Downregulation of β3 integrin by miR-30a-5p modulates cell adhesion and invasion by interrupting Erk/Ets-1 network in triple-negative breast cancer

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Received November 19, 2015; Accepted December 15, 2015

DOI: 10.3892/ijo.2016.3319

Abstract. Integrins are adhesion receptors involved in bidirectional signaling and are crucial for various cellular responses during normal homeostasis and pathological conditions, such as cancer progression and metastasis. In the present study, we demonstrated that blockage of β3 integrin-mediated cell-extracellular matrix interactions restrained triple-negative breast cancer (TNBC) growth, and elevated β3 integrin can trigger the rewiring of Erk/Ets-1 signaling pathways, thereby enhancing cell growth and invasion. Ectopic expression of miRNA has been implicated in the deregulation of integrin expression and activity, blocking of cancer tumor development and progression, and acquisition of metastatic phenotype. miR-30a-5p expression has been implicated in the progression of breast cancer. Overexpression of miR-30a-5p suppressed the proliferation, migration and invasion of breast cancer cells. On the contrary, inhibition of miR-30a-5p promoted the proliferation, migration, and invasion of TNBC cells by suppressing the expression of ERK/Ets-1 signal. An inverse correlation was found between the mRNA expressions of miR-30a-5p and β3 integrin in TNBC samples. Furthermore, bioinformatics analysis revealed the putative miR-30 binding sites in the 3'-UTR of β3 integrin. Results of luciferase assay revealed a strong repression of luciferase activity after transfection with miR-30a-5p and wild-type 3'-UTR of β3 integrin. In TNBC cells, miR-30a-5p promoted an epithelial phenotype and suppressed invasion by specifically targeting β3 integrin subunit to subsequently interdict the β3 integrin/Erk/Ets-1 network.

Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive subcategory of breast cancer that currently lacks well-defined molecular targets for effective targeted therapies. Disease relapse, metastasis and drug resistance render standard chemotherapy ineffective in the treatment of TNBC. The acquisition of metastatic phenotypes by mammary tumors has been linked to the alterations in integrin expression (1,2). High level expressions of many integrins, including α5β1, α6 and αvβ3 have been correlated with tumor progression (3,4).

β3 integrin is frequently overexpressed in tumor cells, including lung cancer, melanoma, glioblastoma and breast cancer cells (5,6). Previous studies coupled β3 integrin to epithelial-mesenchymal transition (EMT) and metastasis; β3 integrin inhibition is a therapeutic target to treat TNBC, attenuates TGF-β-mediated EMT and invasion, and inhibits 3-dimensional organoid growth (7). β3 integrin-mediated adhesion can trigger the activation of numerous signaling intermediates, such as FAK, Src, ILK, PI3K and MAPK (8,9). Evidence that ERK signaling promotes cell proliferation, cell survival and metastasis and that this pathway is aberrantly activated in breast cancer at an overwhelming frequency support current efforts to identify inhibition strategies for this pathway. Thus, finding out whether alterations in β3 integrin affect ERK signaling in tumor cells is crucial to improve strategies for treating or preventing metastatic disease.

MiRNAs are powerful regulators of gene expression in cancer cell invasion and metastasis that downregulate gene expression at the post-transcriptional level (10-12). miR-30a has been identified as one of the crucial regulators for development and progression of breast and prostate cancers by directly targeting MTDH and ERG, respectively (13,14). However, the exact function and underlying mechanisms of miR-30a in the progression of breast cancer still warrant further investigation. In the present study, we focused on the functional analysis of miR-30a-5p, a member of the miR-30 family that is reportedly downregulated in cancer cells.

Our data suggested that the activation of signaling cascades downstream from β3 integrin involved the ERK/Ets-1 pathway.
Results also showed that miR-30a-5p suppressed the proliferation and invasion of breast cancer cells in vitro by directly targeting the β3 integrin and suspended β3 integrin-Erk-Ets-1 loop. Thus, a tumor suppressor role of miR-30a-5p in breast cancer was suggested.

Materials and methods

Patient samples. Breast cancer specimens were obtained from 156 patients at the Weifang Medical University Affiliated Hospital after surgical resection. Twenty para-cancerous tissues were allocated into the negative control group. No patient in the present study received chemotherapy or radiation therapy prior to surgery. This study was approved by the Institutional Review Board of Weifang Medical University Hospital and informed consent was obtained from each patient. All fresh samples were stored at -80°C.

Immunohistochemistry, immunofluorescence and cytoskeleton staining. Labeled streptavidin biotin method was used for immunohistochemistry. After deparaffinization and rehydration, primary antibodies were added for overnight storage at 4°C, and slides were incubated with biotin-labeled secondary antibodies. Finally, the slides were incubated with HRP-streptavidin for 15 min. After DAB staining, the results were graded for intensity (0, 1, 2 and 3 for negative, weak, moderate and strong, respectively). The percentage of positive cells, i.e., 0 and 1 (1-24%), 2 (25-49%), 3 (50-74%) and 4 (75-100%), was determined. Discrepancies were resolved by consensus. The grades were multiplied to determine the scores. Tumor scores were defined by using the following rules: low (score, 0-4) and high (score ≥5). For immunofluorescence, cells were grown on coverslips, fixed in 4% paraformaldehyde, and incubated in a blocking buffer (1% BSA, 0.25% Triton X-100 in PBS, pH 7.4). The cells were then probed with primary antibody and fluorescein-conjugated goat anti-mouse IgG (Beyotime Institute of Biotechnology, Haimen, China). The cells were counterstained with DAPI to label the cell nuclei. Cell cytoskeleton was stained with FITC-phalloidin. The cells were seeded into 24-well culture plates, washed with PBS, fixed with 4% paraformaldehyde and incubated with 0.2% Triton X-100. After blocking with 1% bovine serum albumin, cells were incubated with CY3-phalloidin. Images were captured by confocal fluorescence microscopy.

Cell lines and culture conditions. Breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-468 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were routinely cultured in Dulbecco's modified Eagle’s medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS (Tianjin Hao Yang Biological Manufacture Co., Ltd., Tianjin, China). All cells were cultured at 37°C and 5% CO₂.

Plasmid construction and transfection/infection. The sequences of miR-30a-5p mimic and mock were synthesized according to the method of Baraniskin et al (15) and were ligated into the restriction sites of pCDH-CMV-MCS-EF1-Puro vectors. Lentiviruses were produced by transfecting human embryonic kidney 293T with a 3-plasmid system according to manual instructions. miR-30a-5p inhibitor was synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For β3 integrin overexpression, the cDNA of β3 integrin was cloned into the pcDNA3.1, as previously described, and transfected into human breast cancer cells using Lipofectimine according to the manufacturer’s instructions (16). Total RNA and protein were collected for 2 days post-transfection or viral infection assay.

Quantitative real-time PCR analysis. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and complementary DNA was synthesized using reverse transcriptase (Sangon Biotech Co., Ltd., Shanghai, China). Real-time quantitative PCR reactions were performed using SYBR-Green (Takara Bio, Dalian, China). To analyze mature miR-30a-5p, quantitative PCR (RT-qPCR) was performed using the miScript PCR System (Qiagen, Hilden, Germany). The mRNA levels of β3 integrin, E-cadherin, vimentin, and Zeb1 were quantified by qRT-PCR using Quantitect SYBR-Green PCR kit (Vazyme Biotech Co., Ltd., Nanjing, China). Primers used are described in Table I. Changes in expression were calculated using the ΔΔCt method. We calculated the median expression value from signal values (log₂). Patients were labeled based on higher or lower β3 integrin expression compared with the median value, as follows: individuals with low β3 integrin expression (< median) and those with high β3 integrin expression (≥ median).

Cell proliferation assay and flow cytometric analysis. Cell proliferation was measured via methyl-thiazolyltetrazolium (MTT) assay. Cells were seeded at a density of 5x10⁴/well into 96-well plates and cultured for 24, 48, 72 and 96 h. The cells were then incubated with 20 μl MTT (5 mg/ml) for 4 h at 37°C, and 150 μl dimethyl sulfoxide was added to solubilize the crystals for 10 min at room temperature (RT). The optical density was measured at 540 nm. For cell cycle analysis, the adhered cells were collected by trypsinization at 48 h after transfection. The cells were incubated with propidium iodide (0.05 mg/ml; Sigma) and RNase A (0.1 mg/ml) for 30 min at RT in the dark and analyzed by using BD FACSCalibur flow cytometer and CellQuest software.

Adhesion, wound healing and invasion assays. Cells (0.5x10⁶ cells/well) were added to each well with 5% CO₂, and incubated for 4 h at 37°C. After washing, the attached cells were fixed with 70% ethanol followed by staining with 0.1% crystal violet in 20% ethanol. The stained crystal violet was dissolved in 10% acetic acid, and the absorbance value was measured at 597 nm. The cell matrix adhesion index was calculated as the OD value (test-negative control)/OD value (positive control-negative control). Each test group was assayed in triplicate and repeated at least thrice. For the scratch wound healing assay, cells were cultured in a serum-free medium for 24 h and wounded with pipette tips. Wound closing procedure was observed for 48 h with images taken every 24 h. For the invasion assay, cell invasion through a 3D extracellular matrix (ECM) was assessed using BD Matrigel invasion chambers ((BD Biosciences, Bedford, MA, USA) with 8.0 μm filter membranes. After 24 h, cells invading the lower surface of the filters were fixed, stained and counted. Percentage change
Results

TNBC expresses low levels of miR-30a-5p and high levels of β3 integrin. The expression levels of miR-30a-5p in TNBC breast cancer cell lines (MDA-MB-231 and MDA-MB-486) were much lower than in NTNBC breast cancer cell lines (MCF-7), as shown in Fig. 1A. We compared miR-30a-5p expression levels in para-cancerous tissues of breast cancer and breast cancer patients. Breast cancer tissues had reduced miR-30a-5p transcript levels compared with para-cancerous tissues (Table II for patient characteristics of all donors). Significant differences were observed when comparing TNBC with NTNBC tissues (Fig. 1B and Table II). Further analysis showed that miR-30a-5p expression strongly correlated with histological grade and survival status (Table II).

The β3 integrin expression in TNBC patients was compared with that in NTNBC patients. Notably, β3 integrin mRNA expression was significantly higher in TNBC patients compared with NTNBC patients and para-cancerous tissues (Fig. 1C) (P<0.001). Kaplan-Meier analysis was performed by using the log-rank test to calculate the effect of β3 integrin mRNA expression on TNBC patient survival. High β3 integrin expression was markedly associated with reduced overall survival in TNBC patient subgroups (Fig. 1D). Western blot analyses of protein extracts revealed a significantly higher relative β3 integrin expression in TNBC patients (Fig. 1E).

Given that miRNAs exploit their inhibitory activity at the post-transcriptional level and the reduced miR-30a-5p expression in the TNBC patients, we subsequently verified the expression of miR-30a-5p and β3 integrin in breast tumor tissues. As shown in Fig. 1F, miR-30a-5p expression was lower in TNBC than NTNBC samples. An inverse correlation was found between
mRNA expression of miR-30a-5p and β3 integrin in TNBC patients (Fig. 1G, r²=0.47, P<0.05; Pearson's correlation). These differences in mRNA and protein expression of β3 integrin in breast cancer patients suggested probable post-transcriptional regulation by miR-30a-5p.

Knockdown of β3 integrin results in the alteration of EMT markers. To investigate the role of β3 integrin knockdown on the reversal of EMT phenotype of breast cancer cells, siRNA-targeting β3 integrin was transfected into MDA-MB-231 cells. After 14 days of transfection, the morphology of β3 integrin-silenced MDA-MB-231 was partially changed from elongated fibroblastoid to epithelial cobblestone-like appearance, with the cells appearing to grow in close contact with each other (Fig. 2A). The silencing of β3 integrin in the MDA-MB-231 cells resulted in the elevation of epithelial marker E-cadherin and the downregulation of mesenchymal markers, including Zeb1 and vimentin, at mRNA and protein levels (Fig. 2B and C).

We next investigated whether molecular alterations were present in E-cadherin and vimentin protein by immunostaining. Silencing of β3 integrin showed that E-cadherin protein was significantly upregulated compared with the levels detected in control cells (Fig. 2D). To determine the role of β3 integrin in actin cytoskeletal reorganization, cells with silenced β3 integrin were stained for F-actin and vinculin and showed morphological changes, including formation of protrusions and destruction of actin filaments (Fig. 2E). These results suggest that the β3 integrin is critical for the acquisition of EMT characteristics and that inhibition...
of β3 integrin was able to reverse the EMT phenotype of breast cancer cells.

**miR-30a-5p directly targets the β3 integrin 3' untranslated region (3'-UTR).** To investigate the direct effects of miR-30a-5p on β3 integrin expression in breast cancer cell lines, we performed miR-30a-5p overexpression experiments in MDA-MB-231 cells. Infection of miR-30a-5p mimic into MDA-MB-231 cells increased miR-30a-5p levels (data not shown). Ectopic overexpression of miR-30a-5p resulted in a significantly decrease in β3 integrin mRNA levels, as determined by qRT-PCR (Fig. 3A). This suppression was also found at the protein level, as observed by western blot analysis (Fig. 3B). On the contrary, miR-30a-5p inhibitor restored β3 integrin expression in a dose-dependent manner (Fig. 3C). Online programs (TargetScan, miRBase and PicTar) revealed that a region in the 65-72 β3 integrin 3'-UTR had a perfect complementary matching region in the seed sequence of miR-30a-5p (Fig. 3D and E). This suppression was also found at the protein level, as observed by western blot analysis (Fig. 3B). On the contrary, miR-30a-5p inhibitor restored β3 integrin expression in a dose-dependent manner (Fig. 3C). Online programs (TargetScan, miRBase and PicTar) revealed that a region in the 65-72 β3 integrin 3'-UTR had a perfect complementary matching region in the seed sequence of miR-30a-5p (Fig. 3D and E). To confirm that the silencing of β3 integrin expression is consequent to miR-30a-5p targeting of the 3'-UTR in β3 integrin transcript, the complete 3'-UTR of β3 integrin and corresponding mutant counterparts were cloned into pGL3 firefly luciferase-containing vector (Fig. 3F).

**miR-30a-5p reduces the adhesion capacity of TNBC cells.** Ectopic miR-30a-5p also inhibited the proliferation rate of MDA-MB-231 and MDA-MB-486 cells by inducing cell cycle arrest at the G0/G1 phase, as shown by the MTT assay and flow cytometry (data not shown). To eliminate the potential confounding effect of cell proliferation on cell migration and invasion, the Transwell experiment and scratch assay were conducted in the presence of mitomycin C to subsequently arrest cell proliferation.

β3 integrin signaling is associated with many cellular functions. Integrin-mediated interactions with the extracellular matrix (ECM) are required for attachment, cytoskeletal organization, mechanosensing, migration, proliferation, differentiation and survival of cells. To test whether miR-30a-5p functionally behaves as a tumor suppressor by targeting β3 integrin, we stably overexpressed miR-30a-5p in MDA-MB-231 and MDA-MB-486 using a lentiviral vector. HEK-293T cells were cotransfected with the firefly luciferase-containing vector, Renilla luciferase-containing vector, and pre-miR-30a-5p. The results revealed a strong repression of luciferase activity after transfection with wild-type 3'-UTR of β3 integrin, but not in cells with mutant 3'-UTR (Fig. 3G).

**Table II. Clinicopathological characteristics and miR-30a-5p expression in breast cancer.**

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>Cases (%)</th>
<th>( \log_2 ) (fold of repression) (mean ± SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤45</td>
<td>72 (46.1)</td>
<td>4.17±0.47</td>
<td>0.268^a</td>
</tr>
<tr>
<td>&gt;45</td>
<td>84 (53.9)</td>
<td>4.02±0.51</td>
<td></td>
</tr>
<tr>
<td>Molecular-based classification</td>
<td></td>
<td></td>
<td>0.006^a</td>
</tr>
<tr>
<td>TNBC</td>
<td>45 (28.8)</td>
<td>2.47±0.39</td>
<td></td>
</tr>
<tr>
<td>NTNBC</td>
<td>111 (71.2)</td>
<td>4.51±0.59</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td>0.173^b</td>
</tr>
<tr>
<td>≤2</td>
<td>66 (42.3)</td>
<td>4.37±0.53</td>
<td></td>
</tr>
<tr>
<td>2-5</td>
<td>79 (50.6)</td>
<td>4.05±0.65</td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>11 (7.1)</td>
<td>3.71±0.24</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td>0.003^b</td>
</tr>
<tr>
<td>I</td>
<td>48 (30.4)</td>
<td>4.47±0.32</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>83 (52.5)</td>
<td>4.16±0.53</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>27 (17.1)</td>
<td>2.19±0.22</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td>0.047^b</td>
</tr>
<tr>
<td>I, II</td>
<td>113 (72.4)</td>
<td>4.52±0.42</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>36 (23.1)</td>
<td>4.18±0.36</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>7 (4.5)</td>
<td>3.89±0.32</td>
<td></td>
</tr>
<tr>
<td>Positive lymph nodes</td>
<td></td>
<td></td>
<td>0.001^b</td>
</tr>
<tr>
<td>0</td>
<td>64 (41.0)</td>
<td>4.83±0.32</td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>51 (32.7)</td>
<td>4.31±0.64</td>
<td></td>
</tr>
<tr>
<td>≥4</td>
<td>41 (26.3)</td>
<td>4.19±0.22</td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td></td>
<td></td>
<td>0.003^b</td>
</tr>
<tr>
<td>Alive</td>
<td>114 (73.1)</td>
<td>5.49±0.52</td>
<td></td>
</tr>
<tr>
<td>Deceased</td>
<td>42 (26.9)</td>
<td>2.86±0.36</td>
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</table>

\^aIndependent-samples t-test. \^bOne-way ANOVA test.
Figure 2. Inhibitory effect on EMT in breast cancer cells by knockdown of β3 integrin. (A) The morphology of MDA-MB-231 cells changed from fibroblastoid to epithelial-like appearance after silencing β3 integrin (upper panel). Downregulation of β3 integrin facilitated the adhesion of MDA-MB-231 cells in vitro (lower panel). (B) Silencing of β3 integrin in MDA-MB-231 resulted in the upregulation of E-cadherin expression and downregulation of Zeb1 expression. Vimentin was assessed by real-time RT-PCR. (C) Western blot analysis was performed to determine E-cadherin, vimentin and Zeb-1 expressions. (D) Fluorescence microscopic staining of E-cadherin and vimentin was performed in MDA-MB-231 cells. Nuclear DNA was stained with DAPI. (E) miR-30a-5p-overexpressing cells exhibited protrusion formation and actin filament destruction compared with uninfected cells. *P<0.05.

Figure 3. β3 integrin is specifically targeted by miR-30a-5p. (A) The potential for miR-30a-5p-mediated silencing of β3 integrin by the predicted miRNAs was tested in MDA-MB-231 cells by qRT-PCR. (B) miR-30a-5p overexpression reduced the β3 integrin protein expression of MDA-MB-231 cells. (C) Different transfection concentration of miR-30a-5p gradually increased β3 integrin expression in MDA-MB-231 cells. (D and E) Putative binding sites for miR-30a-5p were predicted by using the PicTar and TargetScan algorithms. (F) Schematic representation of the predicted target site of miR-30a-5p in the wild-type (WT) 3'-UTR of β3 integrin and mutant (MUT) β3 integrin 3'-UTR. Seed matching sequence is indicated. (G) Ectopic miR-30a-5p expression inhibited WT, but not the activity of MUT β3 integrin 3'-UTR, *P<0.05, **P<0.01.
We focused on the effect of miR-30a-5p on the adhesion of TNBC cells by planting cells in plates coated with fibronectin, which could bind to a β3 integrin receptor expressed on the surface of tumor cells (18). As shown in Fig. 4A, the adhesion ability of MDA-MB-231 and MDA-MB-486 cells with miR-30a-5p overexpression showed significantly reduced adherence compared with the control cells. On the contrary, the adhesion ability of cells was lower when miR-30a-5p was inhibited using siRNA than when scramble siRNA was used (Fig. 4B). Moreover, reduction occurrence after transfection with siRNA directed against β3 integrin (siITGB3) elucidated the significance of reduced β3 integrin in overexpressed miR-30a-5p cells (Fig. 4C).

Ectopic expression of miR-30a-5p suppresses cell migration and invasion in vitro. To test whether miR-30a-5p overexpression suppressed tumor migration and invasion, we first examined the morphological changes in miR-30a-5p-overexpressed cells. As shown in Fig. 4D, MDA-MB-231 cells overexpressing miR-30a-5p exhibited epithelial morphology. Cells overexpressing miR-30a-5p showed a non-aggressive appearance and depressed adhesion of cell to fibronectin. Thus, we hypothesized that miR-30a-5p could suppress the migration and invasive behavior of TNBC cancer cells. Then, wound scratch and Transwell assay were employed to detect the migration and invasion after miR-30a-5p manipulation, respectively. We found that miR-30a-5p could significantly suppress migration (Fig. 4E) and invasion (Fig. 4F) in TNBC cells lines. To further investigate whether the inhibitory effect of miR-30a-5p on migration and invasion was mediated by mesenchymal to epithelia transition (MET), we examined the expression of several MET markers. As expected, miR-30a-5p
overexpression increased the expression level of E-cadherin and decreased the expression levels of vimentin and Zeb1 (Fig. 4G). These observations suggested that ectopic expression of miR-30a-5p was able to impede migration and invasion mediated by MET in vitro.

miR-30a-5p interrupts the β3 integrin-Erk-Ets-1 network in triple-negative breast cancer. Integrins elicit a series of transduction events that regulate cell cycle progression and apoptosis in a process known as 'outside-in' signaling. The 'outside-in' β3 integrin-mediated signaling proceeds primarily via the Erk1/2 MAPK pathway in MDA-MB-231 breast cancer cells. Immunohistochemical staining of β3 integrin, Erk1/2, and Ets-1 in breast cancer and normal tissues are shown in Fig. 5A. Results indicated that expression of β3 and Erk1/2 (P<0.05) and β6 and Ets-1 (P<0.01) were positively correlated, as shown in Table III.

To further confirm the association between Erk1/2-Ets-1 phosphorylation and miR-30a-5p inhibition, we collected extracts from si-miR-30a-5p MDA-MB-231 cells. Western blot analysis of the extracts showed a specific enhancement of pErk1/2 (Fig. 5B). As the activities of Ets-1 were enhanced by...
Erk1/2-mediated phosphorylation at threonine 38 for Ets-1 (26), we further examined the phosphorylation levels of Ets-1, showing Erk1/2 activation correlated well with the elevated phosphorylation levels of pEts-1, whereas PD98059 (Erk pathway inhibitor) markedly lowered pEts-1 levels (Fig. 5C). However, Ets-1 phosphorylation was also enhanced (Fig. 5D) with obvious nuclear translocation in ectopic miR-30a-5p-expressing MDA-MB-231 cells (Fig. 5E). Similar correlations between miR-30a-5p overexpression and reduced Erk1/2-Ets phosphorylation were found in MDA-MB-486 cells (data not shown). Thus, β3 integrin induced ERK1/2 phosphorylation, thereby triggering the activation of Ets-1 transcription factors leading to β3 integrin upregulation, whereas miR-30a-5p interrupted the positive feedback loop (Fig. 5F).

Discussion

TNBC has a high incidence of early relapse and metastasis and contributes to poor clinical outcomes. Treatment options are limited for TNBC because endocrine therapy and targeted therapy, which aim directly at human epidermal growth factor receptor-2, are ineffective. Interactions between cells and ECM convey micro-environmental cues that influence cell proliferation, differentiation, adhesion, and migration (19,20). Thus, targeting cell-ECM interactions can become a potential component of an oncologist’s therapeutic arsenal as a novel therapy for TNBC. Integrins, a family of cell adhesion molecules, are involved in a wide range of cell-ECM and cell-cell interactions. Basement membrane proteins interact with mammary epithelial cells via integrins and transmembrane proteoglycans and syndecan, which all couple to the cytoskeleton and assemble signaling platforms to control cell function (21). Integrin and associated intracellular signaling effector expression levels and/or activity are modified in TNBCs, thereby suggesting that the adhesion machinery has a role in malignant transformation and tumor progression (7,22).

Integrin adhesion receptors modulate cell functions, including cell proliferation; hypodermic injection of breast cancer cells stably transfected with β1 integrin into athymic nude mice resulted in decreased size and weight of subcutaneous xenograft tumors (23). Moreover, β1 integrins support outgrowth of metastatic colonies in the lung. In the present study, we demonstrated that ectopic expression of β3 integrin attenuated the proliferation and induced cell cycle arrest at G0/G1 phase in breast cancer cells. This confirms and extends earlier reports where outgrowth of breast cancer cells arrest also showed the role of β3 integrin signaling in the modulation of cell proliferation of breast cancer (24).

β3 integrin has been detected in tumor tissues from patients with melanoma, breast cancer and its expression is particularly pronounced in metastatic tissue (7,25,26). TNBC is a highly aggressive subgroup of breast cancer and currently lacks definite molecular targets for effective targeted therapies. Among the 156 tissue specimens, 57 (36.5%) were positive for β3 expression. A significant statistical difference existed between TNBC and NTNBC groups. Previous studies exploited β3 integrin as a therapeutic target for treating TNBC by delivering siRNA (7,8). Breast cancer cell adhesion and spread are triggered by the contact between β3 integrin and fibrinogen. Blocking β3 integrin with antibodies and siRNA leads to significantly lower adhesion of MDA-MB-231 cells to fibronectin (27). EMT is a crucial procedure in tumor metastasis; therefore, prevention of EMT represents a very promising therapeutic strategy to prevent tumor metastasis (28,29). In the present study, reduced expression of β3 integrin could cause morphological changes in MDA-MB-231 cells, i.e., from fibroblastoid cells to epithelial-like cells with weak invasive capacity. Silencing of β3 integrin promoted MDA-MB-231 cell migration due to the change in the amount of organized actin cytoskeleton. This finding is significant because targeting TNBC by β3 integrin might provide a valuable tool in developing new therapeutic avenues against metastasis.

MiRNAs negatively regulate EMT-related genes at the post-transcriptional level and play critical roles in cancer metastasis. In the present study, we reported that miR-30a-5p is associated with the regulation of EMT. Aberrant expression of miRNA has been implicated in the deregulation of integrin expression and activity. MiRNA-130b suppresses migration and invasion of colorectal cancer cells through downregulation of β1 integrin (30). We found that overexpression of miR-30a-5p in breast cancer cells significantly suppressed β3 integrin in vitro, whereas, miR-30a-5p was inversely correlated with β3 integrin expression in breast cancer tissues. Computational prediction by using the TargetScan software revealed an evolutionarily conserved region in the β3 integrin 3'-UTR mRNA, which has a perfect complementary matching region to the seed sequence of the miR-30a-5p. Luciferase activity assay indicated that miR-30a-5p could bind to the 3'-UTR sequence of β3 integrin mRNA, whereas β3 integrin is a direct target of miR-30a-5p.

Many cellular responses to soluble growth factors, such as EGF, PDGF, LPA and thrombin, depend on the adhesion of cells to substrates via integrins (31). The integrin family represents major receptors that mediate adhesion to the ECM and trigger critical intracellular signaling pathways involved in the invasion and migration (32). Previous studies show that the presence of a positive feedback loop between β3 integrin and Ets-1 (33,34). Thus, β3 integrin might be a novel therapeutic target. Results from our study indicated that the expression level of β3 and Ets-1 was associated with differentiation, TNM stage, and breast cancer classification. Correlation analysis showed that the expression of β3 and Ets-1 were positively correlated. To verify the potential impact of ectopic miR-30a-5p on phosphorylation Ets-1, the activation of the ERK/Ets-1 was determined. Overexpression of miR-30a-5p displayed reduced phosphorylation of Erk1/2 and Est-1, and weakened the nuclear localization of Ets-1 in MDA-MB-231 cells. Furthermore, inhibition of Erk attenuated the silence-mediated upregulation of pEts-1 in miR-30a-5p.

In conclusion, β3 integrin contributed to cancer progression in TNBCs by regulating Erk1/2 and Est-1, which drive malignant cell behavior. This regulation can be exploited in therapeutic strategies to inhibit cancer progression. The present study showed that miR-30a-5p, as a tumor suppressor, is a regulator of β3 integrin replication in breast cancer. This validates the concept of targeting the cross link of β3 integrin/Erk/Ets-1 network as a novel and promising modality to treat TNBCs.
Acknowledgements

The present study was funded by grants from the National Nature Scientific Foundation of China (30901779 and 81471048) and the Natural Science Foundation of Shandong Province (ZR2015HL064).

References


