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Pro-oncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF α in breast cancer

Lianjin Jin^{1,2}, Oliver Wessely³, Eric G. Marcusson⁴, Cristina Ivan⁵, George A. Calin⁵, and Suresh K. Alahari^{1,2,6}

¹Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, LA

²Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, LA

³Department of Cell Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH

⁴Regulus Therapeutics, San Diego, CA

⁵Department of Experimental Therapeutics, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX

Abstract

MicroRNAs (miRs) are a critical class of small (21–25 nucleotides) non-coding endogenous RNAs implicated in gene expression regulation. We identified miR-23b and miR-27b as miRNAs that are highly upregulated in human breast cancer. We found that engineered knockdown of miR-23b and miR-27b substantially repressed breast cancer growth. Nischarin (NISCH) expression was augmented by knockdown of miR-23b as well as miR-27b. Notably, these miRNAs and Nischarin were inversely expressed in human breast cancers, underscoring their biologic relevance. We demonstrated the clinical relevance of the expression of these miRNAs and showed that high expression of miR-23b and miR-27b correlates with poor outcome in breast cancer. Moreover, intraperitoneally delivered anti-miR-27b restored Nischarin expression and decreased tumor burden in a mouse xenograft model of human mammary tumor. Also we report for the first time that HER2/neu (ERBB2), EGF, and TNF α promote miR-23b/27b expression through the AKT/NF- κ B signaling cascade. Nischarin was found to regulate miR-27b/23b expression through a feedback loop mechanism by suppressing NF- κ B phosphorylation. Since anti-miR-27b compounds that suppress miR-27b inhibit tumor growth, the anti-miR-27b appears to be a good candidate for the development of new anti-tumor therapies.

Keywords

Her2; breast cancer; miRNA; Nischarin; therapy

Introduction

MiRNAs are a class of small (21–25 nucleotides) noncoding endogenous RNAs that negatively regulate gene expression by binding to the 3'UTR of target mRNAs, resulting in

⁶Address for Correspondence: S. K. Alahari, Ph.D., CSRB 406, Biochemistry/Stanley S. Scott Cancer Center, LSUHSC, 1901 Perdido Street, New Orleans, LA 70119, Tel 504-568-4734; Fax 504-568-6888; salaha@lsuhsc.edu.

Author Contributions

LJ and SKA conceived, designed, and interpreted the experiments. LJ performed the experiments, GAC and CI performed the statistical analyses, and OW helped with in situ hybridization. EGM provided antisense compounds for therapeutic experiments.

either transcript degradation or translational inhibition (1–3). Since the discovery of miRNAs in 1993, more than 1,500 human miRNAs have been identified. To date, miRNAs have been shown to regulate many cellular processes and pathways that are critical for neoplastic transformation and tumor progression (4–7). MiRNAs have profound positive effects on cancer metastasis; examples include miR-31 and miR-200 family members (8, 9).

Many miRNAs are upregulated or downregulated in tumors compared with normal tissues, supporting their dual role in carcinogenesis as either tumor suppressors or tumor promoters (the latter generally referred to as oncomirs) (10, 11). Examples of tumor-suppressor miRNAs include the Let-7 family, which suppresses the Ras oncogene and is present at low levels in lung cancer, and miR-16, which suppresses the Bcl2 gene as well as multiple other cell-cycle genes and is downregulated in leukemia (12, 13). Conversely, the oncomirs miR-21 and miR-221/222 are upregulated in several cancers and modulate the expression of their targets (14, 15).

Her2/neu (c-erbB-2) is a membrane receptor tyrosine kinase that is amplified or overexpressed in approximately 20% of breast cancers and is associated with increased disease recurrence, tumor invasion, and poor prognosis (16). Some oncomirs, such as miR-21, can be upregulated by Her2/neu signaling and can promote cell invasion in breast cancer (17). Our previous in vitro and in vivo studies identified Nischarin, a novel intracellular protein, as a suppressor of tumor growth and lung metastasis (18). Nischarin is a binding partner for the $\alpha 5\beta 1$ integrin, interacts with members of the PAK family kinases, and thereby regulates the metastatic behavior of tumor cells (19, 20). Here we show that Nischarin expression in breast cancer is regulated by miR-23b and miR-27b binding to the Nischarin 3'UTR region. Both miRNAs are highly expressed in human cancer at least in part due to elevated Her2, EGF, and TNF α signaling. Moreover, suppression of miR-23b/27b activity upregulates Nischarin in breast cancer cells and diminishes tumor growth and metastasis in vivo.

Materials and Methods

Cell culture and reagents

Human breast cancer cell lines MDA-MB-231-4175 (a gift from Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center, New York), MCF7, and MDA-MB-231 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. MCF10A was maintained in MEBM medium supplemented with 5 % fetal bovine serum, 20 ng/ml epidermal growth factor, 0.5 μ g/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μ g/ml insulin. Human breast cancer cell line SKBR3 (ATCC) was cultured in McCoy's 5A medium with 10% fetal bovine serum. BT-474 and ZR75 cells (ATCC) were cultured in RPMI 1640 with 10% fetal bovine serum. Her2/neu agonist, anti-Her2/neu ScFv-TNF α (S147Y) antibody, was a gift from Dr. Sheri Morrison, UCLA. U0126 and LY294002 compounds were purchased from Calbiochem; TNF α and curcumin were from Sigma Aldrich (St. Louis, MO).

Soft agar assay

Anchorage-independent growth of miR-23b stable clones was tested according to our published method (18). The 1-ml culture medium with 0.5% agar was first plated into each well of a 6-well plate. After the agar solidified, each well received another 1 ml of 0.35% agar in culture medium containing 10,000 cells. After 4 weeks, colonies were fixed and stained with 0.1% crystal violet. The number of colonies was determined microscopically by manual counting from triplicate wells for each cell line.

Transwell cell migration and cell invasion assay

Cell migration and cell invasion assays were done essentially as we have described [18]. Briefly, the underside of a transwell insert was coated with fibronectin. Cells were resuspended in serum-free medium and plated onto the upper chamber of the transwell insert; 10% serum medium was added to the lower chamber. In cell-invasion assays, the upper surface was also coated with Matrigel (BD Scientific, CA). Incubation was done for 48 h. At 24 h after incubation, cells that had migrated through the membrane were counted by 0.1% crystal violet staining. The ratio of migrated cells to total cells was plotted (normalized to the vector control).

MTT assay

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. ZR75 cells were transfected with pre-miR-23b/27b (or controls) (Ambion, CA) using Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded at 5,000 cells/well and cultured for various times, after which 10 μ l of MTT (5 mg/ml in PBS) was added to each well. The wells were incubated for an additional 2 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 200 μ l of MTT solvent (4 mM HCl, 0.1% NP-40 in isopropanol). The activity of mitochondria, reflecting cell growth and viability, was evaluated by measuring optical density at 572 nm with a micro-plate reader (Bio-Rad).

Histology and immunohistochemistry

Tissues were fixed in 4% paraformaldehyde for 16 h at 4 °C, embedded in paraffin, sectioned at the level of the tracheal bifurcation at 5-mm intervals, and H&E stained using standard procedures. Proliferation was assessed by staining using antibodies against Ki67 (Abcam, MA). Paraffin-embedded sections were deparaffinized, heated in a microwave in 0.01 M sodium citrate buffer for antigen retrieval, treated with 3% H₂O₂ for 10 min, and rinsed in H₂O and PBS. Sections were blocked in 5% goat serum in PBS, then incubated with anti-Ki67 antibody. Signals were detected with a Vectastain ABC kit (Vector Laboratories, CA) and 3,30-diaminobenzidine (DAB) substrate. Sections were counterstained with hematoxylin and mounted. Ki67-positive cells scored per high-power field from three separate animals (four tumors per animal) and each genotype are represented as the mean percentage of the total number of cells $\pm$ SEM.

Orthotopic mammary transplant and tumorigenesis assay

Female athymic nude (*nu/nu*) mice (ages 4–5 wks) were purchased from NCI and maintained in the Louisiana State University Health Sciences Center's accredited animal facility. We generated mice bearing mammary tumors by transplantation as previously described (18), with the following modifications. We transplanted 500,000 cells into the cleared inguinal fat pads of naïve 3- to 4-week-old athymic female nude mice. Mice were observed for tumor formation. Tumor volume was measured with calipers every 3 days. Mice were killed at 42 days after transplant, or sooner if they had reached the ethical end point of the experiment. All animal studies were performed in accordance with NIH animal use guidelines and a protocol approved by the Louisiana State University Animal Care Committee. Tumor tissue was either embedded in optimal cutting temperature medium or fixed in 4% paraformaldehyde and processed for histologic analysis.

Antagomir treatment of breast tumors

MDA-MB-231-4175 (a subline of MDA-MB-231) human breast cancer cells (5×10^5) were injected into the mammary fat pads of nude mice. We received antagomir-27b and scramble control oligomers from Regulus Therapeutics for in vivo injection. Anti-miR-27b or

scramble control oligomers (25 mg/kg) were injected intraperitoneally daily for 1 week starting 5 days after transplantation and thereafter twice a week for another week (21 days from the day of transplantation). Tumor volume was measured as described. At the end of the experiment, primary tumors were fixed in formalin for further analysis.

Experimental metastasis assay in vivo

Female athymic nude (*nu/nu*) mice (ages 4–5 wks) were injected through tail veins with 1×10^6 exponentially growing 4175 cells with knockdown of miR-23b or miR-27b. Four weeks after injection, the animals were sacrificed. The lungs were harvested and either fixed in ZFIX solution or stained with India ink. Paraffin-embedded lung tissue sections were stained with hematoxylin and eosin. Tumor nodules were counted using a microscope.

Statistical analyses

Results are expressed as means $\pm$ SEM. For comparisons between groups, statistical analyses were performed using the two-tailed paired or unpaired *t*-test (GraphPad Prism, version 5). Differences with *P* values of <0.05 were considered significant.

Survival analysis and correlation/regression analysis were performed using R software (version 2.14.2) (<http://www.r-project.org>). To determine the association of miR-23b/27b/Nischarin (NISCH) expression with survival time, the log-rank test was employed. The Kaplan-Meier method was used to generate survival curves. The expression levels for miR-23b/27b and NISCH as well as survival information for 286 patients with breast cancer were downloaded from the public-access data base Rotterdam (GSE2034). The entire population was randomly divided into two equal sets, the training set and the validation set, for survival analysis. In each set, patients were grouped into percentiles according to miR-23b/27b or NISCH expression. The log-rank test revealed the most significant relationship between miR-23b/27b expression and survival time to be at the 58th percentile. For NISCH, the most significant relationship between expression and survival time was found to be at the 37th percentile. If the patients are divided into two groups with coherent low or high expression of both NISCH and miR-23b/27b, the association between survival and expression becomes stronger.

A Shapiro-Wilk test was used to test whether the data followed a normal distribution. The Pearson correlation test and Spearman's rank-order correlation test were applied to measure the strength of association between the expression of Her2 and Nischarin. A regression model was fitted to determine the nature of the relationship.

(Additional Methods included as Supplemental Data)

Results

Identification of miR-23b

Our previous data indicated that miR-27b functions as an oncogene. miR-27b and miR-23b are encoded within a large miRNA cluster in an imprinted region of chromosome 9 in humans (21). Computer algorithms predicted that the 3'UTR of Nischarin contains regions that match the seed sequences of miR-23b and miR-27b (Fig. 1A and 1B). Since we have a strong interest in Nischarin and miR-23b, we wished to further understand the role of miR-27b and its neighboring miR-23b. We identified a 55-bp region of high homology in the Nischarin 3'UTR shared across humans, mice, rats, and horses but not nonmammalian species. This region corresponds to nucleotides 117–132 of the human Nischarin 3'UTR region. It is similar to the 84% identity we found when comparing the coding portions of the human Nischarin exon (Fig. 1A) with the corresponding sequence from mice, suggesting

that this region of 3'UTR may have functional importance. To confirm this finding, we generated Nischarin 3'UTR wild-type or mutant luciferase reporter constructs and analyzed them by luciferase activity. We found that miR-23b/27b directly bound to the 3'UTR of Nischarin (Fig. 1C, Supplementary Figure S1A). To further confirm this, we investigated whether these miRNAs affect endogenous expression of Nischarin. We performed either ectopic expression of miR-23b/27b by precursor oligomer or silencing of miR-23b/27b by antisense oligomer (Fig. 1D and 1E). All these findings together support the notion that miR-23b/27b directly targets Nischarin and represses its expression.

We used several bioinformatics tools (PicTar, TargetScan, miRanda) to predict the potential target genes of miR-23b. Seventeen genes commonly present in these three databases are putative targets of miR-23b (Supplementary Table S1). We selected six cancer-related genes, and our analysis showed that none of the tested genes are real targets for miR-23b/27b. As shown in Supplementary Figure S1B, only Nischarin and ST14 (as reported by us (21)) were upregulated by these anti-miRNAs.

Ectopic expression of miR-23b/27b promotes in vitro tumorigenic properties

Since miR-23b/27b targets Nischarin, a potential breast cancer tumor suppressor, we suspected that these miRNAs might function as oncomirs. Ectopic expression of miR-23b/27b promoted cell proliferation compared with negative controls (Fig. 2A). To explore whether these effects are due to the reduction of Nischarin, a Nischarin cDNA lacking the 3'UTR region was co-transfected and assayed for cell proliferation. Indeed, Nischarin reduced cell proliferation induced by miR-23b/27b (Fig. 2A). We previously reported that Nischarin suppresses breast cancer cell migration (25). Therefore, we investigated the effect of miR-23b/27b on cell motility using a transwell assay (Fig. 2B) and a scratch-wound assay (Fig. 2C). Ectopic expression of miR-23b/27b in low-invasive ZR75 cells significantly increased migration compared with negative controls. As in the case of proliferation, cells transfected with Nischarin along with miR-23b or miR-27b exhibited reduced cell migration. Together, these findings suggested that ectopic expression of miR-23b/27b promotes cell migration in vitro. Overexpression of Nischarin inhibited migration (18, 22), supporting our hypothesis that the effects of miR-23b/27b are at least in part due to the suppression of Nischarin.

Effects of silencing endogenous miR-23b/27b on cell proliferation, anchorage-independent growth, and cell migration in vitro

Because ectopic expression of miR-23b/27b had dramatic effects on breast cancer cells, we next examined the consequences of silencing endogenous miR-23b/27b. Stably expressed anti-miR-23b/27b constructs were used to knock down miR-23b/27b in the highly metastatic 4175 human breast cancer cell line, which expresses high levels of miR-23b/27b (Supplementary Fig. S1C). Knocking down miR-23b/27b inhibited cell proliferation as compared with that in the scrambled controls (Fig. 2D). Similarly, we noted that cells expressing either anti-miR-23b or anti-miR-27b attenuated anchorage-independent growth (Fig. 2E, 2F). Next, we used transwell and scratch-wound assays to determine the effect on cell migration. To this end, we silenced miR-23b/27b in highly invasive 4175 cells. In both assays, migration was significantly reduced compared with the scrambled controls (Fig. 2G, 2H). Furthermore, silencing of miR-23b/27b inhibited cell invasion in vitro (Supplementary Fig. S2). Knockdown of miRNA is shown in Supplementary Figure S3A. These results indicated that silencing of miR-23b/27b inhibits cell proliferation, anchorage-independent growth, cell migration, and cell invasion in vitro.

Silencing of miR-23b/27b reduces tumor growth and metastases in vivo

The in vitro studies suggested that downregulation of miR-23b/27b expression causes a growth disadvantage. To further investigate this observation, we performed in vivo experiments; 4175 cells transduced with anti-miR-23b/miR-27b or a scrambled control were injected into the mammary fat pads of BALB/c *nu/nu* mice, with measurement of tumor volume starting 5 days later. Tumors from control cells attained a mean volume several times greater than did tumors from anti-miR-23b/27b-expressing cells (Fig. 3A and 3B). This result was also confirmed by staining for cell proliferation using the cell-cycle marker Ki67. Anti-miR-expressing tumors had significantly fewer cells stained for Ki67 staining than the controls (Fig. 3C). Finally, Western blot and qRT-PCR analyses of the tumor tissues confirmed that anti-miR-expressing tumors continued to show reduced expression of miRNAs and increased expression of Nischarin (Fig. 3F and data not shown). This suggested that the observed inhibition of tumor growth is due to downregulation of miRNAs and upregulation of Nischarin expression.

Next, using a lung metastasis model, we assessed whether anti-miRs would also affect metastasis. In this paradigm, tumor cells were injected directly into the bloodstream, bypassing the invasion and intravasation steps. Next, 4175 cells transduced with anti-miR-27b, anti-miR-23b, or scrambled control cells were injected into tail veins of nude mice. As shown in Figure 3D, fewer metastatic lung foci were developed in mice injected with anti-miR-expressing cells than in mice with the scrambled control cells. To further confirm the effect on metastasis, histological analysis of lung tissues was performed. Indeed, few metastases were formed in the lungs of mice injected with antisense-miRNA-transduced cells (Fig. 3E). In contrast, lungs from mice injected with scrambled control cells were heavily infiltrated by metastases.

We previously demonstrated that Nischarin inhibits integrin $\alpha 5$ expression, as well as phosphorylation of PAK1, LIMK, and cofilin (19, 20). Therefore, we wondered whether the same Nischarin signaling cascade operates in these tumors as a result of silencing miR-23b/-27b. Western analyses showed that miR-23b and -27b knockdown in tumor cells decreased expression of LIMK1, PAK1, and cofilin phosphorylation (Fig. 3F). Together, these results suggest that miR-23b and -27b are important regulators of breast tumor growth and metastases. Moreover, this process may be due to enhanced expression of Nischarin and downregulation of PAK signaling.

Therapeutic effect of anti-miR-27b in a mouse breast tumor model

Recent reports have shown that treatment of cancers with miRNA antagonists can reduce tumor burden and metastases (23). Thus, we decided to extend our study to explore whether inhibition of miR-27b has a therapeutic effect on human breast cancer cells. We used a chimeric miRNA antagonist oligomer (anti-miR) that was modified as 2'-O-methoxyethyl (2'-MOE) and 2'-alpha-fluoro united with a phosphorothioate backbone (24). This design potently inhibits miRNA in vivo and has higher affinity and specificity to RNA than 2'-O-methyl (OME) analogues (25). Nude mice were first injected with 4175 human breast cancer cells into the mammary fat pads. Five days later, the mice were given intraperitoneal injections (25 mg/kg body weight per injection) of the chimeric anti-miRs designed to antagonize miR-27b (anti-miR-27b), as well as a control anti-miR of random sequence. The mice were observed for the next 20 days. As shown, treatment with anti-miR-27b led to markedly decreased size (Fig. 3G) and weight (Fig. 3H) of the tumors. We confirmed that these effects were indeed due to the downregulation of miR-27b and upregulation of Nischarin (Supplementary Fig. S3B and S3C). These results show that the anti-miR-27b compound can target solid tumors, indicating that tiny antagomirs inhibiting cancer-associated miRNAs have a high potential in the development of new therapeutic strategies.

Her2/neu signaling regulates miR-23b/27b expression through the AKT pathway

In breast cancer, Her2/neu is associated with increased risk for metastasis and poor prognosis. It has been shown that subsets of miRNA genes, such as miR-17/92, miR-23b/27b/24-1, and miR-30b, are regulated by NF- κ B (26). Since NF- κ B is one of the transcription factors downstream of Her2/neu, we hypothesized that miR-23b/27b expression is regulated by the Her2/neu signaling cascade. To test this, we transiently expressed Her2/neu or an empty vector control in normal breast epithelial cells MCF10A and in poorly invasive MCF7 breast cancer cells (both of these cell types have low expression of Her2 and low expression of miR-23b/27b; Supplementary Fig. S1C and data not shown). In line with our hypothesis, miR-23b/27b expression levels were robustly increased in HER2/neu-expressing cells compared with vector control cells (Fig. 4A). To examine whether this effect is due to active signaling via HER2/neu, we used an anti-Her2/neu ScFv-TNF α (S147Y) antibody (a generous gift from Dr. Sheri Morrison, UCLA), a Her2/neu agonist that stimulates tyrosine phosphorylation of Her2/neu (27). HER2/neu agonist treatment induced miR-23b/27b upregulation in the Her2-positive breast cancer cell line BT-474 (Fig. 4B, C), whereas IgG control had no effect on miR-23b/27b upregulation. In contrast, Nischarin expression was downregulated by HER2/neu agonist treatment (Fig. 4D), suggesting that Her2/neu enhances miR-23b expression and consequently suppresses Nischarin expression.

The RAS-MEK1/2-ERK1/2 and phosphatidylinositol 3-kinase-AKT-NF- κ B pathways are the two major signaling cascades downstream of Her2/neu, and both are activated by the Her2/neu agonist (27). To identify which of the two pathways is responsible for Her2/neu-mediated miR-23b/27b upregulation, we treated BT-474 and SKBR3 Her2/neu-positive cells with Her2/neu agonist along with specific inhibitors of MEK1 (U0126) or PI3 kinase (LY294002). Cells treated with Her2/neu agonist upregulated the miR-23b/27b expression level. Inhibition of MEK1 had no effect on miR-23b/27b induction by the Her2/neu agonist (Fig. 4E). Conversely, inhibition of PI3 kinase activity significantly reduced Her2/neu-dependent miR-23b/27b upregulation (Fig. 4F). As expected, Nischarin expression followed accordingly: it was not affected by U0126 but was increased by LY294002 in a dose-dependent manner (Fig. 4G and 4H). Similar results were obtained with SKBR cells (Supplementary Fig. S4). Together, these findings indicate that the AKT pathway is a key mediator for Her2/neu-dependent miR-23b/27b upregulation.

Growth factors EGF and TNF α induce miR-23b/27b expression

A solid tumor is an ecosystem composed of tumor cells, resident and infiltrating non-tumor cells, and molecules present in proximity to these cells. This ecosystem is collectively described as the tumor microenvironment. It is also characterized by many ligands secreted from the tumor and stromal cells that together either inhibit or promote tumor progression. The tumor microenvironmental (or growth) factors TNF α and EGF can induce NF- κ B (28). We therefore hypothesized that these two ligands also induce expression of miR-23b/27b. To examine this, we treated BT-474 cells with TNF α (10 ng/ml) or EGF (20 ng/ml) for 30 minutes to 2 hours. MiR-23b/27b expression was upregulated by TNF α or EGF treatment in a time-dependent manner (Fig. 5A, B, top and bottom panels). In contrast, Nischarin expression was downregulated by TNF α and EGF treatment (Fig. 5C, 5D, 5E, 5F).

As expected, NF- κ B signaling measured by phospho-p65 was also upregulated and I κ B α was downregulated as a result of TNF α and EGF treatment (Fig. 5E, 5F, 5I, and 5J). To assess whether miR-23b/27b upregulation actually is due to elevation of NF- κ B phosphorylation, we treated cells with curcumin, a well-known inhibitor of NF- κ B. As shown in Figure 5G, 5H, 5I, 5J, curcumin decreased TNF α -induced expression of miR-23b/27b, and Nischarin expression was increased. Identical results were obtained with another

specific inhibitor of IKK (Fig. 5G) as well as another cell line (MDA-MB-231 cells) (Supplementary Fig. S5). Therefore, tumor microenvironmental factors such as TNF α and EGF, as well as Her2/neu, can induce miR-23b/27b expression through the NF- κ B pathway and thereby the consequences of this regulation on Nischarin functions.

Expression of miR-23b/27b as a marker for breast cancer prognosis and metastasis

To examine the relevance of our findings with respect to tumor formation, we examined miR-23b/27b expression in different breast cancer cell lines derived from different stages of the disease. Interestingly, highly invasive cancer cells exhibited higher miR-23b and -27b expression levels than did less invasive cancer cells or nontumorigenic cells (Supplementary Fig. S1C). This result indicates that miR-23b/27b expression level correlates with the metastatic status of breast cancer cell lines.

Having observed that miR-23b and -27b downregulated Nischarin expression in human breast cancer cell lines and that their expression levels correlated with their invasiveness, we wondered whether this regulation also occurs in patient samples. Using qRT-PCR, we examined the expression levels of miR-23b/27b and Nischarin mRNA in human breast cancer and normal tissues. We found that miR-23b/27b levels were significantly higher in cancer tissues (Fig. 6A, 6B; $P < 0.0001$) and that, conversely, Nischarin levels were lower in tumor tissues and higher in normal tissues (Fig. 6C; $P < 0.0001$). We observed similar results when we analyzed data from Oncomine (Karnoub dataset, www.oncomine.org). These results suggest that high expression of miR-23b/27b may be the mechanism that negatively regulates Nischarin expression in breast cancer.

Finally, we wanted to evaluate whether the expression of miR-23b/27b is associated with outcome in breast cancer patients. We downloaded breast cancer gene expression datasets with clinical information from Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/geo/>). The patient population was stratified into two groups from the Rotterdam dataset. We divided tumors into two groups having high or low levels of miR-23b/27b and then examined those groups using univariate Kaplan-Meier survival analysis. We found that patients with elevated miR-23b/27b expression showed statistically significantly shorter recurrence-free survival times compared with patients with low expression levels (Fig. 6D, $P = 0.0018$). In contrast, patients in the group expressing high levels of Nischarin had longer survival times (Fig. 6E, $P = 0.0013$). When the patients were divided in two groups, one group with coherent low miR-23b/27b and high Nischarin expression and the other with high miR-23b/27b and low Nischarin expression, the patients with low miRNA and high Nischarin levels had longer survival times (Fig. 6F, $P = 0.0011$). The observation that patients with low miR-23b/27b and elevated Nischarin expression had significantly longer recurrence-free survival indicates that increased miRNA expression predicts outcome. Since our in vitro data showed that miR-23b/27b expression was induced by Her2, we decided to look at the expression correlation between miR-23b/27b and Her2; we found a significant negative correlation between Nischarin and Her2 (Fig. 6G).

Nischarin regulates miR-23b/27b expression by inhibiting NF- κ B, a feedback regulatory loop

We examined the expression levels of miR-23b and miR-27b in MDA-MB-231 cells expressing Nischarin. To our surprise, expression levels of miR-23b and miR-27b were dramatically low in Nischarin-overexpressing cells compared with the controls (Fig. 7A). To further confirm these results, we investigated the expression of miR-23b and miR-27b in Nischarin-knockdown MCF10A cells; high levels were detected (Fig. 7B). Thus, we hypothesized that this effect may also be due to NF- κ B signaling. Activation occurs via phosphorylation of I κ B α , resulting in the ubiquitin-mediated degradation of I κ B α and the

release of p65 and nuclear translocation of active NF- κ B dimers. Indeed, phospho-p65 and phospho-ERK were downregulated and I κ B α was upregulated in Nischarin-overexpressing MDA-MB-231 cells (Fig. 7C, left panel), whereas the opposite effect was seen in Nischarin-knockdown MCF-10A cells (Fig. 7C, right panel). These findings suggest that Nischarin regulates miR-23b/27b expression by directly inhibiting NF- κ B-p65 phosphorylation in a feedback loop mechanism via a yet unknown regulatory mechanism. In summary, our findings suggest that Her2 can regulate miR-23b/27b expression, which in turn affects Nischarin expression. Also, Nischarin can regulate miR-23b/27b expression (Fig. 7D).

Discussion

MiRNAs inhibit the expression of certain proteins and are important in the development and metastasis of breast cancer (29). However, miRNAs are also promising anti-neoplastic agents (23). Here we report, for the first time, the functional effect of the miR-23b cluster as a novel regulator of tumor growth through its regulation of the tumor suppressor Nischarin. Our findings show that miR-23b and miR-27b, which are located in one genomic cluster (miR-23b/27b/24), are expressed differently in human normal breast and breast cancer tissues. Consistent with our findings, miR-23b dysregulation in cancer has been documented by miRNA profiling data and has been shown to regulate a repertoire of cancer-related genes (30). However, no reports showed whether miR-23b expression is causally involved in breast tumorigenesis. In the current study, gain- and loss-of-function phenotypes of miR-23b demonstrated that miR-23b is a positive regulator of tumor growth and identified Nischarin as a critical target of its activity. Moreover, our study ties in with the regulation of miR-23b in tumor initiation and progression. While it has been reported that miR-23b/27b/24 cluster genes are transactivated via NF- κ B p65 (26), we have now discovered that tumor growth factors EGF and TNF α , as well as Her2/neu, induce expression of miR-23b/27b via the NF- κ B signaling pathway. This positive regulatory pathway is countered by a negative feedback loop that involves Nischarin itself regulating miR-23b/27b expression by inhibiting NF- κ B. Thus, during tumor progression, miR-23b/27b becomes upregulated, causing downregulation of Nischarin and elevation of NF- κ B, followed by an additional increase in miR-23b/27b transcription. Such a scenario has been observed in several biological processes, in which miRNAs have a balancing effect to favor and oppose the same process, thus keeping the system in equilibrium (31). This process could be one of the driving forces for constitutive NF- κ B activation in breast cancer cells, a fact that is supported by the observation that both miR-23b/27b overexpression and low Nischarin expression are detected in breast cancer tissues compared with noncancerous tissues. We showed that in vivo treatment with anti-miR-27b results in attenuated tumor growth. More importantly, to our knowledge, this is the first report of decreased primary breast tumor growth in response to a systemically delivered in vivo treatment inhibiting a miRNA. Future work should build on these findings by determining precisely how these tumor-suppressive pathways are activated by miR-23b/27b repression and how augmented Nischarin expression promotes repression of breast cancer growth in vitro and in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

miR-23b targeted the Nischarin 3'UTR. A, Nischarin 3'UTR in different species (human, chimpanzee, rhesus, mouse, rat, guinea pig, shrew, hedgehog, dog, cat, horse, cow, and armadillo). The box shows a miRNA predicted binding site, starting from 117–132. B, Nischarin 3'UTR region showing potential miR-23b/27b: Alignment of the seed regions of human miR-23b and miR-27b for the Nischarin 3'UTR (NISCH-WT) is shown. Sites of target mutagenesis are underlined (NISCH-MUT). C, Luciferase reporter assay showing the effect of miR-23b on Nischarin: NISCH-WT (gray), NISCH-MUT (black), or vector alone (white) luciferase constructs were co-transfected with pTL-Renilla into 4175 cells with or without anti-miR-23b oligonucleotides. After 48 h, luciferase activity was measured and normalized with Renilla luciferase values. The mean and standard errors from triplicate experiments are indicated. D, Overexpression of pre-miR-23b/27b inhibited endogenous Nischarin expression: MCF-10A cells transfected with 50 nM precursor of miR-23b, miR-27b, or negative control (NC) RNA was used for qRT-PCR (top panel), and proteins were used for Western blotting (bottom panel). qRT-PCR data were normalized with U6 small nucleolus RNA data. Nischarin was detected using Nischarin-specific antibodies. Vinculin was used as a control. E, Antisense miR-23b and -27b enhanced endogenous Nischarin expression: 4175 cells were transfected with 50 nM anti-miR-23b oligonucleotide (anti-23b) or its negative control oligonucleotide (NC). Anti-miR-27b was similarly transfected. RNA was used for qRT-PCR (top panel), and protein was used for Western blotting (bottom panel) as a loading control (bottom). Western blots were quantified for signal intensity, and the numbers are indicated on top of the lanes.

Figure 2.

Effect of overexpression and underexpression of miR-23b/27b on cell proliferation and migration. A, ZR75 cells were transiently transfected with pre-miR-23b, pre-miR-27b, or negative control (NC) oligonucleotides. These cells were cultured for different times and their proliferation examined by MTT assay. In some experiments, Nischarin cDNA was introduced along with miR-23b or miR-27b. B, Migratory capability was examined by transwell assay using the same cells as in A. Representative images showed more migratory cells in ectopic expression for miR-23b or miR-27b than for NC. C, Representative images of ZR75 cells expressing miR-23b/27b showing cell migration for various times after wounding. More cells migrated into the wound at 48 h in ectopically expressing miR-23b or miR-27b cells than in NC cells. D, In 4175 cells stably transfected with scramble or GFP-anti-miR-23b/27b, cell proliferation was examined by MTT assay. The mean and standard error from triplicate experiments are indicated. E, Anchorage-independent growth was examined by soft agar assays. Representative images (top, plate-scanned image; middle, microscopic image, $\times 4$ magnification) of colonies of scramble control and anti-miR-23b/27b are shown. The number of visible colonies was manually counted (bottom). F, Quantitative data for E. G, Stably expressed anti-miR-23b/27b 4175 cells were plated onto transwell inserts. After 24 h, migrated cells were stained with 1% crystal violet (top panel). Knocking down miR-23b/27b significantly decreased cell migration ($P < 0.001$) (bottom panel). The scale bars represent 60 μm . H, Representative images showing stably expressed anti-miR-23b/27b or scramble control cells for various time points after making the wound ($\times 4$ magnification).

Figure 3.

Silencing miR-23b/27b diminished tumor growth and metastasis in vivo. A, Representative images of nude mice with primary tumor growth of miR-23b/27b-knockdown cells in mammary fat pads. Mice were injected with miR-23b or miR-27b knockdown cells and scramble control cells (n = 3 mice per group). B, Graphic representation of tumor volume of mice injected with anti-miR-23b/27b or scramble control cells. Mean and standard errors from triplicate experiments are indicated. C, Representative images showing Ki-67 staining of sections from scrambled control and anti-miR-23b/27b tumors at the end point. Quantification is shown for the percentage of Ki-67-positive cells per field (bottom panel). D, The cells expressing anti-miR-23b/27b and scramble control were injected into mouse tail veins; after 4 weeks, mouse lungs were extracted. Top panel: Representative images of formalin-fixed lungs; bottom panel, lungs stained with India ink. E, Bottom panel shows quantification of metastatic nodules in different lung sections. F, Primary cells were prepared from tumors, and lysates were made from 4175 primary tumor cells alone, 4175-anti-miR-23b primary cells, or anti-miR-27b primary cells immunoblotted with phospho-LIMK, phospho-PAK1, phospho-cofilin, Nischarin, and antibodies (vinculin was used as a

loading control). G, Primary tumor growth of animals treated with anti-miR-27b or control oligomers systemically through intraperitoneal injection for 15 days. Tumor growth was measured for 25 days, and tumor volume was plotted. The mean and standard errors from triplicate experiments are indicated. H, Average tumor weight was plotted at the end point. Tumors dissected from mice are shown in the right panel.

Figure 4.

Her2/neu signaling regulated miR-23b/27b expression through the AKT pathway. A, Her2 expression promoted miR-23b/27b expression. MCF7 or MCF-10A cells were transiently transfected with Her2-expressing vector or vector-alone control. After 48 h, RNA was isolated. qRT-PCR confirmed miR-23b/27b expression. The mean and standard error from triplicate experiments are indicated. B–D, Treatment with Her2/neu agonist. BT474 cells were treated with different concentrations of Her2/neu agonist and checked for miR-23b (B), miR-27b (C), and Nischarin (D) expression by qRT-PCR. Mean and standard error from triplicate experiments are indicated. E, MEK1 inhibitor (U0126) did not affect expression of miR-23b/27b. BT-474 (Her2-positive) cells were treated with Her2/neu agonist (50 nM) and U0126 (10 μM) or DMSO control for 4 h. Expression of miR-23b/27b or Nischarin was tested by qRT-PCR. Western blots showed the expression levels of total ERK, phospho-ERK, and vinculin following different treatments (bottom panel). F, BT-474 cells were

treated with Her2/neu agonist and LY294002 (5, 10, 20 μ M) or DMSO control for 2 h. miR-23b/27b was examined by qRT-PCR. Mean and standard error from triplicate experiments are indicated. G, Western blot showing the effect of MEK1 inhibitor (U0126) on ERK phosphorylation. H, Western blot showing the effect of LY294002 on AKT phosphorylation. Different doses of LY294002 were used. Vinculin was used as a loading control in each Western blot. Western blots were quantified for signal intensity, and the numbers are indicated on top of the lanes.

Figure 5.

Growth factors such as EGF and TNF α induced miR-23b/27b expression. A–D, TNF and EGF enhanced miR-23b expression and decreased Nischarin expression: BT-474 cells were treated with TNF α (10 ng/ml) or EGF (20 ng/ml) for the indicated times. Isolated total mRNA was examined for miR-23b/27b and Nischarin expression by qRT-PCR; U6 snRNA was used as an internal control. The mean and standard error from triplicate experiments are indicated. Expression of miR-23b (top panel) and 27b (bottom panel) is shown in A (TNF α) and B (EGF); Nischarin expression is shown in C (TNF) and D (EGF). E and F, Whole-protein lysates from (A) and (B) were tested for phospho-p65 and Nischarin expression by Western blotting; vinculin was used as a loading control. G and H, Curcumin and IKK inhibitor inhibited miR-23b/27b expression and increased Nischarin expression. DMSO control and curcumin-pretreated cells or IKK inhibitor cells were treated with TNF α (10 ng/ml) for 2 h. Expression of miR-23b/27b (G) and Nischarin (H) was analyzed by qRT-PCR. The mean and standard error from triplicate experiments are shown. I and J, Western blotting was performed on whole-protein lysates from (G) to test phospho-p65 and

Nischarin expression level after curcumin (I) and IKK inhibitor (J) treatment; vinculin was used as a loading control.

Figure 6.

Clinical correlation of miR-23b/27b expression with tumorigenesis in breast cancer patients. A and B, Total RNA from human breast cancer and normal tissues was analyzed by qRT-PCR. qRT-PCR data was normalized with U6 snRNA. miR-23b and miR-27b had significantly higher expression in cancerous tissues (n = 74) than in normal tissues (n = 74) ($P = 0.025$ and $P < 0.001$). C, Expression levels of Nischarin in human cancerous and normal breast tissues were investigated by qRT-PCR; GAPDH was used to normalize the data. The expression level for each cancerous and normal sample is shown. Nischarin had significantly higher expression in normal tissues (n = 74) than in cancerous tissues (n = 74) ($P < 0.001$). D, miR-23b/27b expression levels of patients were averaged, and patients were

then stratified into the high miR-23b/27b or low miR-23b/27b groups. Associated time to survival of the breast cancer patients was then plotted using a Kaplan-Meier survival curve. The median survival times were 66 months for patients with high expression levels of miR-23b/27b and 86 months for those with low expression levels. E, Associated Nischarin message levels and survival in women with breast cancer. Nischarin expression levels from a microarray analysis of breast cancer patients were averaged, and patients were then stratified into either the high or low Nischarin group. Associated time to survival of the breast cancer patients was then plotted using a Kaplan-Meier survival curve. The median survival times were 68 months for patients with low expression levels of Nischarin and 84 months for those with high expression levels. F, Similarly, expression levels of miR-23b/27b and Nischarin were compared. The median survival times were 23 months for patients with high expression level of miR-23b/27b and low expression of Nischarin and 88 months for those with low expression levels of miR-23b/27b and high expression levels of Nischarin. G, Nischarin and Her2 were significantly inversely correlated.

Figure 7.

Nischarin regulated miR-23b/27b expression by inhibiting NF-κB. A, Expression of miR-23b and -27b in Nischarin overexpressing cells. RNA was prepared from cells and qRT-PCR was performed for miR-23b and -27b. B, Expression of miR-23b and -27b in Nischarin-knockdown MCF10A cells. RNA was prepared from cells and qRT-PCR was performed for miR-23b and -27b. C, Immunoblotting with Nischarin-overexpressing cells (MCF-10A) and Nischarin-knockdown cells (MDA-MB-231) with phospho-p65, IκB, phospho-ERK, total ERK, phospho-AKT, and total AKT antibodies. Vinculin was used as a loading control. D, Schematic representation of the signaling cascade showing how TNFα and EGF and Her2 regulate expression of miR-23b/27b. Feedback loop mechanism is also shown, where Nischarin inhibition of NF-κB affects microRNA expression.