours. For Luciferase assay, cells were lysed and the Luciferase assay was carried out using the Dual-Luciferase Reporter Assay System (Promega, USA). The luminescence was measured.

Cell cycle analysis

1×10^6 cells were seeded onto 60 mm dishes in Phenol red-free DMEM/F-12 and DMEM medium containing 5% DCC-FBS, and transfected with adenoviruses expressing GFP or GFP-AIB1 in the next day. After 24 hours, the adenoviruses were removed and the cells washed with PBS, and treated with 20 μM LY294002 for 30 min followed by 24 hours stimulation of E2. Cells were washed once with cold PBS, trypsinized and then pelleted by centrifugation. Pellets were resuspended in solution containing 100 μg of RNAase and 50 μg propidium iodide, and shaken for 30 min at room temperature before analysis by flow cytometry. DNA content was assessed using flow cytometry.

Western blot analysis

Cells were collected with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM

Na₃VO₄, 1% Triton X-100, 2.5 mM Sodium Pyrophosphate, 1 $\mu\text{g}/\text{ml}$ Leupeptin, 1 mM PMSF) after 50 ng/ml IGF-1 treatment for 30 min. Equal amounts of protein (50 μg) were loaded onto 7.5% polyacrylamide gels and then transferred onto nitrocellulose membranes. Membranes were blocked in 10% non-fat milk for 1 hour at room temperature and incubated overnight at 4 °C with either anti-p-AKT antibody (Ser 473, 1:1000) or anti-AKT antibody (1:1000; CST, USA). After three additional washes in TBS containing 0.1% Tween-20, membranes were incubated with 1:2000 HRP-conjugated anti-rabbit IgG (CST, USA) for 1 hour at room temperature and washed three times with TBS containing 0.3% Tween-20 followed by additional three washes in TBS-T containing 0.1% Tween-20. Membranes were then incubated with ECL Western Blotting Detection Kit (Amersham Biosciences, USA) for 1 min and exposed to x-ray film to visualize the proteins.

In vitro AKT kinase assay

Cells were transfected with adenoviruses expressing GFP or GFP-AIB1. After 24 hours, cells were treated with 20 μM LY294002 (BIOMOL Cat# ST-420) for 30 min, followed by 30 min of treatment of 50 ng/ml IGF-1 (BACHEM), and then were lysed using lysis buffer on ice for 30 min. Equal amounts of lysates were pre-cleared with 20 μl protein A/G agarose slurry (Santa Cruz, USA) for 90 min at 4 °C after centrifugation. Immunoprecipitation was carried out using the anti-AKT antibody 1:100 (Cell Signaling Cat#9272) and 30 μl protein A/G agarose slurry at 4 °C overnight. Immune complexes were washed two times with lysis buffer and two times with washing buffer (100 mM Tris-HCl pH 7.5, 0.5 M LiCl, 1 mM DTT, 10 mM MgCl₂), and incubated with 15 μl of kinase reaction buffer (2.5 μg H2B [Roche Cat#223 514], 50 μM cold ATP, 1 mM DTT, 50 μM PKA inhibitor peptide, 10 mM MgCl₂, 5 μCi γ -P32) at 30 °C for 30 min followed by 5 min boiling at 95 °C in 15 μl 2 \times SDS buffer. The samples were quickly centrifuged and loaded onto 15% SDS polyacrylamide gels. The gels were dried for 4 hours at 50 °C and exposed to film.

Chromatin immunoprecipitation (ChIP) assay

Cells were treated with 30 min of 20 μM LY294002 or 10^{-4} M ICI 182 780 followed by 50 ng/ml IGF-1 treatment according time courses. Cross-linking was performed with 1% formaldehyde for 10 min. Cells were washed and harvested using cold PBS containing protease inhibitors. The pellets were centrifuged and resuspended using 200 μl SDS lysis buffer. The chromatin was sheared by sonication (8 pulses, 8 cycles 10 sec each) followed by centrifugation at 13000 rpm for 10 min at 4 °C, 10% of supernatant was used as input and 90% of supernatant was transferred into 1.8ml dilution buffer, pre-cleared for 2 hours at 4 °C using 80 μl protein A/G agarose slurry. Immunoprecipitation was carried out using purified anti-AIB1 antibody (Sigma) and 70 μl protein A/G agarose slurry at 4 °C overnight. The following steps of CHIP assay were performed according the standard protocol (Upstate Cat#17-295). For PCR, 3 μl from 20 μl DNA extraction and PS2 primer (sequences 353F GGCCATCTCTCACTATGAAT, 30R GGCAGGCTCTGTTTGCTTAA) were used for

35 cycles.

Co-immunoprecipitations

8 × 10⁶ of 911 cells were seeded onto 150 mm dishes in Phenol red free DMEM containing 10% DCC-FBS, and plasmids transfection were performed with pAd1020 si-fi-CMV-GFP-hAIB1 20 μg/dish and pLNCX-HA-AKT 20 μg/dish in the next day using Fugene 6 as directed by the manufacturer. After 24 hours of IGF-1 treatment, cells were lysed in cold 500 μl RIPA buffer with protease inhibitors (10 μg/ml PMSF, 1 μg/ml pepstatin and 1 μg/ml Aprotinin). The lysates were sonicated 10 seconds, 5 cycles at 5 pulses, and centrifuged at 14 000 rpm 4 °C for 10 min. 10% of lysates was used as positive control. Lysates were pre-cleared with 30 μl protein A/G agarose slurry for 90 min, and then separated into two tubes (0.5 mg/tube) and immunoprecipitated with anti-AIB1 rabbit serum antibody (1:100), anti-HA (1:100 Convance Cat#MMS-101R) and IgG with 35 μl protein A/G agarose slurry. The immune complexes were then washed with PBS for three times and one more time with RIPA buffer, 25 μl 2 × SDS buffer was added and the samples were heated for 5 min at 97 °C, and separated by 7.5% SDS-page gel. Then, the western blot was performed as described above.

Statistical analysis

Analysis was performed with Statistica 7 statistical software package using student t-test (P<0.05).

Results

AKT and AIB1 interaction is elicited by E2

To test whether AKT has the ability to regulate the AIB1 function, we transfected MCF-7 cells with pERE-Luc, AKT, and/or AIB1 plasmids, or vehicles (CT), and then treated cells with E2 and measured ERE luciferase activity (Figure 1). Results showed that the ERE luciferase activity was strongly stimulated by E2. AIB1 and AKT overexpression alone increased the ERE luciferase activity remarkably in the presence of E2. Intriguingly, when MCF-7 cells transfected with AKT+ AIB1 plasmids in the presence of E2, the luciferase activity was higher than AIB1 and AKT overexpression alone. However, the changes were not detected in the absence

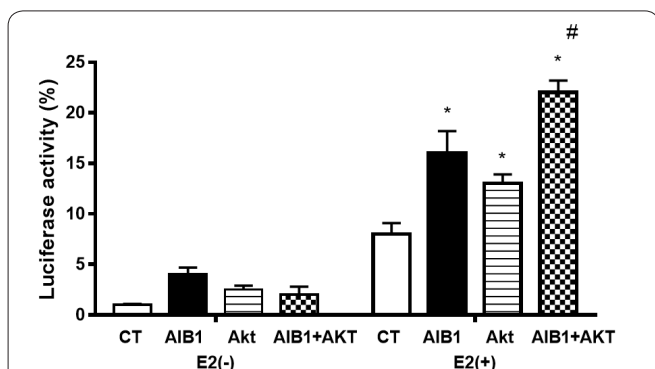


Figure 1. AKT and AIB1 interaction in the presence of estrogen (E2). MCF-7 cells were transfected with plasmids of AIB1 and/or AKT, or vehicles (CT). Next day, cells were treated with or without 10⁻⁹ M 17-beta estradiol (E2(+)) or 100% ethanol (E2(-)) for 24 hours. Then, ERE luciferase activity was measured. E2 can increase ERE luciferase activity. *p<0.05 vs. CT, #p<0.05 vs. AIB1 or AKT alone, by one-way ANOVA.

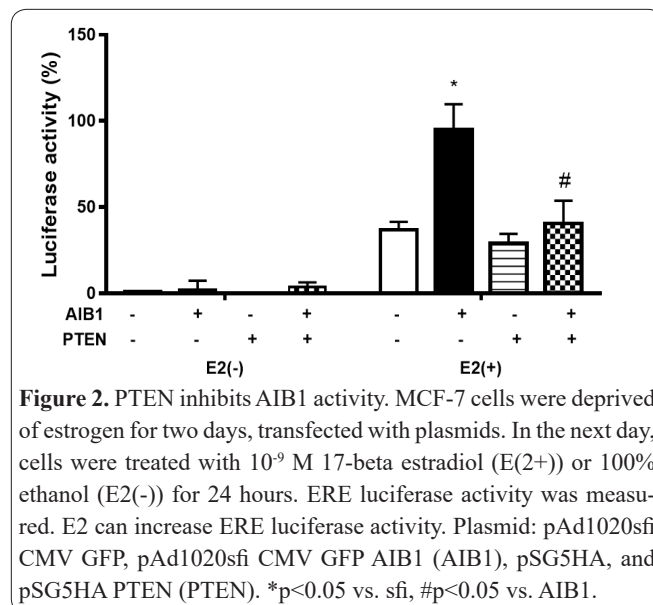


Figure 2. PTEN inhibits AIB1 activity. MCF-7 cells were deprived of estrogen for two days, transfected with plasmids. In the next day, cells were treated with 10⁻⁹ M 17-beta estradiol (E2(+)) or 100% ethanol (E2(-)) for 24 hours. ERE luciferase activity was measured. E2 can increase ERE luciferase activity. Plasmid: pAd1020sf CMV GFP, pAd1020sf CMV GFP AIB1 (AIB1), pSG5HA, and pSG5HA PTEN (PTEN). *p<0.05 vs. sf, #p<0.05 vs. AIB1.

of E2, suggesting that AKT and AIB1 interaction was involved in the E2 action.

PTEN inhibits AIB1 activity in the presence of E2

To gain further insight into AKT regulation on AIB1 activity in the presence of E2, we used PTEN, an inhibitor of PI3K, to identify whether the activation of AIB1 can be blocked by PTEN or not. Cells were transfected with plasmids containing AIB1 and/or PTEN, or vehicle into MCF-7 cells, and treated with or without E2. Overexpression of PTEN obviously inhibits AIB1 overexpression-induced ERE luciferase activity (Figure 2), suggesting that inhibition of the PI3K/AKT signal pathway by PTEN can significantly decrease the AIB1 activity in the presence of E2.

AIB1 overexpression enhances cells in S phase

The PI3K/AKT is the major survival pathway for various cell types and affects cell survival via regulating cell cycle progression. To detect whether the overexpression of AIB1 promotes cell cycle through the PI3K/AKT pathway, ER-positive MCF-7 cells were transfected with adenoviruses expressing GFP-AIB1 or AIB1 siRNA, and then cell cycles were examined in the presence of E2 with or without pretreatment of PI3K inhibitor LY294002. We observed that overexpression of AIB1 promoted S phase entry in cells with or without E2 treatment (Figure 3a). In contrast, silence of AIB1 reduced S phase entry (Figure 3b). LY294002 abolished cell entry into the S phase (Figure 3a). These data indicated that overexpression of AIB1 enhanced S phase entry in ER-positive breast cancer cells through the PI3K/AKT pathway.

AIB1 overexpression increases p-AKT (Ser 473) activity

PI3K/AKT regulates cell survival and cell cycle by phosphorylating substrates. We investigated the effect of AIB1 overexpression on levels of p-AKT and AKT. After transfection, cells were treated with IGF-1 for 30 min. It was noteworthy that AIB1 was strongly overexpressed in cells transfected with adenovirus encoding AIB1 (Figure 4a). In both T47D and MCF-7 cells overexpressed AIB1, p-AKT (Ser 473) expression were

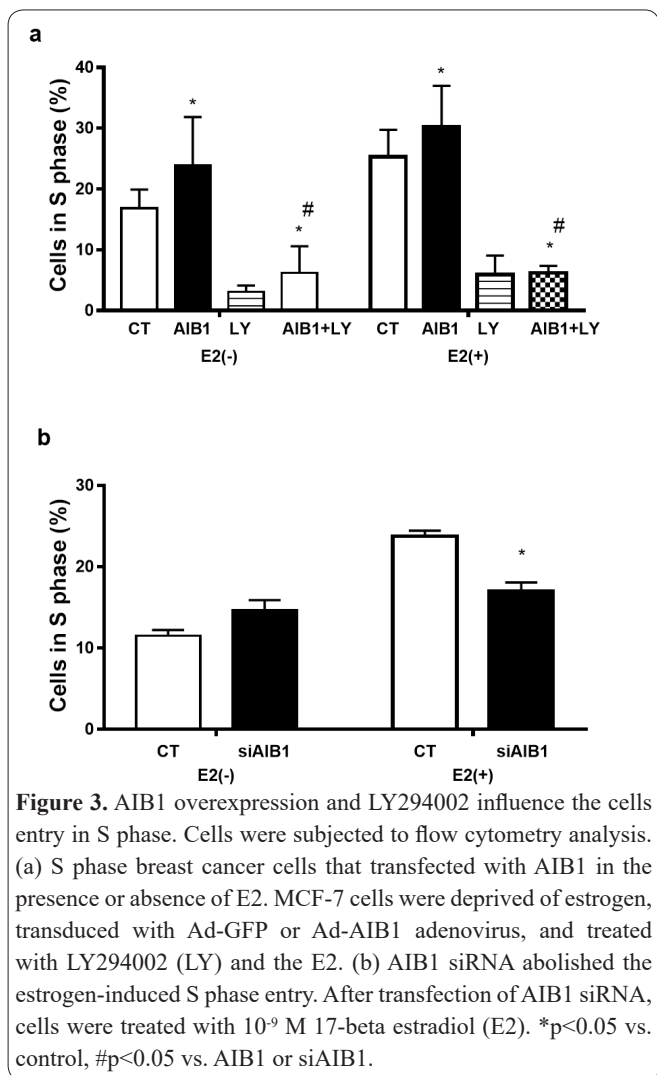


Figure 3. AIB1 overexpression and LY294002 influence the cells entry in S phase. Cells were subjected to flow cytometry analysis. (a) S phase breast cancer cells that transfected with AIB1 in the presence or absence of E2. MCF-7 cells were deprived of estrogen, transduced with Ad-GFP or Ad-AIB1 adenovirus, and treated with LY294002 (LY) and the E2. (b) AIB1 siRNA abolished the estrogen-induced S phase entry. After transfection of AIB1 siRNA, cells were treated with 10^{-9} M 17-beta estradiol (E2). * $p < 0.05$ vs. control, # $p < 0.05$ vs. AIB1 or siAIB1.

significantly increased in presence or absence of IGF-1, and increased more in presence of IGF-1 (Figure 4a).

The AKT phosphorylation activity was also evaluated by kinase assay using H2B as a substrate. The data shown that AIB1 increased AKT phosphorylation activity compared with control, suggesting that AIB1 overexpression can up-regulate AKT function (Figure 4b). However, in the presence of IGF-1 condition, we observed that AIB1 overexpression increases AKT phosphorylation activity (Figure 4b), suggesting that the regulation on AKT activity by AIB1 overexpression involved in IGF-1 pathway.

IGF-1 enhances AIB1-bound PS2 promoter activity through the PI3k /AKT pathway

To determine whether PI3K/AKT is involved in the ER-dependent transcription of PS2 through the coactivator of AIB1, we used IGF-1 to stimulate the LY294002 pre-or not-treated (30 min) MCF-7 cells for 60 min (Figure 5). The whole-cell lysates were immunoprecipitated by AIB1 antibody, DNA fragments bound to AIB1 protein were eluted and amplified by PCR using PS2 promoter. In contrast to the control level, AIB1 recruitment increased with IGF-1 treatment time, and reached peak at 60 min, then decreased (data not shown). Intriguingly, LY294002 significantly inhibited (> 50%) AIB1 recruitment stimulated by IGF-1. Our results indicate that PI3k/AKT signal pathway is involved in AIB1 recruitment and subsequently regulates the ER-dependent transcription activity.

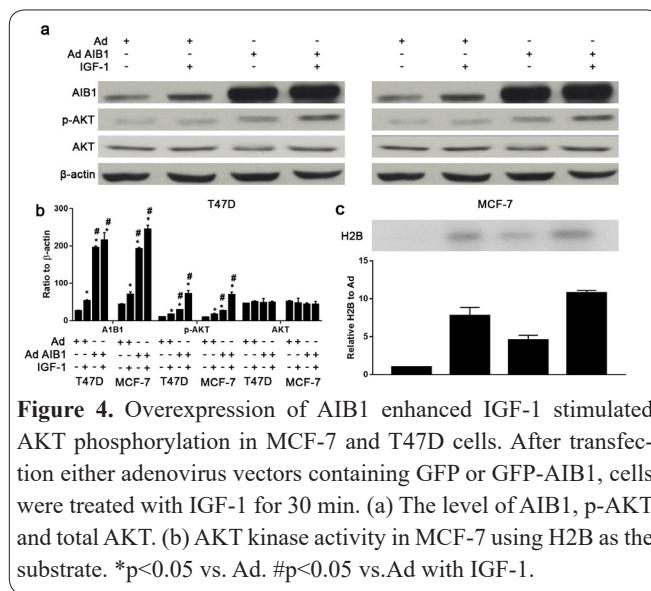


Figure 4. Overexpression of AIB1 enhanced IGF-1 stimulated AKT phosphorylation in MCF-7 and T47D cells. After transfection either adenovirus vectors containing GFP or GFP-AIB1, cells were treated with IGF-1 for 30 min. (a) The level of AIB1, p-AKT and total AKT. (b) AKT kinase activity in MCF-7 using H2B as the substrate. * $p < 0.05$ vs. Ad. # $p < 0.05$ vs. Ad with IGF-1.

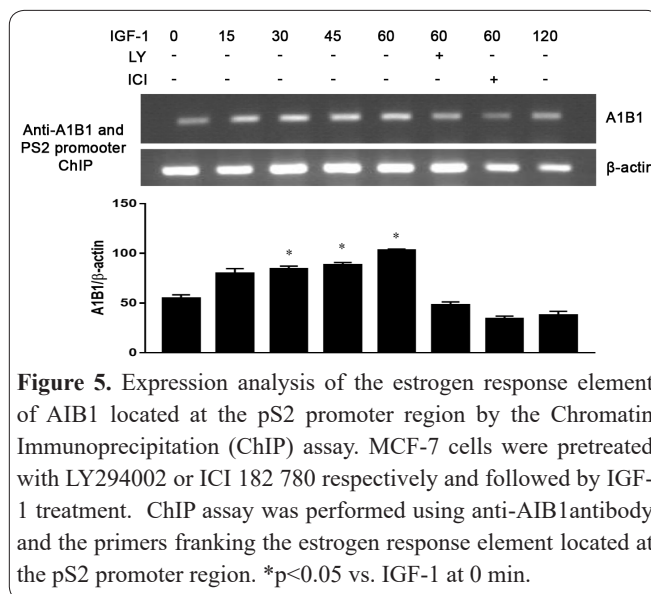


Figure 5. Expression analysis of the estrogen response element of AIB1 located at the pS2 promoter region by the Chromatin Immunoprecipitation (ChIP) assay. MCF-7 cells were pretreated with LY294002 or ICI 182 780 respectively and followed by IGF-1 treatment. ChIP assay was performed using anti-AIB1 antibody and the primers flanking the estrogen response element located at the pS2 promoter region. * $p < 0.05$ vs. IGF-1 at 0 min.

In order to test whether AIB1 recruitment is ER-dependent or not, we also pre-treated the MCF-7 cells with ICI 182,780, an inhibitor of estrogen, for 30 min followed by 60 min treatment with IGF-1, we observed that the AIB1 recruitment stimulated by IGF-1 was obviously blocked by ICI182,780 (Figure 5), suggesting that AIB1 recruitment stimulated by IGF-1 is ER-dependent.

AIB1 and AKT presents in the same complex.

To explore the existence of AIB1 and AKT in the same complex, we co-transfected 911 cells (ER negative) with plasmids containing AIB1 or HA-AKT. After 24h for IGF-1 treatment, we harvested the transfected cells and carried out immunoprecipitation using the anti-AIB1, anti-HA (HA tagged AKT), or IgG Ab as a control to pull down the immune complex. The association between AIB1 and AKT was detected (Figure 6). AKT can be pulled down by anti-AIB1 antibody and AIB1 can also be precipitated by anti-HA (HA-tagged AKT) antibody. In addition, IgG precipitation was negative as expected. The data indicates that AIB1 and AKT can combine *in vivo*, and consequence of its cooperation leads to regulate cell cycle and ER-dependent transcription activity in breast cancer cells. Importantly, ER could promote the formation of this complex.

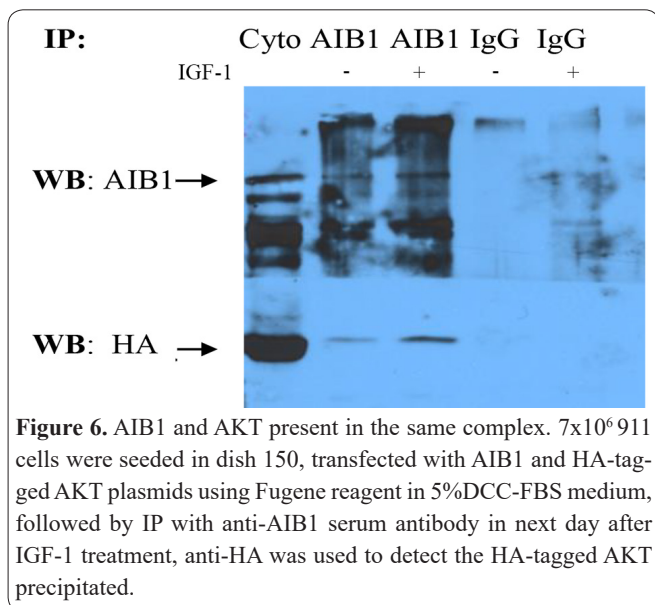


Figure 6. AIB1 and AKT present in the same complex. 7×10^6 911 cells were seeded in dish 150, transfected with AIB1 and HA-tagged AKT plasmids using Fugene reagent in 5%DCC-FBS medium, followed by IP with anti-AIB1 serum antibody in next day after IGF-1 treatment, anti-HA was used to detect the HA-tagged AKT precipitated.

Discussion

It's clear that AIB1 is involved in cell cycle progression via PI3K/AKT pathway. In this study, we demonstrated that AIB1 overexpression can regulate AKT activity, which subsequently contributes to cell cycle and cell growth. Furthermore, we provided evidence that AKT is involved in regulating AIB1 recruitment to the PS2 promoter region in the presence of IGF-1. Coimmunoprecipitation of AIB1 with AKT, and luciferase assay data show the formation of AIB1 and AKT complex. As a result, the interaction between AIB1 and AKT influences cell growth and cell proliferation, as well as ER-mediated gene transcription. These data will help us to understand the mechanisms underlying role of AIB1 and drug resistance in breast cancer cells.

AIB1 is overexpressed in breast cancer cells and its expression correlates with ER-positivity, which is associated with breast cancer prognosis (1, 2). As a nuclear receptor coactivator, AIB1 plays an important role in ER-mediated transcription in part by recruiting CBP/p300 and /or arginine methyltransferase (3, 4). AIB1 is required for normal growth, female reproductive function, and mammary gland development (5). Inactivation of AIB1 in mice reduces mammary epithelial proliferative lesions, suppresses breast tumor formation, and retards breast tumor growth, and decreases metastasis (6). Additionally, there is evidence that p160 coactivators may act on non-nuclear receptor pathways including NF- κ B, VREB and AP-1, potentially broadening the effects of AIB1 overexpression (7). Previous reports showed that AIB1 is a substrate of kinases, such as mitogen-activated protein kinase (MAPK) and I κ B kinase (IKK) (8, 9)(27). This evidence raises the possibility that AIB1 may play an important role in growth factors pathway of regulating ER-mediated transcription other than the manner of phosphorylating ER directly, and may help to explain the mechanism in drug resistance. In this report, we provided evidence that AKT can phosphorylate by AIB1 in vitro and PI3K/AKT pathway is also involved in AIB1 recruitment in some degree, which extends the range of AIB1 function. In terms of non-nuclear receptor pathway, we observed that AIB1 and AKT interaction enhances cell cycle progression.

On one hand, AKT mediates the physiological effects of several growth factors including PDEF, EGF and IGF-1, and activation of AKT pathway results in cellular proliferative as well as antiapoptotic cellular responses (10, 11). On the other hand, growth factors can regulate function of AIB1 (p/CIP) by relocating AIB1 into the nucleus from the cytoplasm in serum-treated cells, which is not altered by inhibitor of MAPK (12). Here, we demonstrated that IGF-1 can regulate AIB1 activity by recruiting AIB1 to the PS2 promoter region through the AKT pathway. This data complements previously described data that IGF-1 regulates ER activity (13, 14), and that IGF-1/AKT/AIB1 maybe the major channel of ER independent for signal transduction in breast cancer cell growth and cell proliferation.

Evidence demonstrates that AKT can regulate ER-alpha function by phosphorylating its AF-1 domain (10, 15, 16). AIB1 is involved in the AKT signaling pathway other than SRC signaling in breast cancer (17). A recent study reported that overexpression of AIB1 (SRC3) increases both cell size and AKT activity, and ability of AIB1 to enhance AKT activity is not dependent on steroid hormones (17). Our data demonstrate that the interaction between AIB1 and AKT occurs in breast cancer cells and enhances ER-mediated ERE reporter gene activity as well as cell cycle S phase entry. Our report describes a novel role for AIB1 function in ER mediated transcription.

Taken together, our data shows that AIB1 overexpression in breast cancer cells increases AKT activity, as well as enhances PI3K/AKT-mediated cell cycle S phase entry. As a positive feedback mechanism, AKT and AIB1 interaction promotes AIB1 recruitment to PS2 promoter region. The coimmunoprecipitation of AIB1 and AKT provides a significant evidence of interaction between AIB1 and AKT in vivo.

Acknowledgements

This research is supported by The National Natural Science Foundation of China, No. 30872700.

Conflict of Interest

None.

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