Int J Clin Exp Pathol 2014;7(11):7563-7570
www.ijcep.com /ISSN:1936-2625/IJCEP0002411

Original Article

Tumor suppressive microRNA-193b promotes breast cancer progression via targeting DNAJC13 and RAB22A

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Received September 9, 2014; Accepted October 31, 2014; Epub October 15, 2014; Published November 1, 2014

Abstract: Breast cancer is still a leading cause of cancer deaths in women. Despite improvements in therapeutic approaches in local control, metastatic relapse is almost always incurable, underlining the importance to better understand the biological bases that contribute to disease progression. In this study, we demonstrated that miR-193b was significantly down-regulated in two primary human breast cancer cell lines (MDA-MB-231 and MCF-7). Reconstitution of miR-193b expression resulted in decreasing cell proliferation, clonogenicity, migration and invasion. By using in silico prediction algorithms approach for target identification, we identified DNAJC13 (HPS40) and RAB22A to be direct targets of miR-193b. Concordantly, Re-expression of miR-193b decreased DNAJC13 (HPS40) and RAB22A expression. Luciferase reporter assays confirmed the direct interaction of miR-193b with both DNAJC13 (HPS40) and RAB22A. Our findings have demonstrated that miR-193b as a novel tumor suppressor plays an important role in breast cancer progression, understanding the mechanisms could account for the aggressive behaviour of breast cancer.

Keywords: Breast cancer, microRNA, target identification

Introduction

Breast cancer is the second leading cause of cancer deaths in women worldwide, with ~1.7 million (11.9%) new cases diagnosed and ~522 000 deaths in 2012. Since 2008, breast cancer incidence has increased by more than 20% and mortality has increased by 14% [1]. With breast cancer screening and therapeutic advances, approximately 80% of patients with a localized disease will experience a prolonged survival. However, early detection of metastatic relapse has not demonstrated evidence of survival benefit, the development of metastatic relapse is almost always incurable [2], underlining the importance to better understand the mechanisms that contribute to disease progression and developing novel therapeutic strategies.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs, which play an essential role in the negatively regulation of gene expression through translational repression or degradation of mRNAs targets [3]. miRNAs have been described to regulate a number of biological processes, including development, differentiation, apoptosis and cancer [4]. MicroRNAs can function either as oncogenes or tumor suppressors and have unique ability to regulate many protein-coding genes [5]. A single miRNA can potentially regulate several hundreds or thousands of genes [6]. In recent years, the role of miRNAs in human cancer has been thoroughly investigated, and many human cancers have aberrant expression of miRNAs. Extensive evidence suggests that some miRNAs may act as selective tools for high-risk patients or miRNAs themselves can be considered as therapeutic targets [4]. Dysregulation of miRNAs in breast cancer has been well documented. miR-10b is the first descripted breast cancer-related miRNAs, which highly expresses in metastatic breast cancer cells and positively regulates breast cancer migration and invasion [7]. MiR-155 and miR-21 have been shown to induce

chemoresistance through their regulation of key resistance-associated proteins [8]. MiR-221/222 has been shown to be key regulators of antiendocrine resistance in vitro through the targeting of cell-cycle inhibitor p27-Kip1 and Era [9]. Dysregulation of miR-193b has been previously reported in lymphoma [10], head and neck squamous cell carcinomas [11], non-small cell lung cancer [12] and prostate cancer [13], however, understanding the interaction between miR-193b and their targets leading to the disease remains largely unknown. Herein, we reported down-regulation of miR-193b in primary human breast cancer cell lines and identified DNAJC13 (HPS40) and RAB22A as novel, direct targets of miR-193b, which in turn, regulated RAS oncogene pathway, highlighting the biological importance of miR-193b in breast cancer progression.

Materials and methods

Cell culture

Human normal mammary gland epithelial cells MCF-10A were cultured in DMEM/HAM F12 supplemented with 5% horse serum, insulin, hEGF, hydrocortisone (Clonetics), and cholera toxin (Sigma). Primary human breast cancer cell lines MCF-7 and MDA-MB-231 were both grown in a-MEM supplemented with 10% fetal bovine serum. All cell lines were maintained at 37°C and 5% CO_2.

Cell transfections

LipofectAMINE 2000 (Invitrogen) was used to transfect the tumor cells with either negative-scramble (SC) control or mimic pre-miR-193b (Ambion) at a final concentration of 40 nmol/L by using the reverse transfection protocol, according to the manufacturer's instructions.

Quantitative real-time PCR for miRNAs and mRNAs expression

Total RNA was isolated using RecoverAll Total Nucleic Acid Isolation kit (Ambion). For mRNAs, the Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen Corp.) according to the manufacturer's recommendations. QRT-PCR analyses were performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA). The primer sequences used in this study were as follow: DNAJC13 (HPS40)-Forward primer: 5'-aagaaaggagtggcc-cgtat-3'; Reverse primer: 5'-agcctgtccactggcta-aga-3' and RAB22A-Forward primer: 5'-ttgta-gttgccattgcagga-3'; Reverse primer: 5'-aggct-gtcttcggagtttga-3'. The relative fold change in RNA expression was calculated using the 2-ΔΔCt method, where the average of ΔCt values for the amplicon of interest was normalized to that of an endogenous gene (GAPDH), compared with control specimens. The expression of miRNA was conducted using Taqman MicroRNA Assay (Applied Biosystems). First, RNAs were reverse-transcribed with a MultiScribe reverse transcriptase by using a stem-loop RT primer specifically hybridises with a miRNA Molecule. Then, The RT products were subsequently amplified with sequence-specific primers using the Applied Biosystems 7900 HT Real-Time PCR system. RNU44 was used as an endogenous control.

Cell proliferation and clonogenic assay

The soluble tetrazolium dye (MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to assess cell proliferation and cytotoxicity of introducing mimic pre-miR-193b in MDA-MB-231 cells. Cells were reverse transfected with SC control, pre-miR-193b or Lipofectamine 2000 and seeded onto 96-well plates (5×10^3 cells per well). Cell viability was measured at 24, 48 and 72 hours after transfection.

A clonogenic assay was used to evaluate the effects of pre-miR-193b on MDA-MB-231 cells. Briefly, transfected cells were seeded into 12-well plates. 48 hours later, cells were harvested, and 500 cells per milliliter were reseeded into 6-well plates in triplicate. After 14 days' incubation, the plates were fixed and stained, and the number of colonies was then counted. The fraction of surviving cells was calculated by comparing with cells treated with SC.

Cell migration and invasion assays

Cell migration and invasion were assessed using BD BioCoat Matrigel Invasion Chambers and Control Inserts (BD Biosciences). The cells were transfected with either pre-miR-193b or SC (40 nM), then seeded on either control

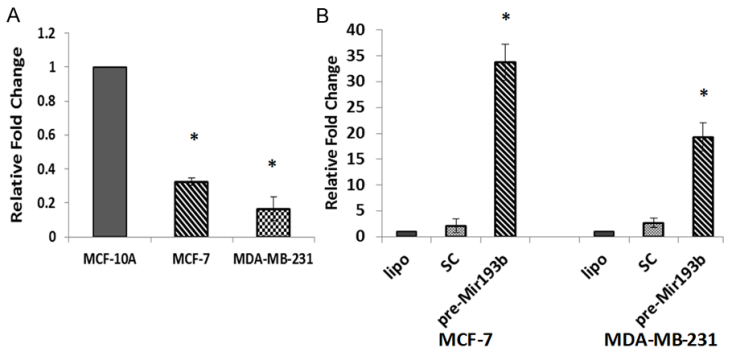

Figure 1. MiR-193b expression in breast cancer cell lines. A. Real-time RT-PCR showed that expression level of miR-193b was significantly low in two breast cancer cell lines-MCF-7 and MDA-MB-231, comparing with normal mammary cell line. B. Mimic pre-miR-193b caused significant increased expression in both cell lines (*$P < 0.01$).

inserts (polyethylene terephthalate membrane) or trans-well chambers with Matrigel. Two ml RPMI supplemented with 15% FBS was added to the lower chamber, served as the chemoattractant. 1×10^5 transfected cells were re-suspended in RPMI plus 1% FBS, added to the upper chamber (0.5 ml). Twenty hours later, migrating or invading cells attached to the lower surface of the membrane insert were fixed and stained, then counted under a microscope. Relative migration was calculated by comparison with cells transfected with the negative control. The percentage invasion was calculated based on the number of cells which have invaded through the Matrigel insert, divided by the number of cells which have migrated through the control insert.

Luciferase reporter assay

Because of the downstream target genes have putative miR-193b binding sites in their 3'-UTR regions, a region of DNAJC13 (HPS40) and RAB22A genes were amplified by PCR and constructed in a pMIRREPORT luciferase vector (Ambion) as a downstream of the firefly *luciferase* gene. A mutant sequence was also cloned as a validation plasmid. Either pMirluciferase or pMir-luciferase_gene specific vectors were co-transfected with pre-miR-193b or SC in MDA-MB-231cell lines. pRL-SV vector (Promega) containing Renilla luciferase was also transfected with each condition as a reference control. At 48 hours post-transfection, luciferase activity of Firefly and Renilla luciferase activities were then measured using the Dual-Luciferase Reporter Assay (Promega).

Statistical analysis

All data was expressed as the mean ± SE; a *P*-value of less than 0.05 was considered to be statistically significant. Statistical Analysis and graphs were completed using Microsoft excel and Graphpad Prism Software (Graphpad Software, Inc).

Results

Expression of miR-193b in breast cancer cell lines

The expression of miR-193b was evaluated in human breast cancer cell lines: MCF-7 and MDA-MB-231, compared to that of the normal mammary epithelial cell line MCF-10A. miR-193b was significantly down-regulated in two cell lines, as shown in **Figure 1A**, 70% of reduction in MCF-7 cells and 82% of reduction in MDA-MB-231 cells, consistent with a previously published protein microarray identification of microRNAs study [14].

Overexpression of miR-193b caused significantly reduction of cell viability and decreasing of clonogenicity

In order to assess the biological significance of miR-196b down-regulation, mimic enable miRNA functional analysis was performed. Cancer cells were transfected with 40 nmol/L scramble controls (SC) or mimic pre-miR-193b. Sustained up-regulation of miR-193b expression was observed at 48 hours after transfection (**Figure 1B**) with either more than 30 folds increasing in MCF-7 cells or 18 folds increasing in MDA-MB-231 cells. Transfection with pre-miR-193b into MDA-MB-231 cells led to significantly decreased cell viability compared to controls at 48 (65%) and 72 (50%) hours post-transfection (**Figure 2A**). miR-196b over expression also resulted in significant reductions in clonogenicity in MDA-MB-231 cells; survival fraction was 22 compared to 45 of SC (**Figure 2B**, **2C**).

Mir-193b regulated cell migration and invasion

To determine if miR-193b was involved in regulating cell migration or invasion, in vitro transwell migration and invasive assays were used.

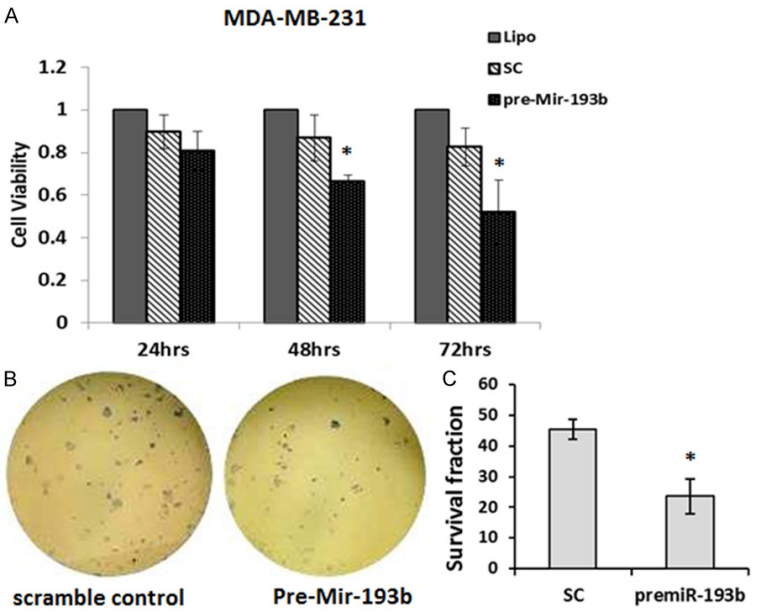

Figure 2. MiR-193b target validations by luciferase assays. A. Construction of pmiR-report-DNAJC13 3' and -RAB22A 3' UTR vectors. B. Relative lucifierase activity after co-transfection with pmiR-DNAJC13 3' UTR or pmiR-DNAJC13 Mutant vectors. C. Relative luciferase activity after co-transfection with pmiR-RAB22A 3' UTR or pmiR-RAB22A Mutant vectors. *$P < 0.05$. Both experiments were performed on MDA-MB-231 cells twice.

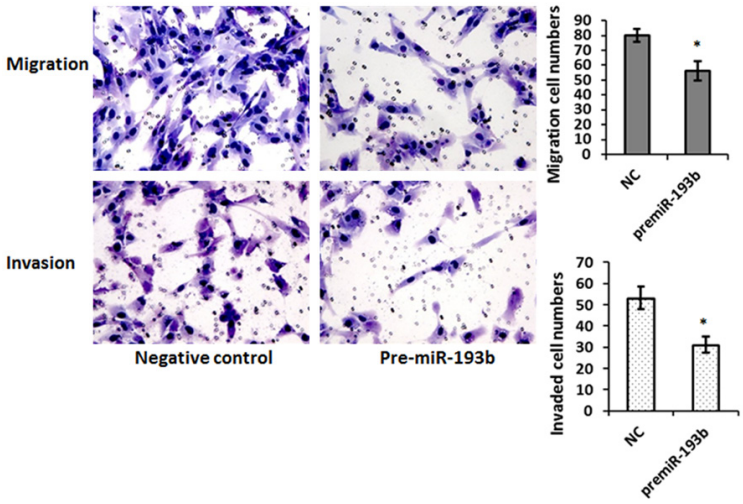

Figure 3. Up-regulation of miR-193b reduced cell migration and invasive. Up---Representative images of migration assay indicated that the reduced ability of MDA-MB-231 cells to migrate after transfection with pre-miR-193b compared to negative scrambled (40 nM). Bottom--Representative images of invasive assay indicated that the reduced ability of MDA-MB-231 cells to invade after transfection with the pre-miR-193b compared to negative scrambled (40 nM).

Compared with their corresponding negative controls, transfection with pre-miR-193b significantly reduced migration of MDA-MB-231 cells by 72%. Moreover, pre-miR-193b transfection resulted in reduction in invasion of MDA-MB-231 cells, as shown in **Figure 3**.

Mir-193b potential mRNA targets identification

To characterize the mechanism by which miR-193b regulation in breast cancer, we used 6 miRNA target-prediction software --miRanda, miRDB, miRWalk, PICTAR5, RNA-22 and TargetScan to predict potential miR-193b targets. Three top mRNAs were selected to further validation (**Figure 4A**). DNAJC13 (Hsp40 or RME-8) is a DNA Domain-Containing Protein, a critical regulator of Hsp70; ERBB4 (Viral Oncogene Homolog-Like 4) relate to cell mitogenesis and differentiation; RAB22A (member RAS oncogene family) plays a role in endocytosis and intracellular protein transport, as shown in **Figure 4A**.

Initially, we assessed the basal levels of DNAJC13, ERBB4 and RAB22A expression by using qRT-PCR methods in two breast cancer cell lines (**Figure 4B**). DNAJC13 and RAB22A were significant up-regulated in both MDA-MB-231 and MCF-7 cells, compared with normal control (NC). However, ERBB4 was only up-regulated in MDA-MB-231 cells but was not shown the consistence in MCF-7 cells, showing down-regulation compared to normal. After transfected pre-miR-193b (40 nM) to those breast cancer cells, DNAJC13 and RAB22A expression levels were reduced in both cell lines, comparing to normal (**Figure 4C**).

Next, to determine whether miR-193b regulates DNAJC13 and RAB22A by binding to its 3' UTR, we constructed several reporter vectors carrying the predicted binding site(s) down-

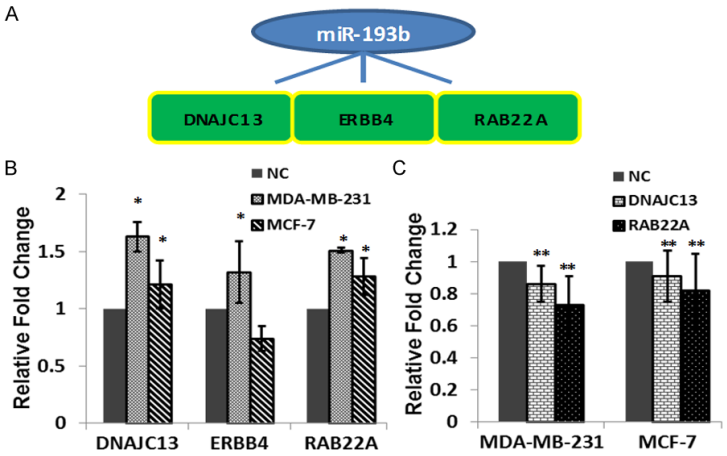

Figure 4. MiR-193b targets identication. A. MiRDB and Targets can identify 3 up-regulated genes as potential targets for MiR-193b. B. Bastal mRNA expression of DNAJC13, ERBB4 and RAB22A was assessed using qRT-PCR in two breast cancer cell lines. *P < 0.01. C. qRT-PCR showed down regulation of DNAJC13 and RAB22A after transfection of pre-miR193b in both MCF-7 and MDA-MB-231 cells, respectively, **P < 0.05.

stream of a firefly *luciferase* gene in the pMIR-Report vector as shown in **Figure 5A**. For the luciferase assay, MDA-MB-231 cells were co-transfected with pre-miR-193b (or a scramble control) and pmiR-DNAJC13-3' UTR (or a mutated pmiR-DNAJC13-3' UTR). The luciferase reporter that contained the DNAJC13 3' UTR was significantly suppressed by pre-miR-193b, whereas the mutated reporter was not affected (**Figure 5B**). Similar results were obtained when we used pmiR-RAB22A-3' UTR (or a mutated pmiR-RAB22A) (**Figure 5C**) on the cells.

Discussion

It is becoming increasing evidence that miRNAs play important roles in mediating tumor development and progression, through the regulation of their respective target genes, leading to overexpression of tumor-promoting genes, or down-regulation of tumor-suppressing genes, thereby driving tumor progression [5, 15, 16]. In this study, we observed that miR-193b was significantly down-regulated in breast cancer cell lines, which promoted tumour cell proliferation, migration and invasion. In order to identify potential mechanisms of miRNA regulation, two potential miR-193b transcriptional downstream target genes were identified. DNAJC13 and RAB22A were involved in the complexity mechanism of breast cancer development, metastasis and progression.

MiR-193b locates on chromosome 16p11.13, a region not previously described to be amplified or lost in breast cancer. MiR-193b deregulation is a common event in both benign and malignant human breast tumors [17]. miR-193b was first described as a potent ERalpha-regulating miRNA and most closely associated with the repression of known estrogen-induced genes [18]. This led to the identification of multiple genes which were targeted by miR-193b, suppressing of the local production of estrogens and other steroid hormones [19]. miR-193b expression has been shown significantly lower in ERα-negative than in ERα-positive tumors [20]. miR-193b is also a negative regulator of the uPA gene, which associate with clinical metastasis in primary breast tumors [21].

However, the role of miR-193b in human malignancies appears to be controversial. MiR-193b was reported increasing in serum of ppancreatic neuroendocrine neoplasms [22]. Over expression of miR-193b has been described previously in HNSCC via down regulation of neurofibromin 1 to enhance tumor progression [11]. These are not corroborated based on our data (**Figure 1A**). As many miRNAs, it is highlight the fact that miR-193b can function as either an oncogenic or a tumor suppressor genes. Besides the ERa related genes and uPA, DNAJC13 (Hsp40) and RAB22A have been identified as novel mRNA targets of miR-193b in our study. DNAJC13 (Hsp40), a homologue of bacterial DNAJ heat shock protein, is a family member of powerful chaperones. There are over 41 members in their family and they reside at distinct intracellular locations [23]. Despite their physiologic roles is not quite clear, recent study has revealed involvement of some of the DNAJ family members in various types of cancers. DNAJC13 depletion decreases EGFR levels in breast cancer cell lines, influencing EGFR at the level of endosomes, could be a potential regulatory target, particularly in ErbB2-positive breast cancers [24]. However, the mechanism by which DNAJC13 may function in endosomal trafficking and EGFR degradation is not current-

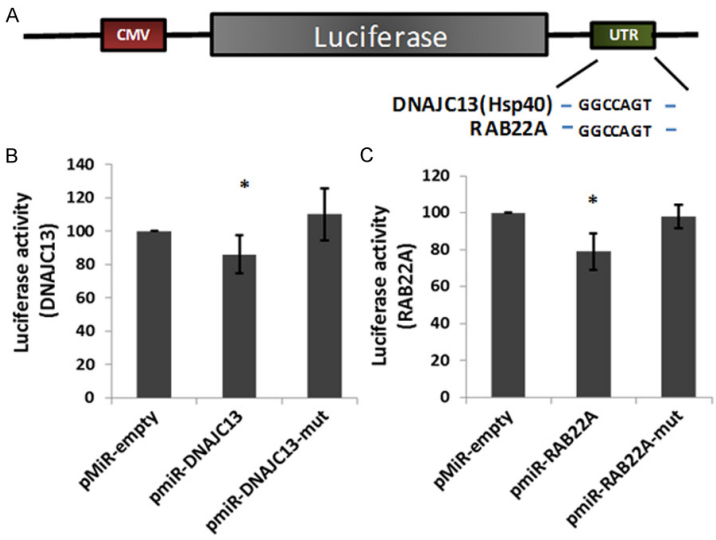

Figure 5. MiR-193b target validations by luciferase assays. A. Construction of pmiR-report-DNAJC13 3' UTR and -RAB22A 3' UTR vectors. B. Relative luciferase activity after co-transfection with pmiR-DNAJC13 3' UTR or pmiR-DNAJC13 Mutant vectors. C. Relative luciferase activity after co-transfection with pmiR-RAB22A 3' UTR or pmiR-RAB22A Mutant vectors. *$P < 0.05$. Both experiments were performed on MDA-MB-231 cells twice.

ly understood. Fujibayashi et al. indicated that DNAJC13 may regulate membrane trafficking through early endosomes [25]. In this study, as an alternate mechanism for DNAJC13, we provide evidence for deregulation of DNAJC13, via miR-193b, in breast cancer.

RAB22A, a member of RAS oncogene family, is amplified or overexpressed in some tumors. Integration of gene-expression with DNA copy-number data reveals that RAB22A role as an oncogene, with high-level amplifications [26]. In hepatocellular carcinoma, it has been shown up-regulative properties [27]. By using the human HG-U133A GeneChip, RAB22A has shown positive expression at the regions of chromosomal breakpoints in malignant melanoma [28]. Moreover, RAB22A has been shown to be associated with early and late endosomes and co-localizes with autophagic vacuoles [29]. Other mechanisms leading to RAB22A overactivation in tumor cells include the interacts with EEA1 and controls endosomal membrane trafficking [26]. Our current study demonstrated that RAB22A was a downstream transcriptional target of miR-193b in breast cancer as shown in **Figure 4B** and **Figure 5C**. We observed an inverse relationship between RAB22A and miR-193b expression, whereby suppression of miR-193b led to an increase in RAB22A in breast cancer cells, which involved in the selective endosomal sorting of molecules like CD44 and CD98, which infect cell-cell and cell-extracellular interactions to propagate downstream of RAS signalling [30].

In conclusion, our experiments have shown that miR-193b is a novel tumor suppressor in breast cancer through regulation of at least the transcription factor DNAJC13 and the expression of oncogene RAB22A. The resulting phenotype of miR-193b down-regulation includes increased cell proliferation, clonogenicity, migration and invasion. These findings suggest other mechanisms such as, miR-193b~DNAJC13 or miR-193b~RAB22A axis, which could account for the aggressive behaviour of breast cancer.

Acknowledgements

This study was supported in part by China-Japan Hospital Research Foundation, Jilin University PhD Student Foundation.

Disclosure of conflict of interest

None.

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