MicroRNA-224 targets RKIP to control cell invasion and expression of metastasis genes in human breast cancer cells

Lin Huang, Ting Dai, Xi Lin, Xiaohui Zhao, Xiuting Chen, Chanjuan Wang, Xinghua Li, Huiyong Shen, Xi Wang

State Key Laboratory of Oncology in Southern China, Department of Breast Surgery, Cancer Center, Sun Yat-sen University, Guangzhou, Guangdong 510060, China
Department of Orthopedics, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510120, China
Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, China
State Key Laboratory of Oncology in Southern China, Department of Ultrasound, Cancer Center of Sun Yat-sen University, Guangzhou, Guangdong 510060, China

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The Raf kinase inhibitor protein (RKIP) is a tumor suppressor that protects against metastasis and genomic instability. RKIP is downregulated in many types of tumors, although the mechanism for this remains unknown. MicroRNAs silence target genes via translational inhibition or target mRNA degradation, and are thus important regulators of gene expression. In the current study, we found that miR-224 expression is significantly upregulated in breast cancer cell lines, and especially in highly invasive MDA-MB-231 cells, compared to normal breast epithelial cells. In addition, miR-224 inhibits RKIP gene expression by directly targeting its 3’-untranslated region (3’-UTR). Moreover, metastasis, as assayed by Transwell migration, 3D growth in Matrigel, and wound healing, was enhanced by ectopic expression of miR-224 and inhibited by miR-224 downregulation. Promotion of metastasis in response to miR-224 downregulation was associated with derepression of the stroma-associated RKIP target genes, CXCR4, MMP1, and OPN, which are involved in breast tumor metastasis to the bone. Taken together, our data indicate that miR-224 play an important role in metastasis of human breast cancer cells to the bone by directly suppressing the RKIP tumor suppressor.

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1. Introduction

Breast cancer is the most common female malignancy, accounting for approximately 22% of all new cancer cases worldwide, with more than 1.05 million new cases every year. Globally, 0.45 million patients died from breast cancer annually, accounting for 13.7% of female cancer deaths [1,2]. In the developing world, including China, breast cancer incidence has been rising in recent years [3]. Breast cancer is prone to metastasis and secondary sites include the lung, liver, bone, and brain. Metastasis can occur many years after the removal of the primary tumor, reducing the survival rate from 85% for early detection to 23% for patients with lung or bone metastasis, and is therefore the main cause of death for breast cancer patients [4]. Currently, the mechanisms controlling metastasis are poorly understood and treatments for metastatic late-stage breast cancer are inefficient and mainly palliative [5]. Hence, it is of great clinical value to understand the molecular mechanisms involved in primary tumor cell invasion and spread to distant sites, such as bone, and thus identify molecular targets for new therapies.

Several regulatory pathways, such as MAPK (MAP kinase) and Akt/protein kinase B (PKB), are important regulators of cell invasion and metastasis in malignant tumors [6,7]. Thus, inhibition of one or more key components of these signaling pathways may provide new therapeutic breakthroughs in cancer treatment. One therapeutic strategy for metastatic tumors is to target metastasis suppressor genes. Raf kinase inhibitory protein (RKIP, also named PEBPI) a metastasis tumor suppressor that regulates MAPK and NFκB signaling pathways [8–10]. Accumulated evidence has demonstrated that RKIP-mediated tumor suppression specifically affects metastatic but not primary tumors in prostate and breast tumor xenografts [11,12]. Moreover, RKIP expression is significantly reduced or absent in a variety of cancer cells, including breast, esophageal, gastric, and prostate cancer [11–14]. Taken together, these studies highlight RKIP as a general suppressor of metastasis.

Metastasis is a complex process involving a series of steps: cellular epithelial–mesenchymal transition leading to basement...
invasion, intravasation into blood or lymph vessels, extravasation from vessels, mesenchymal–epithelial transition, and metastatic colonization of distal tissues [15]. RKIP repression of genes implicated in the development of bone metastasis, Chemokine (C-X-C motif) receptor 4 (CXCR4), the matrix metalloproteinase1 (MMP1), the integrin-binding glycoprotein osteopontin (OPN), is reported to be mediated by MAPK inhibition and enhanced transcription of the mRNA let-7 [12,16–19]. MMP1 has important functions in facilitating colonization in bone via expression of bone extracellular matrix degrading enzymes, OPN-mediated osteoclast activation and adhesion to the bone surface, and CXCR4 targets specific homing of tumor cells to the bone microenvironment [20]. In conclusion, CXCR4, MMP1, and OPN are important downstream targets of the RKIP pathway that inhibits bone metastasis, and their expression forms the bone metastasis signature. MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that inhibit gene expression at the post-transcriptional level, and their expression forms the bone metastasis signature. MicroRNAs (miRNAs) are involved in regulating various processes, including the proliferation, invasion, metastasis, and prognosis of cancers such as breast, lung, and prostate cancer, and glioma [23–25]. Breast cancer progression requires altered expression of multiple oncopgenes and tumor suppressors, and it is likely that miRNAs play a pivotal role in the regulation of these genes [26,27]. miRNAs are now known to be linked to breast cancer metastasis: some have been identified as metastasis promoters (Promega, Madison, WI, USA) and pGFP-C3 (Clontech, Mountain View, CA, USA) plasmids. The primers used were: RKIP-3′-UTR-wt-

2. Materials and methods

2.1. Cell culture

Normal breast epithelial cells (NBEC) were obtained from Clonetics-Biowhittaker (Walkersville, MD, USA) and cultured in KSFM medium (Clonetics-Biowhittaker). Breast cancer cell lines MCF-10A, MDA-MB-468, T47D, MCF-7, MDA-MB-453, MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in D-Medium (Clonetics-Biowhittaker). Breast cancer cell lines MCF-10A, MDA-MB-468, T47D, MCF-7, MDA-MB-453, MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in D-Medium (Clonetics-Biowhittaker) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen) at 37°C/C176°C/C176 barometric atmosphere containing 5% CO2.

2.2. Plasmids and transfection

The RKIP 3′-UTR was PCR amplified from NBEC RNA and cloned into the SacI/XmnI sites of the pGL3-control luciferase reporter (Promega, Madison, WI, USA) and pGFP-C3 (Clontech, Mountain View, CA, USA) plasmids. The primers used were: RKIP-3′UTR-wt-

2.3. Western blotting

Western blot analysis was performed according to standard methods using anti-RKIP, anti-MMP1, anti-OPN, anti-CXCR4, and anti-green fluorescent protein (GFP) antibodies (Cell Signaling, Danvers, MA, USA). Membranes were stripped and reprobed with an anti-α-tubulin monoclonal antibody (Sigma, St. Louis, MO, USA) to control for loading.

2.4. RNA extraction and real-time quantitative PCR

Total miRNA was purified from cultured cells and using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions and cDNA was synthesized from 5 ng of total RNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). mi-R224 expression was quantified using a miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems). miRNA expression levels were defined based on the threshold cycle (Ct), and relative expression levels were calculated as 2−[(Ct of miR-224)−(Ct of U6)] and normalised to U6 small nuclear RNA using the formula 2−[(Ct of miR-224)−(Ct of U6)].

Real-time reverse transcription PCR (RT-PCR) was performed using the Applied Biosystems 7500 Sequence Detection system using the following primers: MMP1, (forward, 5′-TGTGGACC ATGCATTAGAAG-3′; reverse, 5′-TCTGCTTGACCCATCAGACC-3′); OPN, (forward, 5′-ACGGAGACCATGCAGAGAC-3′; reverse, 5′-TCTGCTTGACCCTCAGAGCC-3′); CXCR4, (forward, 5′-CACAGTGATGCAAGTG-3′; reverse, 5′-CAGGTTCCATGATGAG-3′); and CXCR4 (forward, 5′-CACAGTGATGCAAGTG-3′; reverse, 5′-CAGGTTCCATGATGAG-3′). Expression data were normalized to the geometric mean expression of the housekeeping gene, GAPDH (forward, 5′-GACTCATGACCA CAGTCCATGC-3′; reverse, 5′-AGAAGCTGGAGGATGCTTGCTG-3′) and calculated using 2−[(Ct of MMP1, OPN, or CXCR4)−(Ct of GAPDH)], where G, represents the threshold cycle for each transcript.

2.5. Three-dimensional spheroid invasion assay

Cells (1 × 104) were trypsinized and seeded in 24-well plates coated with Matrigel (2%; BD Biosciences), and medium was changed every second day. Microscope images were taken at 2-days intervals for 8 days.

2.6. Transwell matrix penetration assay

Cells (1 × 104) were plated into the upper chamber of the Bio-Coat Invasion Chambers (BD, Bedford, MA) containing a polycarbonate Transwell filter coated with Matrigel and incubated at 37°C/C176°C/C176 for 22 h, followed by removal of cells remaining inside the upper chamber using cotton swabs. Cells that had invaded the lower membrane surface were fixed in 1% paraformaldehyde, stained with hematoxylin, and counted (ten random fields per well at 100 x magnification). Cell counts are expressed as the mean number of cells per field of view. Three independent experiments were performed and the data are presented as mean ± standard deviation (SD).
2.7. Statistical analysis

A two-tailed Student’s t-test was used to evaluate the significance of the differences between two groups; p values <0.05 were considered significant.

3. Results

3.1. MiR-224 is upregulated in highly invasive MDA-MB-231 breast cancer cells

Real-time RT-PCR analysis revealed that miR-224 expression was markedly increased in highly invasive MDA-MB-231 cell lines, and increased to a lesser extent in other breast cancer cells, compared to NBEC (Fig. 1A). This data indicates that miR-224 is strongly upregulated in highly invasive breast cancer cells.

3.2. MiR-224 expression levels correlate with invasiveness in breast cancer cells

To investigate the function of miR-224 in breast cancer, we transfected the hsa-miR-224 mimic into MDA-MB-231 and MCF-7 breast cancer cells and measured the effect on cell invasion. MCF-7 and MDA-MB-231 are breast cancer cell lines: MCF-7 cells were isolated from an in situ breast adenocarcinoma and MDA-MB-231 cells were isolated from a distant metastatic site. MiR-224 overexpression in MDA-MB-231 and MCF-7 cells following transfection was shown by real-time RT-PCR (Fig. 1B). The invasive capacity of transfected cells was then examined using the Transwell–Matrigel penetration assay, which showed that transfection with miR-224 in MDA-MB-231 and MCF-7 cells increased the number of cells that penetrated the gel–membrane barrier (Fig. 1C; p < 0.01). Furthermore, in a three-dimensional (3D) spheroid invasion assay, miR-224-transfected cells had more outward projections compared with control cells (Fig. 1D), thus displaying an altered morphology typical of highly invasive cells. Moreover, cell migration measured using the wound healing assay was increased in MDA-MB-231 and MCF-7 cells transfected with miR-224 mimic than in negative controls (Fig. 1E). These results demonstrate that miR-224 upregulation promotes invasion in breast cancer cells.

We next transfected a miR-224 inhibitor into highly invasive MDA-MB-231 breast cancer cells, which led to significant repression of miR-224 (Fig. 2A; p < 0.01). Consistent with our previous findings, the Transwell–Matrigel penetration assay indicated that fewer MDA-MB-231 cells transfected with miR-224 inhibitor than transfected negative controls could pass through the gel–membrane barrier (Fig. 2B, p < 0.01). Furthermore, the 3D culture in Matrigel and the wound healing assays showed that the invasive ability of MDA-MB-231 cells was significantly decreased following transfection with the miR-224 inhibitor (Fig. 2C and D). Taken together, our data suggest that miR-224 controls the invasive ability of MDA-MB-231 breast cancer cells.

3.3. MiR-224 directly targets the RKIP 3’-UTR in breast cancer cells

A previous study revealed that RKIP transcriptionally represses a series of genes relevant to bone metastasis, including CXCR4, MMP1, and OPN [12,16–19]. Interestingly, our bioinformatic

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Fig. 1. MiR-224 upregulation promotes breast cancer cell metastasis. (A) Real-time RT-PCR analysis of miR-224 expression in the highly invasive breast cancer cell line MDA-MB-231 and other breast cell lines, including NBEC, MCF-10A, MCF-7, MDA-MB-453, T47D, MDA-MB-468. (B) Real-time RT-PCR analysis of miR-224 expression in indicated cells. (C) Transwell assays indicate that invasiveness of miR-224-transfected cells is increased compared to negative control (NC)-transfected cells. Cells that penetrated the gel–membrane barrier are counted. (D) 3D growth capacity is increased in miR-224-overexpressing MDA-MB-231 and MCF-7 cells. (E) Wound healing is increased in MDA-MB-231 and MCF-7 cells transfected with miR-224 mimic compared to negative controls (NC). Bars represent the mean ± SD of three independent experiments; *p < 0.05.
analysis indicated that miR-224 may directly target RKIP 3′-UTR (Fig. 3A). RKIP is downregulated in the highly invasive breast cancer cell line MDA-MB-231 (data not shown), in which miR-224 is upregulated (Fig. 1A). We observed that ectopic miR-224 expression decreased RKIP protein levels in MDA-MB-231 cells (Fig. 3B and C), supporting our identification of RKIP as a potential miR-224 target gene. In addition, miR-224-dependent RKIP downregulation was associated with significant increases in MMP1, OPN, and CXCR4 expression (Fig. 3B).

To confirm that miR-224 inhibition of RKIP is mediated by binding to the 3′-UTR, we cloned the RKIP 3′-UTR into both GFP (pEGFP-C3) and luciferase (pGL3) reporter plasmids, and investigated the effect of miR-224 and miR-224 inhibitor on reporter activity. GFP fluorescence was dramatically reduced by ectopic expression of miR-224 in MDA-MB-231 cells transfected with the GFP reporter, compared to those transfected with a control plasmid, suggesting that miR-224 specifically targets the RKIP 3′-UTR (Fig. 3C). Furthermore, miR-224 transfection reduced the luciferase activity of the RKIP 3′-UTR luciferase reporter plasmid in MDA-MB-231 breast cancer cells in a dose-dependent manner (Fig. 3D). MiR-224 repression of the RKIP 3′-UTR was abrogated by point mutations in the miR-224 seed region of the RKIP 3′-UTR (Fig. 3D). Moreover,
transfection of a miR-224 inhibitor increased basal activity of the pGL3-RKIP-3’-UTR reporter in MDA-MB-231 cancer cells in a dose-dependent manner (Fig. 3E). These results confirm RKIP to be a miR-224 target.

Importantly, RKIP overexpression and miR-224 inhibition could both reduce mRNA levels of the RKIP targets CXCR4, MMP1, and OPN (Supplemental Fig. S1A and B).

3.4. MiR-224-induced breast cancer cell metastasis is modulated by RKIP

To investigate the role of RKIP in miR-224-induced cell invasion, RKIP cDNA (lacking the 3’-UTR), RKIP-3’-UTR (including the 3’-UTR) expression plasmids, and control vector were transfected into MDA-MB-231 cells. Expression of MMP1, OPN, and CXCR4 mRNA was reduced more greatly in cells cotransfected with miR-224 and RKIP, compared to cells transfected with miR-224 and RKIP-3’-UTR (Fig. 4A). Furthermore, MDA-MB-231 cell invasion, assayed by Transwell migration, 3D Matrigel culture, and wound healing, was significantly inhibited following cotransfection of miR-224 and RKIP compared to cells cotransfected with miR-224 and RKIP-3’-UTR or with miR-224 and vector (Fig. 4B–D). In conclusion, our data suggest that miR-224-induced breast cancer cell invasion is directly mediated by RKIP suppression.

4. Discussion

In the past decade, the importance of tumor–stroma interactions for the progression of primary tumors to an aggressive and
invasive phenotype has been recognized. Through an interdiscipli-
inary approach, RKIP was identified as an inhibitor of invasion
and metastasis through the regulation of stroma-associated genes
[13]. RKIP binds to Raf-1 and inhibits Raf-1-mediated MEK
phosphorylation [34–36] and can regulate various cellular processes,
in particular metastasis [36]. In breast cancer, RKIP suppresses
metastasis to the bone by regulating CXCR4, MMP1, and OPN
[13]. In the current study, we identified the RKIP tumor suppressor
gene as a putative miR-224 target using bioinformatic analysis,
and confirmed that miR-224 can target the RKIP 3′-UTR in breast
cancer cells. miRNAs are important regulators of many biological
processes [37,38] and miR-224 expression is frequently upregu-
lated in various human tumor types, including colorectal cancer,
hepatocellular cancer (HCC), and renal cancer [39–41]. In addition,
miR-224 is significantly overexpressed in highly invasive and met-
astatic pancreatic ductal adenocarcinoma (PDAC) and CD40 protein
levels are significantly reduced in these tumors when miR-224 is
upregulated, suggesting that CD40 is a target of miR-224 [15].
Moreover, miR-224 expression in clear cell renal cell carcinoma
is increased more than fourfold compared to control samples
[40]. Furthermore, miR-224 is one of the most highly differentially
expressed miRNAs in methotrexate-resistant cells, and its over-
expression induces the resistant phenotype in HT29 colon cancer
cells [41]. Taken together, these studies suggest that miR-224 is
an onco-miRNA. In agreement, we found that miR-224 upregulation
to breast cancer invasion and also indicate that miR-224 may
function as an onco-miRNA.

However, the mechanism of miR-224 upregulation in breast
cancer remains unclear. Increased miR-224 expression in HCC
tumors correlates with overexpression of the EP300 transcriptional
coactivator and increased EP300 binding to the Xq28 locus, where
the miR-224 gene is located [39]. These findings suggest that
miR-224 is upregulated through an epigenetic mechanism. An
alternative explanation is that gene amplification of the Xq28 locus
upregulates miR-224. However, further research is required to
determine whether these or other mechanisms regulate miR-224
expression in breast cancer.

In summary, the key finding of our current study is that
miR-224 upregulation increases MDA-MB-231 breast cancer cell
invasion by inhibiting RKIP. We have therefore shown that
miR-224 plays an essential role in regulating breast cancer cell
invasion and metastasis and may function as an onco-miRNA.
Understanding the precise role of miR-224 in breast cancer pro-
gression will not only advance our knowledge of breast cancer
biology but may also verify miR-224 as a novel therapeutic target
for the treatment of breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in

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