Silencing of the annexin II gene down-regulates the levels of S100A10, c-Myc, and plasmin and inhibits breast cancer cell proliferation and invasion

Jian Zhang, MD, Bianqin Guo, MD, Yan Zhang, PhD, Ju Cao, PhD, Tingmei Chen, PhD.

ABSTRACT

Objectives: To explore the roles of annexin II in breast cancer progression, and to study the effect of annexin II on breast cancer cell proliferation and invasion.

Methods: This study was conducted in the Key Laboratory of Diagnostic Medicine Designated by the Ministry of Education, Chongqing Medical University, Chongqing, China from December 2006 to January 2009. First, we employed Western blot and reverse transcriptase polymerase chain reaction to detect the expression of annexin II and S100A10 in a panel of well-characterized human breast cancer cell lines, and investigated the localization of annexin II and S100A10 by use of immunofluorescence. We then silenced the expression of annexin II in MDA-MB-435s, which was found to over express annexin II, using the chemically-synthetic annexin II small interfering RNA (siRNA) duplexes (including 3 groups: blank MDA-MB-435s cells, cells transfected with negative control siRNA, and cells transfected with annexin II-siRNA). Finally, the cell proliferation, invasion, and plasmin generation were assayed, and the cellular levels of S100A10 and c-Myc were also detected. All the tests were repeated 3 times.

Results: Annexin II and S100A10 were over expressed in invasive human breast cancer cell lines. The siRNA targeting annexin II of MDA-MB-435s cells did not only decrease annexin II messenger RNA and protein levels, but also down-regulated the levels of S100A10, and c-Myc. The treated cells were remarkably blocked in the G0/G1 phase, and cells in the S/G2-M phase decreased. Additionally, the treatment with siRNA resulted in reduction of plasmin generation as well as a loss of the invasive capacity of breast cancer cells.

Conclusion: Annexin II might be a key contributor to breast cancer proliferation and invasion.


From the Department of Orthopedics (Zhang J), the First Affiliated Hospital of Chongqing Medical University, and the Department of Clinical Hematology (Guo, Zhang Y, Cao, Chen), Key Laboratory of Diagnostic Medicine Designated by the Ministry of Education, Chongqing Medical University, Chongqing, China.

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Address correspondence and reprint request to: Dr. Tingmei Chen, Department of Clinical Hematology, Faculty of Laboratory Medicine, Chongqing Medical University, 1 Yixue Yuan Road, Yuzhong District, Chongqing 400016, China. Tel. +86 (23) 68485184. Fax. +86 (23) 68485005. E-mail: chentingmei@sohu.com
Annexin II is a calcium (Ca$^{2+}$)-dependent phospholipid-binding protein, and exists as a monomer as well as a heterotetramer (AlII), which is composed of 2 copies of a 36 kilodalton (kDa) heavy chain (annexin II, ANX2/p36) and 2 copies of the 11 kDa light chain (S100A10/p11). Typically, the annexin II and S100A10 monomer localize intracellularly, whereas the membrane-associated A2 is part of a heterotetrameric complex. Annexin II is involved in diverse cellular processes such as cell motility, linkage of membrane-associated protein complexes to the actin cytoskeleton, endocytosis, fibrinolysis, ion channel formation, and cell matrix interactions. In addition to these membrane-related events, annexin II is also known to be not only a novel RNA-binding protein, but also associated with nuclear processes. There is a growing body of evidence suggesting the elevated expression of annexin II in different types of cancers, and indicating a role for annexin II in tumor cell proliferation, invasion, and metastasis. It has been reported that AlII on the cell surface served as a receptor/binding protein for tissue plasminogen activator (tPA) as well as its substrate, plasminogen (PLG), and strongly yields the active serine proteases plasmin, which may result in activation of metalloproteases (MMPs). In addition, AlIIt can also provide a structural linkage between proteases and potential substrates on the cell surface to facilitate extracellular matrix (ECM) degradation and tumor invasion and migration. Although it is convincing that membrane-associated AlII appears to be involved in these processes, the exact roles of annexin II and S100A10 remain unclear. The subunit S100A10 belongs to the S100 family of proteins, and it modulates some of the biological properties of another subunit, annexin II. For example, S100A10 was required for Src kinase-mediated tyrosine phosphorylation of A2, which translocated both proteins to the cell surface. Complex formation with S100A10 also increased the affinity of annexin II for calcium and phospholipid, thereby directing it to the membrane surfaces. Interestingly, some studies demonstrated that the membrane-associated annexin II subunit failed to bind t-PA or PLG, but bound plasmin, while the S100A10 subunit bound t-PA, PLG, and plasmin, and it seemed that the S100A10 subunit functioned as a PLG receptor. Contrary to these reports, some reports proposed that the phospholipid-binding sites of annexin II served to anchor AIIt to the extracellular surface of the plasma membrane. Others showed that annexin II was a key regulator of cellular levels of the S100A10 protein by a post-translational mechanism; S100A10 was expressed at very low levels in the absence of A2 both in vitro and in vivo, and annexin II stabilized intracellular S100A10 through direct binding, thus masking an autonomous S100A10 polyubiquitination signal that triggered proteasomal degradation. Herein, to explore the roles of annexin II and S100A10 in breast cancer progression, we studied expressions and roles of annexin II and S100A10 by use of a panel of well-characterized human breast cancer cell lines.

**Methods.** This study was conducted in the Key Laboratory of Diagnostic Medicine Designated by the Ministry of Education, Chongqing Medical University, Chongqing, China from December 2006 to January 2009. All tests were approved by the Ethical Committees of Chongqing Medical University.

**Cell culture.** The human breast cancer cell lines MDA-MB-435s and MDA-MB-231 were obtained from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. The ZR-75-30 and MCF-7 cell lines were donated by Chongqing Medical University, Chongqing, China. These cell lines were grown at 37°C in Dulbecco's modified eagles medium (DMEM) with 10% fetal bovine serum (FBS) (Sijiqing Biological Engineering Material Co, Hangzhou, China) and in a humidified 5% CO$_2$ incubator. All cells were washed 3 times in pH 7.4 phosphate buffer solution before harvesting for different experiments.

**Reverse transcriptase polymerase chain reaction (RT-PCR).** Total RNA was extracted using RNArose (Watson Biotechnologies, Shanghai, China). Purified RNA was reverse-transcribed using the 2-step RT-PCR system (Takara Co, Dalian, China). Subsequently, cDNA was PCR-amplified using annexin II, S100A10, and β-actin cDNA-specific primers. The band density was measured by the GEL DOC 2000 system (Bio-Rad Technologies, Hercules, CA, USA). The special primers for annexin II (forward: 5'-ACTTTGTATGCTGAGC-GGGATG-3', reverse: 5'-CGAAGGCAATATCCT- GTCTCTGTG-3', 126bp); for S100A10 (forward: 5'-GCTCATGAAATCCTTCTATGGG-3', reverse: 5'-AGCAGAAGGGAAAGAAGTAGGC-3', 119bp); and for β-actin (forward: 5'-GACCTTGATATTCCCTATATGGG-3', reverse: 5'-AGCAGAAGGGAAAGAAGTAGGC-3', 119bp) were designed and synthesized by TaKaRa (TaKaRa Co, Dalian, China).

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SYBR real-time fluorescent quantitative PCR. Real-time fluorescent quantitative PCR was prepared using the SYBR premix Ex Taq kit (TaKaRa Co., Dalian, China) according to the manufacturer’s protocol, and amplification was performed on an ABI Prism 7000 detection system (Applied Biosystems, Foster, CA, USA) according to the conditions recommended by the manufacturer. The data was analyzed by ABI Prism 7000 Sequence Detection System software (Applied Biosystems, Foster, CA, USA). The mRNA levels of targets (annexin II, S100A10) were analyzed using the comparative threshold cycle (Ct) method, and presented as 2^{ΔΔCt} normalized to the endogenous reference (β-actin).23 The human annexin II, S100A10 and β-actin primers of real-time fluorescent quantitative PCR were the same as RT-PCR.

Western blot. The total protein was extracted from the breast cancer cells using RIPA lysis buffer with a protease inhibitor, phenylmethanesulphonyl fluoride, 25 micrograms protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% for annexin II, c-Myc, β-actin, and 15% for S100A10) and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Technologies, Hercules, CA, USA). Membranes were blocked in 5% non-fat dried milk and probed with antibodies against annexin II, S100A10, and β-actin (Santa Cruz, CA, USA). Following incubation with horseradish peroxidase conjugate antibodies, proteins were visualized with an enhanced chemiluminescence system according to the manufacturer’s instructions (Beyotime, Jiangshu, China). The band density was measured by the GEL DOC 2000 system (Bio-Rad Technologies, Hercules, CA, USA).

siRNA transfection. In this study, we used a validated siRNA duplex to target 3 annexin II variants sequence (NM_001002858, NM_001002857, and NM_004039), sense: 5’-GCAGCAUGCAAGAGAGAdTdT-3’, anti-sense: 5’-UGUCUCUGUAUGCGUGCTdTdT-3’. A scrambled siRNA duplex was used as a negative control (siRNA-NC) (RiboBio, Guangzhou, China). Cells were split into 6-well culture plates at 2.0×10⁴ cells per well. When cells confluent arrived at 60%, the transfection was carried out. The siRNA complexes were removed after transfection for 8 hours and replaced with DMEM supplemented with 10% FBS. Cells were analyzed at 48 hours post-transfection.

Flow cytometric analysis. The MDA-MB-435s cells were seeded at 2.0×10⁴ per well in 6-well plates and transfected at 75 nmol/L siRNA when cell confluent arrived at 60%. At 48 hours post-transfection, the cells were harvested, then fixed in 70% ethanol for 12 hours at 4°C, and stained with propidium iodide (Sigma, St. Louis, MO, USA) for cell cycle analysis.

Cell proliferation assay in vitro. The cell viability was measured by methylthiazol tetrazolium (MTT) assay. The MDA-MB-435s cells were seeded at 5×10³ per well in 96-well flat-bottom plates. After transfection for 24 hours, 48 hours, 72 hours, and 96 hours, 20 μL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) was added to each well, the cells were incubated for another 4 hours, and 150 μL dimethyl sulfoxide (Sigma, St. Louis, MO, USA) was added, and then lysed for 15 minutes. The absorbance value (A) was measured on an automated 96-well plate reader (Bio-Rad Technologies, Hercules, CA, USA) at 490 nm.

Plasmin generation assay. The MDA-MB-435s cells were seeded at 5×10⁴ per well in a 96-well flat-bottom plates and transfected. After transfection for 48 hours, cells were washed twice with DMEM (without phenol red; Life Technologies, Carlsbad, CA, USA), then 200 μl of reaction buffer (50% [vol/vol] 0.05 units/ml PLG [Calbiochem, LaJolla, CA, USA] in DMEM [without phenol red], 40% [vol/vol] 50 mM Tris-HCl buffer pH 8.2, and 10% [vol/vol] 3 mM Chromozyme PL [Calbiochem, San Diego, CA, USA] in 100 mM glycine solution) was added. The plates were then incubated for 8 hours, at which time the color absorbance was measured by an automated 96-well plate reader at 405 nm. The absorbance value (A) represented the amounts of plasmin generated.24 Percent inhibition of plasmin generation was calculated as follows: A (blank cells or negative control cells) – A (siRNA-annexin II cells)/ A (blank cells or negative control cells).

Chamber invasion assay. The cells invasive capacity was evaluated using a 12 μm pore size millicell chamber (Millipore, Billerica, MA, USA). The upper surface of the polycarbonate membrane was coated with 50 μl matrigel (1:6 [vol/vol] 1 mg/ml matrigel [BD Biosciences, San Jose, CA, USA] in DMEM [without serum]) and the lower surface with 30 μl fibronectin (10 μg/ml Sigma, St. Louis, MO, USA). After rehydration of membrane, the millicell chamber was placed within a 24-well chamber filled with 600μl NIH-3T3 conditioned medium. The 2×10⁵ cells were suspended with 400 μl free serum DMEM medium with or without 0.2 μM PLG and planted into the upper chamber. After incubation for 24 hours, the upper surface was scraped to remove non-invasive cells. Invaded cells on the bottom were fixed with 95% ethanol and stained with Hematoxylin & Eosin. For quantification, the average numbers of invasive cells per field were assessed by counting 5 random fields at 200× magnification under a light microscope. The numbers of penetrated cells represented invasive capacity.

Statistical analysis. The data were statistically valued by ANOVA and presented as mean ± SD.
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from 3 independent experiments. Probability values of less than 0.05 were considered significant. All analyses were carried out using the Statistical Package for Social Sciences Version 10 (SPSS Inc, Chicago, IL, USA).

**Results.** Overexpression of annexin II, S100A10 in human breast cancer cell lines. Annexin II and S100A10 were studied in 4 well-characterized breast cancer cell lines, and the invasive capacity of these breast cancer cell lines was confirmed by millicell chamber assay (data not shown). Western blot and RT-PCR analysis disclosed that annexin II and S100A10 were transcriptionally and translationally overexpressed in MDA-MB-231, MDA-MB-435s, and ZR-75-30 (Figures 1a & 1b), and they localized to the cytoplasm and plasma membrane of the breast cancer cells (data not shown). Moreover, there was an apparent correlation between annexin II, S100A10 levels, and the invasive potential of these cell lines.

Silencing the annexin II gene in MDA-MB-435s cells by siRNA. We silenced expression of Annexin II in MDA-MB-435s, which was found to overexpress annexin II in our study by using the chemically-synthetic annexin II siRNA duplexes. It was shown that annexin II mRNA was reduced 5 folds (Figure 2a), and protein levels were reduced 3 folds compared with...
Table 1 • Effect of annexin II siRNA on cell cycle of MDA-MB-435s.

<table>
<thead>
<tr>
<th>Groups</th>
<th>G0/G1 (±)</th>
<th>S (±)</th>
<th>G2/M (±)</th>
</tr>
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<tbody>
<tr>
<td>B</td>
<td>(48.99 ± 4.61)</td>
<td>(34.12 ± 1.78)</td>
<td>(16.89 ± 1.25)</td>
</tr>
<tr>
<td>N (48 hours)</td>
<td>(68.49 ± 4.21)</td>
<td>(18.71 ± 1.99)</td>
<td>(12.80 ± 1.18)</td>
</tr>
</tbody>
</table>

% - percentage of cell cycle phase, *p=0.0128 compared with B and N. B - blank MDA-MB-435s cells, N - cells transfected with negative control siRNA, R - cells transfected with annexin II siRNA.

Table 2 • Effect of annexin II siRNA on invasive ability of MDA-MB-435s.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasminogen (-)</th>
<th>Plasminogen (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>135 ± 5.56</td>
<td>215 ± 5.96</td>
</tr>
<tr>
<td>N</td>
<td>130 ± 4.35</td>
<td>209 ± 4.95</td>
</tr>
<tr>
<td>R (48 hours)</td>
<td>98 ± 2.70*</td>
<td>130 ± 3.21**</td>
</tr>
</tbody>
</table>

* p=0.0135, ** p=0.0089 compared with B and N. B - blank MDA-MB-435s cells, N - cells transfected with negative control siRNA, R - cells transfected with annexin II siRNA.

negative control cells and blank controls (Figure 2b). The knockdown of annexin II resulted in down-regulation of S100A10 and c-Myc proteins, but had no effect on S100A10 mRNA level.

The effect of annexin II siRNA on proliferation and cell cycle of MDA-MB-435s cells. The MTT assay showed that compared with the blank and negative control groups, the cells proliferation was remarkably inhibited at 48 hours and 96 hours, with the highest inhibitory rate of 51.32% ± 3.35% at 48 hours post-transfection (Figure 3). Flow cytometry indicated the treated cells were blocked in the G0/G1 phase, along with a decreased number of cells in S and G2/M phases (Table 1).

Role of annexin II in the invasion of MDA-MB-435s cells. The results indicate that compared with blank and siRNA negative control, treatment with annexin II siRNA significantly decreased the numbers of invasive cells whether in the presence of PLG (Figure 4a) or in the absence of PLG (Figure 4b). Furthermore, in the presence of PLG, both the siRNA-treated cells and control cells demonstrated increased numbers of invasive cells compared with that of the absence of PLG (Table 2).

Plasmin generation assay. As expected, the annexin II siRNA treating cells down-regulated plasmin generation (Figure 5).

Discussion. Although prior studies described that annexin II was found in a diverse range of tumors including lung cancer, renal cell carcinoma, and pancreatic carcinoma, the mechanisms by which annexin II regulates tumor progression, metastasis, and angiogenesis are poorly elucidated. It is known...
that annexin II appears to be involved in most cellular processes in its tetrameric form (annexin II). (S100A10). Typically, annexin II and S100A10 localize intracellularly, and the formation of an S100A10 - annexin II complex results in its plasma membrane trans localization. To explore the roles of annexin II and S100A10 in breast cancer, we employed well-characterized invasive and non-invasive human breast cancer cell lines, including MDA-MB-231, MDA-MB-435s (highly invasive breast cancer cells), ZR-75-30 (poorly invasive breast cancer cell line), and MCF-7 (non-invasive breast cancer cell line). Western blot and RT-PCR analysis showed that annexin II and S100A10 were transcriptionally and translationally overexpressed in invasive breast cell lines (MDA-MB-231, MDA-MB-435s, ZR-75-30). Although both annexin II and S100A10 were overexpressed in invasive breast cancer cell lines, we thought that annexin II should be a more crucial molecule involved in breast cancer progression.

Firstly, the cellular distribution of annexin II was more diffuse than S100A10. Annexin II and S100A10 localized intracellularly as a monomer, and the formation of the AIIt complex resulted in its cytoplasmic face and submembranous cytoskeleton. More importantly, the annexin II monomer appeared to be nuclear, and was regarded as an effector of DNA synthesis. Additionally, annexin II regulated cellular levels of the S100A10 protein by a post-translational mechanism, and stabilized intracellular S100A10 through masking an autonomous S100A10 polyubiquitination signal that triggered proteasomal degradation. Finally, annexin II was also a novel RNA-binding protein that binds directly to c-Myc mRNA and up-regulates c-Myc protein, which was believed to participate in most aspects of cellular function, including replication, growth, metabolism, differentiation, and apoptosis. Owing mostly to its more multiple intracellular functions, it is convincing that knockdown of annexin II gene should be a better strategy.

Our data showed that knockdown of annexin II of MDA-MB-435s resulted in down-regulation of annexin II and S100A10 proteins, but had no effect on S100A10 mRNA level, which indirectly verified previous observations that annexin II induced a stabilization of S100A10 by blocking S100A10 polyubiquitination signaling and a post-translational mechanism. In addition, knockdown of annexin II resulted in c-Myc reduction. On the basis of its roles in cell replication, growth, metabolism, differentiation, and apoptosis, it is reasonable to speculate that c-Myc might be responsible for the annexin II siRNA treated cells proliferation inhibition as well as cell cycle arrest. In addition to c-Myc, annexin II actin cytoskeleton distribution, and effector of DNA synthesis might account for the treated cells proliferation inhibition and cell cycle arrest.

One of the most important properties of metastatic cells is their ability to degrade and move through extracellular ground substance. Tumor cell migration involves attachment of tumor cells to the underlying basement membrane, local proteolysis, and permeates the proteolytically modified region. There is extensive evidence suggesting that the plasminogen/plasmin system plays a critical role in tumor progression, angiogenesis, invasion and metastasis. Plasminogen is an inactive enzyme which is cleaved by PLG activators on the cell surface, and converts to the active plasmin which may lead to activation of MMPs, latent growth factors, and proteolysis of membrane glycoproteins and degradation of ECM. It has been reported that AIIt on the cell surface served as a receptor/binding protein for tPA as well as its substrate, PLG, and involved in cell surface plasin generation. This contributed to ECM degradation, cellular invasion and tumor metastasis. Here, we take advantage of annexin II siRNA to test whether annexin II promotes the activation of PLG in breast cancer. The MDA-MB-435s cells were transfected with annexin-siRNA for 48 hours, and then subjected to plasmin generation assay. The data revealed that the treated cells down-regulated plasmin generation, along with the decreased numbers of invasive cells in matrigel invasion assay. In addition, in the presence of PLG, the invasive cells were more, than in the absence of PLG. It suggested downregulation of annexin II resulted in invasion assay. In addition, in the presence of PLG, the invasive cells were more, than in the absence of PLG.
in blockade of PLG conversion to plasmin, and the mechanism by which annexin II promoted breast cancer invasion might be related to stimulate PLG to convert into plasmin. However, it was not further elucidated in our study, whether the active serine proteases plasmin results in the activation of MMPs.

In conclusion, annexin II was selectively overexpressed in breast cancer cells. Silencing the annexin II gene by siRNA contributed to the inhibition of breast cancer cells proliferation, invasion, and plasmin generation. Therefore, we suggested annexin II could be of value as a therapeutic target in breast cancer.

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References


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