Abstract. Accumulating evidence has shown that miR-196a plays an important role in tumorigenesis and tumor progression in various types of cancer. miRNA profiling studies have suggested that miR-196a is highly overexpressed in breast cancer. However, the functional mechanism of miR-196a in breast cancer remains unclear. In the present study, we first showed that the expression of miR-196a was significantly upregulated in human breast cancer samples and breast cancer cell lines. Using a loss-of-function approach, we showed that the downregulation of miR-196a inhibited the proliferation of breast cancer cells in vitro and in vivo. Ubiquitin-conjugating enzyme E2C (UBE2C) gene as a cellular proto-oncogene, which was overexpressed and positively correlated with miR-196a expression in breast cancer tissues, was identified as a direct target of miR-196a. Moreover, in order to investigate whether miR-196a regulated cell growth in breast cancer cells by targeting UBE2C, rescue studies were performed in breast cancer cells. The restoration of UBE2C by transfecting UBE2C cDNA in anti-miR-196a-transfected breast cancer cells rescued the suppression of cell proliferation. In conclusion, the present study showed that miR-196a promoted cell proliferation by targeting UBE2C in breast cancer. Thus, miR-196a may be a potential oncogene in breast cancer and a promising therapeutic target in breast cancer treatment.

Introduction

MicroRNAs (miRNAs) are a family of small (~22 nucleotides), non-coding RNA molecules that regulate gene expression by targeting promoters or mRNAs for transcriptional inhibition or translational repression (1). miRNAs have important regulatory functions in various biological processes, such as cell proliferation and differentiation, and inhibition of apoptosis (2). Evidence suggests that some miRNAs are involved in carcinogenesis and tumor progression, acting as tumor suppressors or oncogenes, and have become potential biomarkers for cancer diagnosis, therapy and prognosis (3).

Breast cancer is the most common invasive cancer in women, and has become the leading cause of cancer-related mortality in women worldwide, accounting for 22.9% of invasive cancers in women and 16% of all female cancers (4). Despite significant advancements in the diagnosis and treatment of breast cancer, approximately one third of patients develop and subsequently succumb to metastatic breast cancer (5). Therefore, it is extremely important to define the exact etiology of breast cancer. Breast cancer is considered a highly heterogeneous disease at the molecular and clinical level, comprising various types of neoplasms and involving different profile changes in the mRNA and miRNA expression (6).

miRNA studies in breast cancer have yielded noteworthy findings, which showed great potential for the development of novel biomarkers and therapeutic targets using miRNA (7). The miR-196 gene family is located in the intergenic regions of homeobox (HOX) clusters within the genome of vertebrates. Three miR-196 genes have been indentified, including miR-196a-1, miR-196a-2 and miR-196b genes. The miR-196a-1 and miR-196a-2 genes transcribe the same functional mature miRNA sequence,3'-GGGUUGUUGUACUUUGAUGGAU-5', i.e., miR-196a (8). Recently, miRNA profiling studies have shown that miR-196a is highly overexpressed in several tumor tissues, including breast cancer (9-12). Furthermore, evidence suggests that miR-196a plays an important role in the development, immunity and tumor pathogenesis by targeting specific genes (9). However, the functional mechanism of miR-196a remains to be elucidated, especially in breast cancer.
In the present study, we found that the expression of miR-196a was significantly upregulated in breast cancer tissues from 34 patients. Using a loss-of-function approach, we showed that the downregulation of miR-196a inhibited the proliferation of breast cancer cells in vitro and in vivo. Furthermore, the ubiquitin-conjugating enzyme E2C (UBE2C) gene, which was overexpressed and positively correlated with miR-196a expression in breast cancer tissues, was identified as a direct target of miR-196a. The present results may elucidate the functions of miR-196a and its roles in breast cancer carcinogenesis.

Materials and methods

Tissue samples and cell lines. Sixty breast cancer tissues and adjacent normal breast tissues were obtained from the Department of General Surgery, Shanghai Hospital (Shanghai, China) for RT-qPCR analysis. The histopathological diagnosis of breast cancer was confirmed by the Department of Pathology in the hospital according to the criteria of the World Health Organization. Tissue samples were surgically removed and immediately snap-frozen in liquid nitrogen. The experiments were approved by the Ethics Committee of Biomedicine Research, Second Military Medical University. Informed consent was obtained from all patients. The patients had no history of radiotherapy or chemotherapy prior to surgery. The patient clinicopathological characteristics are provided in Table I. Additionally, six normal breast tissues were obtained from adjacent breast tissues of contusion and laceration in traumatic breast injury patients. The MCF-7, T47d, BT549 and HBL-100 breast cancer cell lines and human MCF-10A breast cell line used in the present study were purchased from the Cell Bank of the Chinese Academy of Sciences and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). HEK-293 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM containing 10% FBS. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

RNA isolation and RT-qPCR. Total RNA was isolated from breast cancer tissues, adjacent normal breast tissues, breast cancer cell lines and human breast cell lines using TRIzol according to the manufacturer’s instructions. Detection of miR-196a expression was performed by RT-qPCR as previously described (13). U6 small RNA was used as an internal control for the normalization and quantification of miR-196a expression. The RT-qPCR-based detection of UBE2C was performed and β-actin was used as an internal control. Experiments were performed in triplicate. The primers are shown in Table II.

Oligonucleotides and plasmid transfection. RNA oligos were chemically synthesized and purified by the Shanghai GenePharma Co., Ltd. (Shanghai, China). Human miR-196a

Table I. Patient clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients (n=60)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (year)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>&gt;40</td>
<td>51</td>
<td>85</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2</td>
<td>7</td>
<td>11.7</td>
</tr>
<tr>
<td>&gt;2 and ≤5</td>
<td>49</td>
<td>81.6</td>
</tr>
<tr>
<td>&gt;5</td>
<td>4</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table II. Primers used in RT-qPCR analysis and luciferase reporter assays.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-196a RT</td>
<td>5'-GTGTATCCAGTGCAGGTGCAGGATTTCCAGCAGTACACGACCCAAC-3'</td>
</tr>
<tr>
<td>miR-196a F</td>
<td>5'-AATGTAAGTGTGTTCATGGT-3'</td>
</tr>
<tr>
<td>miRNA Universal R</td>
<td>5'-GTGCAGGGTGTCGGAGTT-3'</td>
</tr>
<tr>
<td>U6 F</td>
<td>5'-GTGCTCGCTTTCCAGCACATATA-3'</td>
</tr>
<tr>
<td>U6 R</td>
<td>5'-AAAAATGTTAGAAGGCTACGGATT-3'</td>
</tr>
<tr>
<td>β-actin F</td>
<td>5'-CAGCAAGCAGGATGTAGCAG-3'</td>
</tr>
<tr>
<td>β-actin R</td>
<td>5'-GAAAGGTTGTAACGGACTAA-3'</td>
</tr>
<tr>
<td>UBE2C F</td>
<td>5'-TCTGCGGATAAAAAGGGATT-3'</td>
</tr>
<tr>
<td>UBE2C R</td>
<td>5'-GTTGAGCTGGTTCTGATGC-3'</td>
</tr>
<tr>
<td>UBE2C (reporter) F</td>
<td>5'-AAAATGATCACCACCCACCGTGACC-3'</td>
</tr>
<tr>
<td>UBE2C (reporter) R</td>
<td>5'-GGAAGCCTTTCCTTCAGGATGCC-3'</td>
</tr>
<tr>
<td>UBE2C (pGL3) F</td>
<td>5'-AAAGGTACCATGCTTCCCAAACCGA-3'</td>
</tr>
<tr>
<td>UBE2C (pGL3) R</td>
<td>5'-AAACTCGAGTACGGGCTCCTGAGTACC-3'</td>
</tr>
</tbody>
</table>

RT, reverse-transcription primer; F, forward; R, reverse.
inhibitors included a nucleotide sequence of 5'-CCC AAC ACG AUG AAA CUA CCU ATT-3'. The nucleotide sequence of negative control inhibitors was 5'-AAU UCU CCG AAC GUG UCA CTT-3'. The transfections were performed with INTERFERin siRNA transfection reagent (Polyplus-Transfection Inc., Illkirch-Graffenstaden, France). The final concentration of miRNA was 50 nM.

To generate pGL3-UBE2C constructs, the coding DNA sequence fragment of UBE2C was amplified by primers (Table II) from normal human cDNA and inserted into the KpnI and XhoI sites of the pGL3 construct. The constructs were verified by direct sequencing. Transfections were performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The final concentration of plasmids was 100 ng.

**Luciferase reporter assays.** A luciferase reporter vector was constructed by cloning human UBE2C mRNA sequence into the pMIR-Report construct (Ambion, Austin, TX, USA). The UBE2C mRNA fragment (from 428 to 481) was amplified and cloned into the luciferase reporter via the SpeI and HindIII sites. The relevant primer sequences are provided in Table II. HEK-293 cells were plated in a 48-well plate and co-transfected with 50 nM single-stranded miRNA mimics or negative control oligonucleotides, 50 ng of firefly luciferase reporter and 10 ng of pRL-TK (Promega, Madison, WI, USA) using the INTERFERin reagent (Polyplus-transfection). The cells were collected 36 h after the previous transfection and analyzed using the Dual-Luciferase reporter assay system (Promega).

**Cell proliferation assay.** Cell proliferation was measured using the CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The cells were plated into each well of a 96-well plate and transfected with miRNA inhibitors or negative control inhibitors. On the day the cells were collected, 10 µl CCK-8 was added to 90 µl of culture medium. The cells were subsequently incubated for 2 h at 37°C and the optical density was measured at 450 nm. Three independent experiments were performed.

**Tumorigenicity assays in Nod/Scid mice.** The mice received humane care according to the guidelines of the Nantong University Animal Care and Use Committees. miR-196a inhibitors and NC-transfected MCF-7 cells (1x10⁶) were suspended in 150 µl PBS and then injected subcutaneously into either side of the posterior flank of the same Nod/Scid mice at 6 weeks of age. Six Nod/Scid mice were included and tumor growth was examined every three days over a course of 4 weeks. Tumor volume (V) was monitored by measuring the length (L) and width (W) of the tumor with calipers and was calculated using the formula: 

\[ V = \frac{L \times W^2}{2} \times 0.5 \]

**Western blot analysis.** Proteins were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was blocked with 5% non-fat milk and incubated with anti-UBE2C antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-β-actin antibody (Sigma, San Diego, CA, USA) at 4°C overnight. The membranes were washed three times with PBS plus 0.1% Tween-20 and then incubated with a goat anti-mouse secondary antibody (Pierce, Rockford, IL, USA). The proteins were detected using ECL reagents (Pierce).

**Statistical analysis.** Statistical analyses were carried out using the SPSS 16.0 statistical software package. Data are presented as mean ± standard deviation (SD). Data were subjected to Student's t-test. The Wilcoxon signed-rank test and Spearman’s correlation coefficient was used for the comparison of tumor volumes. P<0.05 was considered to indicate a statistically significant result.

**Results**

**miR-196a is overexpressed in breast cancer.** To determine the expression levels of miR-196a in breast cancer tissues and cell lines, total RNAs were isolated from breast cancer samples and adjacent normal tissues, the normal breast cell line and breast cancer cell lines. The clinicopathological characteristics of 60 patients are shown in Table I. The expression levels of miR-196a were determined by RT-qPCR and normalized to an endogenous control (U6 RNA). As shown in Fig. 1A, the miR-196a expression was significantly higher in breast cancer tissues than that in adjacent normal tissues. We also found that miR-196a expression was significantly increased in the four breast cancer cell lines, compared with normal breast tissues and the normal MCF-10A breast cell line (Fig. 1B). Thus, miR-196a was abnormally overexpressed in human breast cancer samples and breast cancer cell lines.

Downregulation of miR-196a inhibits the proliferation of breast cancer cells in vitro and in vivo. To investigate the regulatory mechanisms and the biological significance of the upregulation of miR-196a in breast cancer, a cell proliferation assay was performed during tumorigenesis. miR-196a inhibitors or negative control inhibitors (NC) were transiently transfected into MCF-7 and T47d breast cancer cell lines with higher endogenous expression levels of miR-196a (Fig. 1B). The expression levels of miR-196a were examined by
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RT-qPCR after transfection. The results indicated that miR-196a expression in MCF-7 and T47d cells were significantly decreased following the transfection of miR-196a inhibitors (Fig. 2A). Results of the CCK-8 and cell proliferation assays showed that the downregulation of miR-196a in MCF-7 and T47d cells resulted in a significant reduction in the ability of cell proliferation. The proliferation rate was suppressed in MCF-7 and T47d cells following transfection with miR-196a inhibitors, and the inhibitory efficiencies were 33.3 and 32.2% at 72 h, respectively (Fig. 2B).

Furthermore, we used an in vivo model to evaluate the effect of tumorigenicity after the suppression of miR-196a expression. miR-196a inhibitors and NC-transfected MCF-7 cells were injected subcutaneously into either side of the posterior flank of the same Nod/Scid mice. In this tumorigenicity assay, five mice were included and tumor growth was examined every three days over a course of 4 weeks. Compared with NC transfectants, miR-196a inhibitor-transfected cells revealed a significant reduction in the tumor size (Fig. 3), suggesting a potential tumor suppressive effect after the downregulation of miR-196a expression.

miR-196a upregulates UBE2C expression at the post-transcriptional level by targeting the UBE2C coding region. As miRNAs regulate gene expression mainly through adjustment of the translation level of target genes, the candidate target genes of miR-196a that function in breast cancer pathogenesis were then investigated. We assessed the CLASH data (14), and found that ~149 genes were targeted by miR-196a in HEK-293 cells. Of these genes, we focused on the cell cycle-associated gene UBE2C, which is involved in many processes including cell proliferation, migration and survival. UBE2C is correlated with tumor malignancy and regarded as a strong proto-oncogene, and it has been detected to be upregulated in many types of cancer including breast cancer (10,15). From the CLASH data in HEK-293 cells, the potential targeting sequence for miR-196a with a calculated activation energy of -13.8 kcal/mol.
is present within the protein coding region of UBE2C mRNA from 428 to 481. To verify whether miR-196a regulated the expression of the UBE2C gene, luciferase reporter assays in HEK-293 cells were performed. We constructed luciferase reporter plasmids with or without miR-196a targeting the sequence of UBE2C mRNA (Fig. 4A), which were co-transfected with miR-196a inhibitors or negative control inhibitors in HEK-293 cells. Luciferase activity in transfected cells was measured after transfection for 48 h. Our results showed that luciferase activity was decreased significantly in HEK-293 cells transfected with the reporter plasmid with targeting sequence of UBE2C mRNA and miR-196a inhibitors, whereas there was no change in luciferase activity in cells transfected with the reporter plasmid without targeting sequence of UBE2C mRNA (Fig. 4B). Subsequently, we examined whether the endogenous UBE2C in breast cancer cells was regulated similarly. MCF-7 and T47d cells were transfected with miR-196a inhibitors or negative control inhibitors. The expression levels of UBE2C mRNA and protein were examined by RT-qPCR and western blotting, respectively. The levels of UBE2C mRNA and protein were substantially downregulated by miR-196a inhibitors (Fig. 4C and D). The results demonstrated that UBE2C was a direct target of miR-196a and positively regulated by miR-196a in breast cancer cells.

UBE2C mRNA levels are upregulated and positively associated with miR-196a in breast cancer tissues. The above results demonstrated that UBE2C was positively regulated by miR-196a in breast cancer cells. We examined the correlation between miR-196a expression levels and UBE2C mRNA levels in breast cancer tissues. An RT-qPCR assay was performed to detect the mRNA expression of UBE2C in the same breast cancer samples as shown in Fig. 1A and normalized to β-actin. As shown in Fig. 5A, UBE2C mRNA levels were significantly increased in breast cancer tissues vs. adjacent normal tissues, which is in concordance with a previous study (15). After normalization to the expression value of normal tissues, RNA levels of miR-196a and mRNA levels of UBE2C in breast cancer tissues were analyzed by Pearson's correlation coefficient analysis. UBE2C mRNA levels were positively correlated with miR-196a expression levels in breast cancer tissues (r=0.7382, P<0.001) (Fig. 5B). Collectively, these results suggested that high endogenous miR-196a targeted and upregulated the expression of UBE2C and contributed to the progression of breast cancer.

Restoration of UBE2C expression rescues miR-196a-induced breast cancer cell proliferation. We confirmed that inhibition of miR-196a suppressed the growth and proliferation of breast cancer cells and UBE2C was a direct target of miR-196a. To investigate whether miR-196a regulated cell growth in breast cancer cells by targeting UBE2C, rescue studies were performed in breast cancer cells. Inhibition of miR-196a in MCF-7 and T47d cells significantly decreased the protein expression of UBE2C and suppressed cell proliferation, while the restoration of UBE2C by transfecting UBE2C cDNA in anti-miR-196a-transfected cells rescued this suppression (Fig. 6). These results demonstrated that miR-196a regulated breast cancer cell growth by targeting UBE2C.

Discussion

In the present study, we found that miR-196a expression was upregulated in breast cancer samples and cell lines compared with that in the normal breast samples and cell lines, which are consistent with findings of a previous study (10). We also demonstrated that downregulation of miR-196a significantly decreased the proliferation ability of the MCF-7 and T47d breast cancer cells in vitro and suppressed tumor growth in vivo. Moreover, we identified UBE2C as a direct and functional target of miR-196a in breast cancer tissues and cells.
Consistent with our results, miR-196a has been suggested to function as an oncogene because its expression is upregulated in several cancer cells, including esophageal cancer (17), malignant melanoma (18), lung (11), gastric (12), colorectal (19,20) and breast (10) cancer. miR-196a is overexpressed in esophageal cancer and promotes cell proliferation, anchorage-independent growth and suppresses apoptosis by directly regulating the Annexin A1 (ANXA1) gene (17). Overexpression of miR-196a in colorectal cancer promoted cancer cell detachment, migration, invasion and decreased its chemosensitivity, and increased the development of lung metastases by activating the Akt signaling pathway (19). It has been shown that a high expression of miR-196a contributes to gastric carcinogenesis by downregulating its target gene p27kip1 (12). miR-196a also promotes the proliferation and invasion of non-small cell lung cancer cells by targeting the HOXA5 gene (11). Taken together, these results suggest that miR-196a is upregulated in various types of cancer and may act as an oncogene in cancer growth and development. It has reported that an individual miRNA is capable of regulating a number of distinct mRNAs. Thus, we hypothesized that miRNA-196a may act on several target genes rather than a single target. In the present study, we identified UBE2C as one of the potential target genes of miR-196a and found that miR-196a could target the coding DNA sequence and upregulate the expression of UBE2C, which was in agreement with previous studies (21-23). However, the underlying functional mechanism of the positive regulation of UBE2C by miR-196a in breast cancer remains to be determined.

UBE2C, a member of the E2 ubiquitin-conjugating enzyme families, is required for the destruction of mitotic cyclins and regulation of the anaphase-promoting complex and for cell-cycle progression (16). Overexpression of UBE2C causes chromosome mis-segregation and alters the cell cycle profile, which may facilitate cell proliferation (24). Overexpression of UBE2C has been detected in many types of human cancers, including aggressive thyroid, ovarian and breast carcinomas, colon cancer, and lymphomas, and is correlated with tumor malignancy (15,25,26). However, the underlying molecular mechanism of UBE2C upregulation in breast cancer is not well known. Previous reports have shown that UBE2C expression always accompanied the expression of the tumor initiator HER2 (human epidermal growth factor receptor 2), and breast microcalcifications lesions with a receptor 2), and breast microcalcification lesions with a high mRNA expression of HER2 also showed a high mRNA expression of UBE2C in breast core biopsies, which have demonstrated the close association between the expression of UBE2C and HER2 (15). In the present study, we found that UBE2C is highly expressed in breast cancer tissues and cell lines and the expression of UBE2C was positively associated with miR-196a in breast cancer tissues. We have demonstrated that miR-196 was able to target and upregulate the expression of UBE2C in breast cancer cells. Moreover, rescue studies were performed in breast cancer cells. The restoration of UBE2C by transfection of UBE2C cDNA in anti-miR-196a-transfected breast cancer cells rescued the suppression of cell proliferation. These results demonstrate that miR-196a regulated breast cancer cell growth by targeting UBE2C. We hypothesize that miR196a upregulates UBE2C expression and then UBE2C regulates the expression of the tumor initiator HER2, resulting in the occurrence of breast cancer.

In summary, the results of the present study have shown that miR-196a is upregulated in breast cancer tissues, and that the downregulation of miR-196a inhibited the proliferation of breast cancer cells in vitro and in vivo. miR-196a directly targets UBE2C and the expression level of UBE2C is positively associated with miR-196a expression in breast cancer. These data suggest that miR-196a is a potential oncogene in breast cancer and a promising therapeutic target in the treatment of breast cancer.

Acknowledgements

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References


