miR-125b Is Methylated and Functions as a Tumor Suppressor by Regulating the ETS1 Proto-oncogene in Human Invasive Breast Cancer

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Abstract

The microRNA miR-125b is dysregulated in various human cancers but its underlying mechanisms of action are poorly understood. Here, we report that miR-125b is downregulated in invasive breast cancers where it predicts poor patient survival. Hypermethylation of the miR-125b promoter partially accounted for reduction of miR-125b expression in human breast cancer. Ectopic restoration of miR-125b expression in breast cancer cells suppressed proliferation, induced G1 cell-cycle arrest in vitro, and inhibited tumorigenesis in vivo. We identified the ETS1 gene as a novel direct target of miR-125b. siRNA-mediated ETS1 knockdown phenocopied the effect of miR-125b in breast cell lines and ETS1 overexpression in invasive breast cancer tissues also correlated with poor patient prognosis. Taken together, our findings point to an important role for miR-125b in the molecular etiology of invasive breast cancer, and they suggest miR-125b as a potential theranostic tool in this disease. Cancer Res; 71(10): 3552–62. ©2011 AACR.

Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs that modulate gene expression by binding to the 3’-untranslated region (3’-UTR) of target mRNA, and promoting RNA degradation, inhibiting mRNA translation, and affecting transcription (1). Growing evidence indicates that miRNAs play important roles in biological processes including development (2), cell proliferation, apoptosis (3), and differentiation (4). miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may function as either tumor suppressors or oncogenes (5).

miR-125b, a brain-enriched miRNA, is evenly distributed between neurons and astrocytes (6). Recent reports suggest that miR-125b might act as an oncogene or as a tumor suppressor, depending on the cellular context. Deregulation of miR-125b has been observed in invasive breast cancer, ovarian carcinoma, hepatocellular carcinoma, and thyroid carcinoma (7–10), and it is also associated with clinical outcome in liver cancer patients (9). Expression of miR-125b is elevated in pancreatic cancer, oligodendrogial tumors, prostate cancer, myelodysplastic syndromes, and acute myeloid leukemia (11–14). miR-125b was reported to suppress cell proliferation by downregulation of ERBB2 and ERBB3 in breast cancer, but to promote proliferation by downregulation of Bak1 in prostate cancer cells (15). miR-125b was also reported to negatively regulate p53, and suppress p53-dependent apoptosis in both zebrafish and humans (16).

In this study, we presented evidences that miR-125b was hypermethylated and functioned as a tumor suppressor by regulating the ETS1 proto-oncogene in invasive breast cancer.

Materials and Methods

Tissue specimens and TMAs construction

All the breast cancer tissues were obtained from Sun Yat-sen University Cancer Center, and classified according to the American Joint Committee on Cancer (AJCC) and tumor node metastasis (TNM) classification system (17). For miR-125b quantitative analysis, formalin-fixed paraffin-embedded tissues (FFPET) of 105 invasive breast cancers and 40 paired normal adjacent tissues (NAT, >2 cm from cancer tissue) were used. For DNA methylation and miR-125b quantitative analysis, as well as in situ hybridization (ISH) analysis, fresh samples from 9 invasive breast cancers and paired NATs were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive breast cancer were used.
Research Ethics Committee of SYSUCC (reference number: YP-2009174). Detailed patients recruitment and methodologic issues are described in Supplementary data.

The clinicopathological characteristics and follow-up data of the patients were summarized in Supplementary Table S1.

Locked nucleic acid–based ISH

Detailed methods are described in Supplementary data.

Quantitative real-time PCR analysis for miR-125b

Total RNAs were isolated from FFPETs using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Quantitative analysis of miR-125b expression in 105 invasive breast cancer tissues was assayed using a Hairpin-it miRNA real-time PCR Quantitation Kit (GenePharma). Quantitative real-time PCR (qRT-PCR) analysis was carried out on an ABI 7900HT instrument (Applied Biosystems). Each sample was analyzed in triplicate, and U6 snRNA was used for normalization. The quantity of miR-125b in each invasive breast cancer sample relative to the average expression in 40 NATs was calculated using the equation: relative quantity (RQ) = $2^{-\Delta\Delta CT}$ (19). The primers were in Supplementary Table S2.

Bisulfite sequencing and methylation-specific PCR

The primers for bisulfite sequencing and methylation-specific PCR (MSP) used in this study were designed by MethPrimer (20), and detailed methods are described in Supplementary data.

Cell culture and treatments

Human breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-453) were purchased from Cell Bank of Chinese Academy of Sciences and used within 2 months after resuscitation of frozen aliquots. MDA-MB-435 breast cancer cell line was kindly provided by Dr. Xiao-Ming Xie (Department of Breast Oncology, Sun Yat-sen University). Cell lines were authenticated on the basis of viability, recovery, growth, morphology, and isoenzymology by the provider. Culture conditions are described in Supplementary data.

For 5-aza-2'-deoxycytidine (5-aza-CdR) treatment, human breast cancer cell lines MCF-7, MDA-MB-435, MDA-MB-231, and MDA-MB-453 were seeded at $2 \times 10^5$ per well in 6-well plates and cultured with 5 $\mu$mol/L 5-aza-CdR (Sigma-Aldrich) for 72 hours, respectively. The medium containing drug was replaced every 24 hours. RNA was isolated and qRT-PCR was carried out to evaluate the restoration of miR-125b after 5-aza-CdR treatment.

For cells transfection, pre–miR-125b precursor molecules and negative control (pre-control) were bought from Ambion Inc. ETS1-siRNAs and control-siRNA were from GenePharma. The sequences of siRNAs were listed in Supplementary Table S2. Transfection of pre–miR-125b, pre-control, ETS1-siRNA, and control-siRNA were used with Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Transfected cells were incubated at 37°C for 24 hours, followed by incubation with complete medium. Cells were harvested at indicated time points.

| Table 1. Relationship between miR-125b expression level and clinicopathologic parameters of invasive breast cancer |
|-------------------------------------------------|-----------|-----------------|----------------|--------|
| Variable                                       | Number of cases | miR-125b, %     | P           |
| Age, yr                                        |           | High expression | Low expression |        |
| ≥48                                            | 54        | 29 (53.7)       | 25 (46.3)    | 0.846  |
| <48                                            | 51        | 26 (51.0)       | 25 (49.0)    |        |
| Pathologic grade                               |           |                 |              |        |
| I                                              | 23        | 15 (65.2)       | 8 (34.8)     | 0.237  |
| II, III                                       | 82        | 40 (48.8)       | 42 (51.2)    |        |
| Clinical stage                                 |           |                 |              |        |
| I, II                                         | 83        | 46 (55.4)       | 37 (44.6)    | 0.241  |
| III                                           | 22        | 9 (40.9)        | 13 (59.1)    |        |
| Lymph node status                              |           |                 |              |        |
| Metastasis                                     | 62        | 26 (41.9)       | 36 (58.1)    | 0.017  |
| No metastasis                                  | 43        | 29 (67.4)       | 14 (32.6)    |        |
| ER status                                      |           |                 |              |        |
| Negative                                       | 54        | 26 (48.1)       | 28 (51.9)    | 0.436  |
| Positive                                       | 51        | 29 (56.9)       | 22 (43.1)    |        |
| PR status                                      |           |                 |              |        |
| Negative                                       | 46        | 22 (47.8)       | 24 (52.2)    | 0.437  |
| Positive                                       | 59        | 33 (55.9)       | 26 (44.1)    |        |
| CerbB2 status                                  |           |                 |              |        |
| 0, 1+                                          | 66        | 39 (59.1)       | 27 (40.9)    | 0.105  |
| 2+, 3+                                         | 39        | 16 (41.0)       | 23 (59.0)    |        |
Cell proliferation assay and colony-forming assay
Detailed methods are described in Supplementary data.

Flow cytometry
Detailed methods are described in Supplementary data.

In vivo tumorigenicity assays
Detailed methods are described in Supplementary data.

cDNA microarray analysis
Total RNA was isolated at 12- or 24-hour posttransfection from MCF-7 or MDA-MB-435 cells, using Trizol reagent (Invitrogen). The mRNA expression profile was carried out using a human genome oligo array service V1.0 (catalogue no. 220010; CapitalBio). Each sample was analyzed once, and data preprocessing, normalization, and filtering were conducted as previously described (21). All microarray data had been deposited to the Gene Expression Omnibus public database with accession number GSE22546.

Validation of cDNA microarray
qRT-PCR was conducted as previously described (22) and primers were in Supplementary Table S2. Relative expression was calculated using the equation \( R = 2^{-\Delta\Delta CT} \) (19).

Luciferase reporter assay
Detailed methods are described in Supplementary data.

Immunoblot analysis
Antibodies used for immunoblots were RPS6KA1 (1:1,000 dilution; Cell Signaling Technology), C9orB6 (1:200 dilution; Abcam), MAN1B1 (1:200 dilution; Santa Cruz Biotechnology), ASB13 (1:500 dilution; Abcam), MKNK2 (1:500 dilution; Abcam), UBE2E3 (1:500 dilution; Abcam), ETS1 (1:1,000 dilution; Abcam), and anti-GAPDH antibody (1:3,000 dilution; Abcam). All protein bands were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunohistochemistry staining
Dako Real Envision Kit (K5007, Dako) was used in IHC staining analysis. Hormonal receptors were evaluated with the 1D5 antibody for estrogen receptor \( \alpha \) (Er\( \alpha \)) and antibody PGR-1A6 for the progesterone receptor (PR; Dako). CerbB2 was detected with CB11 (Dako). ETS1 was detected with a primary antibody ETS1 (1:500 dilution; Abcam). Three observers independently determined consensus scoring of IHC staining using a semi-quantitative estimation, as described previously (23). Samples with scores lower than the median score were grouped as low protein expression (24).

Statistical analysis
Data were analyzed using SPSS16.0 software (SPSS Inc.). Significant associations between miR-125b expression and clinicopathological parameters were assessed using a \( \chi^2 \) test. Survival curves were plotted by Kaplan–Meier analysis and compared by the log-rank test. Cox regression analysis was carried out to assess the significance of variables for survival. Data were expressed as mean \( \pm \) SD, and \( t \) test was used to determine the significance of differences between 2 groups. All tests carried out were 2 sided. \( P < 0.05 \) was considered statistically significant.

Results

miR-125b expression is downregulated in invasive breast cancer, and correlated with poor prognosis and lymph node metastasis
A decrease in miR-125b staining intensity was observed in invasive breast cancer tissues, compared with corresponding NATs by chromogenic in situ hybridization (CISH; ref. Fig. 1A). Parallel detection by fluorescence in situ hybridization (FISH) is shown in Supplementary Fig. S1. The expression of miR-125b was detected by qRT-PCR in the FFPEts of 105 invasive breast cancers and 40 NATs (Fig. 1B). Consistent with the results of CISH and FISH, miR-125b expression levels in breast cancers (\(-2.69 \pm 0.49, \text{mean} \pm \text{SD}\)) were significantly lower than that in NATs (\(1.10 \pm 0.27, \text{mean} \pm \text{SD}\); \(P < 0.001\), Fig. 1B).

One hundred five invasive breast cancer tissues were used to analyze the correlation between miR-125b expression and clinicopathological characteristics of invasive breast cancer (Table 1). Low miR-125b expression correlated with lymph node metastasis (\(P = 0.017\)), and no correlation was observed between miR-125b expression and age, pathologic grade, clinical stage, ER status, progesterone receptor (PR) status, or CerbB2 status.

The 5-year overall survival rate of the 105 invasive breast cancer patients was 68.6% (Fig. 1C). The 5-year survival rate of breast cancer patients with low miR-125b expression was 53.8% (\(n = 50\), which was significantly lower than that in patients with high miR-125b expression (81.8%, \(n = 55\); \(P = 0.002\), Fig. 1C). When patients were stratified by lymph node status, low miR-125b expression exhibited a significant correlation with poor survival of patients without lymph node metastasis (\(P = 0.003\), Fig. 1C). However, among the patients with lymph node metastasis, miR-125b expression did not show a statistical correlation with survival (\(P = 0.232\), Fig. 1C). In addition, in the breast cancer patients with early clinical stage stratum, low CerbB2 stratum, PR-positive stratum or treatment with surgery, and adjuvant therapy stratum, miR-125b expression exhibited a significant correlation with survival (Supplementary Fig. S2).

Univariate Cox proportional hazard regression analysis revealed that low miR-125b expression was a significant predictive factor for poor prognosis (HR = 2.98; \(P = 0.004\)). Multivariate Cox proportional hazard regression analysis revealed that low miR-125b expression (HR = 2.60; \(P = 0.012\)) was also a significantly unfavorable independent prognostic factor in breast cancer patients (Supplementary Table S3).

miR-125b is methylated in breast cancer
Recent studies showed that the methylation status of distal Cpg-rich regions correlated with the expression pattern of miRNAs in cancer (25). Here, we defined a Cpg-rich region as island length more than 100 bp, GC percent more than 50, and observe/expect more than 0.60. Two Cpg-rich regions within...
Figure 1. miR-125b was overexpressed in invasive BC and correlated with poor prognosis. A, detection of miR-125b by CISH at 400× magnification. Positive in situ hybridization signals are in blue. miR-125b expression levels in invasive BC tissues is much lower than in corresponding NATs. B, RT-PCR shows that expression of miR-125b is reduced in invasive BC tissues compared with NATs (P < 0.001). C, Kaplan–Meier survival curves and log-rank test for BC patients with high or low miR-125b expression and negative or positive lymph node status. BC, breast cancer.
2,000 bp upstream of the miR-125b-1 gene were found by using MethPrimer with the default criteria of the online tool (20). Recently, researchers revealed that the transcription factor NF-κB p65 binding site of the promoter element of miR-125b-1 resided in −1,059 to −1,050 region of the miR-125b-1 gene (26). Given that NF-κB p65 binding site of the promoter element of miR-125b-1 is close to CpG-rich region 1 (Fig. 2A), we carried out bisulfite sequencing to assess CpG methylation status.

Figure 2. miR-125b was methylated in invasive BC tissues and BC cell lines. A, methylation status of 8 CpG sites within the miR-125b-1 gene promoter was analyzed by bisulfite sequencing. Five clones were picked out and sequenced for 2 BC tumors (BC1 and BC2), 2 NATs (NAT1 and NAT2), and 2 BC cell lines (MDA-MB-435 and MCF-7). Open and filled circles represented unmethylated and methylated CpG sites, respectively. Circles were partially filled according to the percentage of methylation of the CpG site. The frames showed the CpG pairs covered by MSP primers. B, MSP and qRT-PCR analysis were used to determine the methylation status and expression of miR-125b in BC cell lines MCF7, MDA-MB-435, MDA-MB-231, and MDA-MB-453. C, relative expression of miR-125b in BC cell lines MCF7, MDA-MB-435, MDA-MB-231, and MDA-MB-453, which were treated with 5 μmol/L 5-aza-CdR. D, MSP and qRT-PCR analysis were used to determine the methylation status and expression of miR-125b in 9 BC tumors (BC1–9) and 9 NATs (NAT1–9), then correlation of methylation status and expression of miR-125b was analyzed. M, methylated alleles; U, unmethylated alleles; BC, breast cancer.
within an ~843 to ~1052 region upstream of miR-125b-1 in 2 invasive breast cancer tissues, paired NATs, as well as 2 breast cancer cell lines MCF-7 and MDA-MB-435 cells (Fig. 2A). CpG-rich region 1 within upstream of miR-125b-1 was found to be heavily methylated in the 2 breast cancer cell lines and invasive breast cancer tissues, while NATs showed absence of methylation (Fig. 2A).

To investigate whether hypermethylation status of miR-125b-1 could affect miR-125b expression in breast cancer, 4 breast cancer cell lines MCF-7, MDA-MB-435, MDA-MB-231, and MDA-MB-453 were treated with a demethylating agent 5-aza-CdR, then methylation status and expression of the miR-125b were analyzed by MSP and qRT-PCR, respectively. We found that miR-125b expression in the MCF-7, MDA-MB-435, and MDA-MB-231 cell lines, which showed hypermethylation in the promoter of miR-125b-1, were restored after treatment with the demethylating agent 5-aza-CdR for 72 hours. However, MDA-MB-453 cell lines with unmethylation in the promoter of miR-125b-1, 5-aza-CdR treatment did not cause an obvious increase in miR-125b level (Fig. 2B and C).

To determine the correlation between DNA methylation status and expression of the miR-125b in clinical samples, the methylation status of miR-125b-1 promoter and expression of miR-125b were analyzed in 9 invasive breast cancer tissues and paired NATs by MSP and qRT-PCR, respectively. The promoter of miR-125b-1 was hypermethylated in 55.6% (5/9) invasive breast cancer tissues; however, unmethylation in the promoter of miR-125b-1 was observed in all of 9 NATs. Furthermore, miR-125b levels in tissues with miR-125b promoter hypermethylation were significantly lower than those without miR-125b promoter hypermethylation (P < 0.05, Fig. 2D). Taken together, these data suggested that hypermethylation of the CpG-rich region 1 within upstream of miR-125b-1 might be involved in miR-125b downregulation in invasive breast cancer.

**miR-125b suppresses cell proliferation and clonogenicity, and induces G1 cell-cycle arrest**

Overexpression of miR-125b in MDA-MB-435 and MCF-7 cells transfected with pre-miR-125b was confirmed by qRT-PCR and FISH (Supplementary Fig. S3). miR-125b overexpression in MDA-MB-435 and MCF-7 cells led to a decrease in growth and proliferation (P < 0.05, Fig. 3A). Evaluation of the effect of miR-125b on the clonogenicity of the 2 breast cancer cell lines found that miR-125b-transfected cells displayed obvious colony inhibition compared with control transfectants (Fig. 3B). These results indicated that miR-125b may play an important role in tumor growth inhibition.

The cell-cycle distribution of transfected cells was investigated using flow cytometry. The proportion of G1 phase cells increased to 66.4% ± 2.9% in MDA-MB-435 cells transfected with pre-miR-125b, compared with 57.9% ± 4.8% in pre-control-transfected cells, at 48 hours after transfection (Fig. 3C). Similarly, the proportion of G1 phase cells increased...
by 5.6% in MCF-7 cells transfected with pre–miR-125b compared with pre-control cells (Fig. 3C). No effect was observed on apoptosis or migration when MDA-MB-435 and MCF-7 cells were transfected with pre–miR-125b (Supplementary Fig. S4).

miR-125b suppresses tumorigenicity in vivo

To address the potential effects of miR-125b in vivo breast cancer cell growth, equal numbers (5 × 10⁶) of MDA-MB-435 cells transfected with pre–miR-125b or the pre-control were injected into female nude mice. As shown in Figure 3D, MDA-MB-435 cells transfected with pre–miR-125b showed significantly reduced tumor growth compared with those transfected with pre-control. On day 18, the mean tumor volume in the pre–miR-125b transfected group (253.9 ± 183.7 mm³) was smaller than that in the pre-control transfected group (486.0 ± 146.4 mm³, P < 0.05). Moreover, the mean tumor weight of the pre–miR-125b group (0.085 ± 0.079 g) was lower than that of the pre-control group (0.165 ± 0.083 g, P = 0.045). These in vivo studies indicated that miR-125b could inhibit the tumorigenicity of MDA-MB-435 cells in nude mice.

miR-125b target gene screening and prediction

Differential genes influenced by miR-125b in breast cancer were evaluated by cDNA microarray analysis using CapitalBio chips. MDA-MB-435 and MCF-7 cells were transfected with pre–miR-125b or pre-control, and then microarray analyses were carried out. We found 53 genes (22 upregulated and 31 downregulated) that were differentially expressed in both cell lines (Fig. 4A). Among these 53 candidate genes, 6 genes, which were predicted by at least one of TargetScan 5.1, miRBase Targets V.5, miRNAMap 2.0, PicTar, and miRanda 3.0 programs, were selected for further validation by qRT-PCR. Consistent with the microarray results, UBE2E3 showed increased mRNA levels, while MAN1B1, C9orf86, MKNK2, ASB13, PRS6KA1, and ETS1 protein detected by immunoblot from BC cell lines transfected with pre-miR-125b and pre-control.
miR-125b directly inhibits ETS1 expression through the 3′-UTR

To determine whether ETS1 responded to miR-125b through direct 3′-UTR interaction, we cloned the 3′-UTR of ETS1 into a reporter plasmid downstream of the luciferase gene to generate pMIR-report-ETS1 3′-UTR and pMIR-report-vector. These plasmids, or miR-125b precursor or precursor control and pCMV-Renilla (internal control) were transiently transfected into HEK 293T cells. At 48 hours after transfection, a dual-luciferase reporter assay system was used to detect luciferase expression. Overexpression of miR-125b using a miRNA precursor resulted in a significant decrease in luciferase expression in pMIR-report-ETS1 3′-UTR-transfected cells, but not in pMIR-report-vector-transfected cells. In addition, transfection of pMIR-report-ETS1 3′-UTR resulted in a decrease in luciferase expression (precursor control) compared with cells transfected with the pMIR-report-vector under the same conditions, suggesting a negative effect of the endogenous miR-125b on the ETS1 3′-UTR (Fig. 5A). Taken together, these results indicated that ETS1 was a direct target of miR-125b.

Knockdown of ETS1 suppresses cell growth, proliferation, and induces G1 cell-cycle arrest in vitro

To address the function of ETS1 in breast cancer, MCF7 and MDA-MB-435 cells were transfected with ETS1-specific siRNAs. Immunoblot analysis indicated that when cells were transfected with an ETS1-specific siRNA (100 nmol/L), at 48 hours posttransfection the ETS1 protein was decreased significantly both in MCF7 and MDA-MB-435 cells (Fig. 5B). Knockdown of ETS1 led to inhibition of cell growth and proliferation both in MCF-7 and MDA-MB-435 cells (P < 0.05, Fig. 5C). A colony-forming assay was carried out to evaluate the effect of ETS1 on the clonogenicity ability of breast cancer cells. ETS1-specific siRNA-transfected MCF-7 and MDA-MB-435 cells displayed fewer and smaller colonies compared with control siRNA transfectants (Fig. 5C). Flow cytometry analysis revealed that the proportion of G1 phase cells increased to 75.7% ± 0.5% in MCF-7 cells transfected with ETS1-specific siRNA, compared with 60.5% ± 1.5% in control siRNA-transfected cells. The proportion of G1 phase cells increased to 66.4% ± 1.4% in MDA-MB-435 cells transfected with ETS1-specific siRNA, compared with 52.3% ± 3.2% in control siRNA-transfected cells (Fig. 5D).

Figure 5. ETS1 is a direct target of miR-125b and knockdown of ETS1 suppresses cell growth, proliferation, and induces G1 cell-cycle arrest. A, pMIR-Report vectors containing the 3′-UTR of wild-type ETS1 mRNA (ETS1) or no 3′-UTR (Vector), and miR-125b precursor or precursor control were cotransfected into HEK293T cells. The overexpression of miR-125b by the miR-125b precursor resulted in a significant decrease in luciferase signals from ETS1, but not vector-transfected cells. B, MCF-7 and MDA-MB-435 cell lines were transfected with ETS1-siRNA (100 nmol/L) or control siRNA (100 nmol/L). Cell lysates were generated at 48 hours posttransfection, followed by immunoblot to determine ETS1 expression, with GAPDH as the loading control. C, the functional role of ETS1 on breast cancer cell growth was analyzed by MTT and colony formation at very low cell density. D, cell-cycle distribution monitored by flow cytometry.
ETS1 is overexpressed in invasive breast cancer and correlated with poor prognosis of breast cancer patients

To investigate ETS1 protein expression in invasive breast cancer, IHC was carried out to detect ETS1 expression in invasive breast cancer tissues. ETS1 was found predominantly in the cytoplasm of breast cancer tumor cells (Fig. 6A). Patients were divided into high ETS1 expression (*n* = 118, score > 6) and low ETS1 (*n* = 103, score ≤ 6) expression. No significant correlation was observed between ETS1 and clinicopathological parameters (Supplementary Table S4). The 5-year overall survival rate of the 221 breast cancer patients was 80.5%. The 5-year overall survival rate of patients with high ETS1 expression (71.0%, *n* = 118) was significantly lower than those with low ETS1 expression (91.1%, *n* = 103; *P* < 0.001, Fig. 6B). When patients were stratified by clinical stage, Kaplan–Meier survival estimates revealed that early stage (I and II) patients with high ETS1 expression (78.6%, *n* = 94) had inferior survival to those with low ETS1 expression (93.0%, *n* = 87; *P* < 0.001, Fig. 6B). Similarly, late stage (III and IV) patients with high ETS1 expression (40.9%, *n* = 24) had inferior survival to those with low ETS1 expression (80.8%, *n* = 16; *P* < 0.001, Fig. 6B). In patients subjected to only surgery stratum and adjuvant therapy stratum, ETS1 levels exhibited a significant correlation with survival (Supplementary Fig. S5).
Univariate Cox proportional hazard regression analysis revealed that high ETS1 expression was a significant predictive factor for poor prognosis (HR = 3.69, P = 0.001). Multivariate Cox proportional hazard regression analysis revealed that high ETS1 expression (HR = 3.92, P < 0.001) was also a significantly unfavorable independent prognostic factor (Supplementary Table S5).

Discussion

Consistent with previous report of miR-125b in breast cancer (7), we showed that miR-125b was frequently downregulated in invasive breast cancer tissues, and downregulation of miR-125b was associated with poor prognosis of the patients. These results suggested that miR-125b downregulation may play an important role in the development and progression of invasive breast cancer. However, the underlying mechanism responsible for miR-125b dysregulation in invasive breast cancer is still unknown. Recent studies showed that miRNAs can be dysregulated by promoter methylation in human malignancies (27, 28). In this investigation, we computationally mapped a CpG-rich region in the promoter region of the miR-125b-1. Hypermethylation of miR-125b-1 promoter was found in breast cancer, and it was correlated with low levels of miR-125b expression. Among 4 cases of invasive breast cancer tissues without methylation in CpG-rich region 1 within upstream of miR-125b-1, the expression of miR-125b in 3 cases was still downregulated, compared with paired NATs, respectively. Taken together, these data revealed that DNA methylation of CpG-rich region 1 partially accounted for the reduction of miR-125b expression in invasive breast cancer, and meanwhile other unknown factors might be also involved in the downregulation of miR-125b in breast cancer.

The role of miR-125b in malignancies is controversial. miR-125b acts as a tumor suppressor in breast cancer and hepatocellular carcinoma (7, 9), but acts as an oncogene to promote proliferation in prostate cancer (13). Consistent with previous findings in breast cancer (13), our functional studies indicated that overexpression of an miR-125b precursor in MCF-7 and MDA-MB-435 breast cancer cell lines significantly suppressed proliferation and colony formation ability of the cells in vitro, and inhibited tumor growth in vivo of mice xenografts.

Dysregulation of the cell cycle is involved in cell proliferation and tumorigenesis. Growing numbers of miRNAs have been implicated in the regulation of the cell cycle, for instance, the miR-15a/16-1 cluster affects the G0/G1–S phase transition in B cells (29). miR-125b was reported to induce cell-cycle arrest at G1/S transition by suppressing CDK6 and CDC25A expression in CD133-positive U251 cells (30). In this study, overexpression of an miR-125b precursor increased G1 cell-cycle arrest in breast cancer cell lines, consistent with previous studies (30).

Identification of miR-125b target genes is critical for understanding its role in tumorigenesis, and is important for defining novel therapeutic targets. To date, ERBB2/ERBB3, Bak1, CYP24, NR2A, TNF-α, Bmf, Smo, and p53 have been identified as targets of miR-125b (13, 15, 16, 31–35). We report that ETS1 is a target gene of miR-125b. ETS1 is a member of the ETS family of transcription factors, located on the long arm of chromosome 11 (11q23–q24), which is involved in regulating a wide variety of biological processes, including cellular growth, migration, and differentiation (36–38). ETS1 is required for angiogenesis under both physiological and pathophysiological conditions, such as chronic inflammatory reactions and tumor-associated angiogenesis. High ETS1 expression is associated with invasion and metastasis, and predicts poorer prognosis of cancer patients (39, 40). This study revealed that ETS1 was overexpressed in invasive breast cancer, and overexpression of ETS1 predicted poor prognosis. Moreover, our data provide the first evidence that miR-125b decreases protein levels of ETS1 by targeting its 3′-UTR. Functional studies by knockdown of ETS1 phenocopied overexpression of miR-125b in breast cancer cells, resulting in G1 cell-cycle arrest, cell growth suppression, proliferation inhibition in vitro, and suppression of the tumorigenicity of mice xenografts, suggesting that miR-125b acted as tumor suppressor by regulating the ETS1 target gene in the development and progression of breast cancer. miR-125b overexpression or siRNA-mediated downregulation of the target gene ETS1 is a potential breast cancer therapy.

In conclusion, we report that miR-125b expression was downregulated in invasive breast cancer tissues and hypermethylation of miR-125b promoter partially accounted for the reduction of miR-125b expression. miR-125b is a tumor suppressor in the development and progression of invasive breast cancer, as it suppressed tumorigenesis, and induced G1 cell-cycle arrest through its target gene ETS1. Low expression of miR-125b and overexpression of its target gene ETS1 predicted poor survival of breast cancer patients. These data suggest that miR-125b plays an important role in breast cancer carcinogenesis, and may have prognostic or therapeutic implications for the clinical management of breast cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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