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Ratio of miR-196s to HOXC8 mRNA Correlates with Breast Cancer Cell Migration and Metastasis

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Abstract

Expression profiling has identified metastasis-associated miRNAs but technical limitations hinder the discovery of metastasis-suppressing miRNAs. In this study, we sought metastasis-suppressing miRNAs by functional screening. Individual miRNAs were lentivirally introduced into metastatic MDA-MB-231 breast cancer cells and analyzed for effects on cell migration, a critical step in cancer metastasis. Among 486 miRNAs screened, 14 were identified that included all of the members of the miRNA-196 family (miR-196a1, miR-1962 and miR-196b). Enforced expression of miR-196a1/2 or miR-196b abrogated *in vitro* invasion and *in vivo* spontaneous metastasis of breast cancer cells, indicating that members of miR-196 family are potent metastasis suppressors. We found that miR-196 inhibited expression of the transcription factor HOXC8. Functional linkage was implied by siRNA-mediated knockdown of HOXC8, which suppressed cell migration and metastasis, and by ectopic expression of HOXC8, which prevented the effects of miR-196 on cell migration and metastasis. Unlike other metastasis-associated miRNAs that have been described, the expression of the miR-196s was not correlated to breast cancer cell migration or the metastatic status of clinical breast tumor specimens. Instead, we detected an excellent correlation between the ratio of miR-196 to HOXC8 messages and the migratory behavior of breast cancer cell lines as well as the metastatic status of clinical samples. Our findings identify miRNA-196s as potent metastasis suppressors and reveal that the ratio of miR-196s to HOXC8 mRNA may be an indicator of the metastatic capability of breast tumors.

Keywords

miRNA; HOXC8; cell migration and metastasis

INTRODUCTION

Tumor metastasis is a multi-step process involving tumor cell migration, protease production and colonization to the distant tissues (1). MicroRNA (miRNA), a class of small RNAs that suppress gene expression post-transcriptionally by base-pairing with the 3′-untranslated regions (UTRs) of target mRNA (2), has been implicated to play an active role in cancer progression and metastasis (3-5). For example, miR-10b, miR-373 and miR-520c

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promote cancer metastasis (6,7) while miR-31, miR-126 and miR-335 act as metastasis suppressors (8,9). Most of these known metastasis-associated miRNAs were identified through analyzing the difference in miRNA expression profiles/expression patterns between metastatic and non-metastatic samples with the aid of microarray-based technology. However, technical limitations of this technology may hinder the effort to identify all miRNAs functionally important for metastasis process.

In this study, we sought to identify metastasis-inhibitory miRNAs through a functional screening. We introduced individual miRNAs into metastatic breast cancer MDA-MB-231 cells and the effect of each miRNA on cell migration was subsequently determined by Transwell migration assay. This screening identified 14 migration-inhibitory miRNAs that included previously reported metastasis-suppressive miRNAs (miR-31, miR-126 and miR-335) and also all three members of miR-196 family (miR-196a1, miR-196a2 and miR-196b). Forced expression of miR-196a1, miR-196a2 or miR-196b diminished *in vitro* invasion and *in vivo* metastasis of breast cancer cells, suggesting that members of miR-196 family are potent metastasis suppressors. To define the mechanism-pertinent to miR-196 function, we showed that 1) enforced miR-196 expression downregulated HOXC8 expression; 2) knockdown of HOXC8 significantly reduced breast cancer cell migration, *in vitro* invasion and *in vivo* spontaneous metastasis; and 3) ectopic expression of HOXC8 transgene reversed miR-196 action. To our surprise, the levels of miR-196s (miR-196a and miR-196b together) correlated to neither cell motility nor metastasis status. Instead, we detected an excellent correlation between the ratio of miRNA-196s to HOXC8 mRNA and migration of established breast cancer cell lines as well as metastasis status of clinical breast tumor specimens. Our functional screening has allowed us to identify miRNAs that would otherwise be overlooked in miRNA expression profile analysis. Importantly, this study suggests that the ratio of miRNA to its target mRNA, rather than the levels of miRNA alone, should be taken into consideration when predicting miRNA functional outcome.

MATERIALS AND METHODS

Cells and antibodies

MDA-MB-231 and MDA-MB-436 cells were cultured in DMEM supplemented with 10% FBS. BT-549, MCF-7, MDA-MB-468, SK-BR3, T47D and ZR-75-1 lines were cultured in DMEM supplemented with 10% FBS plus 10μg insulin. SUM-159 line was maintained in Ham's F12 medium supplemented with 5% FBS, 5μg insulin and 1μg hydrocortisone. All these lines were obtained from ATCC within last 6 months and were further tested 1 month before the experiments for authentication. The authentication methods included morphology analysis, growth curve analysis and *Mycoplasma* detection and were performed according to the ATCC cell line verification test recommendations. Anti-HOXC8 mAb (Cat # 400003224-B01; titer: 1:1,000) was purchased from Abnova. Anti-HOXA5 polyclonal antibody (Cat # sc-81289; titer: 1:500), anti-HOXA7 polyclonal antibody (Cat # sc-81290; titer: 1:250), anti-HOXA9 polyclonal antibody (Cat # sc-81291; titer: 1:250) and anti-βactin mAb (Cat # sc-47778; titer: 1:1,000) were obtained from Santa Cruz Biotechnology.

Functional miRNA screening

All 486 human miRNA-containing lentiviral vectors were obtained from BioSettia. In these vectors, miRNA precursors and approximately 100bp upstream and downstream flanking genomic sequences were cloned into a self-inactivated lentiviral vector. To identify miRNAs that affect cell migration, MDA-MB-231 cells were infected with each of these individual miRNA lentiviral expression vectors for 2 days. As the lentiviral vector contains puromycin gene, cells were treated with 2μg/ml puromycin for another 2 days. Cells were detached, resuspended in serum-free medium and analyzed for cell migration using the 96-well

formatted cell migration assay system (Cell Biolabs). To confirm the specificity of miRNA effect, cells with enforced miRNA expression were transfected with 0.2μM respective Anti-miRs using Lipofectamine2000 (Invitrogen) for 24 hrs. Cells were replenished with fresh medium and cultured for another 2 days followed by the analysis of cell migration. All Anti-miRs were purchased from Invitrogen and detailed information about these Anti-miRs can be found at www.invitrogen.com/ordermirna.

Transwell migration assays

Cell migration was performed as previously described (10,11). Briefly, the undersurface of Transwells (8μm pore size; Costar) was coated with 10μg/ml collagen I overnight at 4°C. Cells were detached with PBS containing 10mM EDTA, then resuspended in serum-free medium at density of 10^6 cells/ml and 100μl cell suspension added to the upper chamber of the Transwells. After 6-hr migration period, the remaining cells in the upper chamber were removed with cotton swabs and the cells on the undersurface of Transwells fixed with 5% glutaraldehyde solution. Cells were stained with crystal violet solution and the number of migratory cells was calculated by counting three different fields under a phase-contrast microscope.

In vitro invasion assay

Cell invasion was performed as previously described (12). Cells were detached and resuspended in serum-free medium. Cells (1.5×10^5 cells/well) were then plated into the Matrigel-coated invasion chambers (Cell Biolabs) and allowed to invade for 24 hrs. The remaining cells in the chambers were removed by cotton swabs and the invading cells on the lower surface of the chambers were stained with Quick-Diff staining solution. The number of invading cells was calculated by counting three different fields under a phase-contrast microscope.

Tumor outgrowth and spontaneous lung metastasis

All animal work was done in accordance with an approved protocol by the Medical College of Georgia Institutional Animal Care and Use Committee. Both *in vivo* tumor outgrowth and lung metastasis assay were performed as previously described (12). Briefly, MDA-MB-231 cells (10^6 cells in 0.1 ml PBS) were injected orthotopically into the fourth mammary fat pad of athymic female nude mice between 4-6 weeks old (Harlan Laboratories) and animals were monitored for 7 weeks to determine *in vivo* tumor outgrowth. Tumor outgrowth was determined by measuring xenografts externally in two dimensions using a caliper and tumor volume (V) was calculated by equation: $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of a xenograft. To determine spontaneous tumor metastasis, mice were sacrificed at 7 weeks after injection. Lungs were removed, then fixed in 4% PBS-buffered paraformaldehyde and processed for paraffin-embedded sectioning. The sections were subjected to H&E staining and the metastatic lesions were visualized under a light microscope.

HOXC8 mRNA 3′-UTR luciferase reporter gene construct and the analysis of luciferase activity

The 3′-UTR of human HOXC8 mRNA was generated by RT-PCR using total RNA isolated from MCF-7 cells and PCR fragment was subcloned into pMIR luciferase report vector (Applied Biosystems). Primers for synthesizing 3′-UTR of human HOXC8 mRNA are forward: GAACTAGTGCAAAAAAGAAAGACCCCCCCCCCCTTAGCAACTCCCTTG and reverse:
GAAAGCTTTTTTCTTCTTCTTTTTATTATTATGACTCACGTATACAATAC. To determine miR-196/HOXC8 interaction, reporter gene constructs were co-transfected into

MDA-MB-231 cells with expression vector encoding miR196a2 or miR196b for 2 days. Expression vector encoding Renilla luciferase was included in transfection for standardization. Duel luciferase system (promega) was used to measure luciferase activity according manufacturer's protocol.

Construction of HOXC8 shRNA and HOXC8 transgene expression lentiviral vectors

To construct HOXC8 shRNA vectors, web-based Invitrogen Block-It program was used to designed HOXC8 shRNA sequence. The oligonucleotides containing HOXC8 shRNA sequences were synthesized and inserted into pLV-shRNA vector (BioSettia). HOXC8 shRNA1 and 2 sequences are 5′-GCAATATCCCGACTGTAAATC-3′ and 5′-GCCTCATGTTTCCATGGATGA-3′ respectively. To construct HOXC8 transgene lentiviral expression vector, the entire human HOXC8 coding sequence was subcloned into pCDH-CMV-MCS-EF1-copGFP (System Biosciences).

qRT-PCR

Total RNA extracted from cultured cells or frozen tissues were used for cDNA synthesis with random primer. The levels of miR-196a, miR-196b and HOXC8 mRNA were analyzed with miR-196a, miR-196b TaqMan® microRNA Assay Kit and TaqMan® HOXC8 probe (Applied Biosystems) respectively. The levels of GAPDH mRNA were also measured and used as the internal normalization factor.

Histochemistry of HOXC8 in human breast cancer and normal breast samples

Human primary breast cancer and normal breast tissues were collected in compliance with a protocol approved by the Medical College of Georgia Institutional Oversight Advisory Board and informed consent was received from all subjects. Sections were made from frozen tissues and used for immunohistochemistry with HOXC8 mAb as previously described (13). The intensity of HOXC8 immunostaining was graded based on the percentage of cells displaying HOXC8 staining and average 1,000 cells were counted for each sample. "-" represents negative HOXC8 staining; "+", "++" and "+++" represent <25%, 25~50% and >50% cells positive for HOXC8 staining respectively. The positive HOXC8 staining was confirmed by competition with HOXC8 peptide (Abnova).

Statistical Analysis

Statistical analyses were performed on data collected from at least three independent experiments. Statistical analyses of cell migration and invasion were done by student *t* test (two-sided). Independents *t* test was used to analyze the significance between the value of lg(miR-196s/HOXC8) and clinicopathological parameters. The differences were considered statistically significant when $P < 0.05$.

RESULTS

Members of miR-196 family suppress cell migration, *in vitro* invasion and metastasis

As cell migration is one of the most critical steps in cancer metastasis, we sought to identify metastasis-suppressing miRNAs by screening for those miRNAs capable of inhibiting cell migration. Individual primary miRNAs were lentivirally introduced into metastatic breast cancer MDA-MB-231 cells and the population of each transduced cells was subjected to 96-well formatted Transwell migration assay for initial screening (Fig.1A). The identified migration-suppressive miRNAs were then subjected to 24-well formatted transwell migration assay for confirmation (Fig.1A). Out of 486 miRNAs screened, 14 miRNAs significantly inhibited cell migration (Fig.1B). Among them, miR-31, miR-126 and miR-335 were previously reported as metastasis suppressors (8,9). To verify the remaining 11

miRNAs, we treated the transfected cells with either control or the respective Anti-miRs (inhibitory antisense molecules for miRNAs) followed by 24-well formatted transwell migration assay (Fig.1A). The treatment of Anti-miRs, but not the control, restored over 80% of cell migration in their respective miRNA-transduced cells (Fig.1C and Fig.1D). These results suggest that these miRNAs specifically inhibit cell migration.

We focused our study on miR-196 because all three members of miR-196 family (miR-196a1. miR-196-a2 and miR-196b) specifically inhibited cell migration (Fig.1C). We generated miR-196 mutants by either reversing the nucleotide sequence in 2-7 position of mature miRNA sequence or introducing A/U→U/A and G/C→C/G mutation in the same nucleotide position (Fig.2A). MDA-MB-231 cells were lentivirally transduced with these mutant miR-196s and subsequently analyzed for cell migration using Transwells. None of the mutants altered cell migration while wild-type miR196s potently suppressed cell migration (Fig.1A). Similarly, wild-type miR-196a2 and miR-196b, but not their mutants, also inhibited cell migration in MDA-MB-436 and SUM-159 cells (Fig.2B). These results thus further confirm the specificities of miR-196 to inhibit cell migration.

We next investigated the effect of miR-196s on invasive properties of breast cancer cells by analyzing *in vitro* invasion using Matrigel invasion chambers. Enforced miR-196a2 and miR196b expression inhibited *in vitro* cell invasion of MDA-MB-231, MDA-MB-436 and SUM-159 cells (Fig.2C). To examine the effect of miR-196s on tumor outgrowth, control MDA-MB-231 cells (empty vector) or MDA-MB-231 cells with enforced miR-196a2 or miR-196b expression were orthotopically injected into the mammary fat pad area of female athymic nude mice. Palpable tumors were developed within 7 days in all groups and daily monitoring up to 7 weeks revealed no statistically significant difference in tumor outgrowth between control and miR-196 groups (Fig.S1), suggesting that miR-196 did not affect *in vivo* tumor outgrowth. In subsequent experiment, we also excised lungs from euthanized mice. The excised lungs were processed for hematoxylin and eosin (H&E) histological staining and observed for metastatic lesions under microscope. Metastatic lesions were detected on the lungs in 70% of mice receiving control cells (7 out 10 mice) (Fig.2D and Table S2). In contrast, metastatic lesion was not detected in mice receiving MDA-MB-231 cell with enforced miR-196a2 or miR-196b expression (Fig.2D and Table S1). These results demonstrate the ability of miR-196s to suppress cancer cell invasion and metastasis.

Members of miR-196 family downregulate HOXC8 levels

To characterize the mechanism associated with miR-196 action, we used a web-based miRNA target prediction program TargetScanHuman 5.1 (14,15) to search potential miR-196 targets. We focused on HOXC8, HOXA5, HOXA7 and HOXA9 genes as the predicted miR-196 target site(s) is present in their 3′-UTRs (Fig.3C and Fig.S2A) and their expression was previously shown to be downregulated by miR-196s during vertebrate embryogenesis (16). Immunoblotting with HOXC8 mAb showed that HOXC8 was significantly downregulated by wild-type miR-196a2 or miR-196b but not their mutants (Fig.3A). In contrast, the levels of HOXA5 or HOXA9 (HOXA7 was undetectable) were not affected by miR-196a2 or miR-196b (Fig.S2B). To determine whether HOXC8 is a direct target of miR196s, we linked 3′-UTR of HOXC8 mRNA to the downstream of luciferase gene in pMIR reporter plasmid. Co-transfection experiments showed that both miR-196a2 and miR-196b, but not their mutants inhibited luciferase activity in MDA-MB-231 cells (Fig.3B). Since 3′-UTR of HOXC8 mRNA contains 5 potential miR196 target sites (Fig. 3C), we examined the relative contribution of these sites by introducing mutations to disrupt base-pairing between miR-196 and the 3′-UTR of HOXC8 mRNA. Mutations in any of these sites only slightly impaired miR-196-induced inhibition in luciferase activity and additional mutations led to accumulative inhibition (Fig.3D). Mutation in all five sites completely abolished miR-196s' ability to inhibit luciferase activity (Fig.3D). These results

suggest that each of these predicted sites contributes equally to miR-196-mediated HOXC8 reduction. As miRNA can regulate target expression either through the mechanism of mRNA degradation or translational repression (17), we isolated total RNA from control and miR-196-transduced MDA-MB-231 cells followed by qRT-PCR to measure the levels of HOXC8 mRNA. Although the levels of miR-196a1, miR-196a2 and miR-196b were greatly elevated in the respective transduced cells over the control cells (Fig.S3A and S3B), the levels of HOXC8 mRNA were not significantly altered (Fig.S3C). These results show that members of miR-196 family inhibit HOXC8 expression through the mechanism of translational repression.

Metastasis-suppressive role of miR-196 is functionally linked to HOXC8

To determine the potential functional link between miR-196 and HOXC8, we designed two effective HOXC8 shRNAs (Fig.S4A) and lentivirally introduced them into MDA-MB-231, MDA-MB-436 and SUM-159 cells. Knockdown of HOXC8 decreased both cell migration (Fig.4A) and *in vitro* cell invasion in all three lines (Fig.4B). In parallel, we orthotopically injected control (luciferase shRNA) or HOXC8-knockdown MDA-MB-231 cells into nude mice. Silencing HOXC8 exhibited no obvious effect on *in vivo* tumor outgrowth (Fig.S4B). However, H&E staining of the lung sections from the euthanized mice indicated that knockdown of HOXC8, but not luciferase shRNA, prevented spontaneous metastasis (0 out 10 mice in HOXC8 shRNA group *vs* 7 out 10 mice in luciferase shRNA group) (Fig.4C and Table S1). These results suggest that HOXC8 plays an essential role in cancer cell invasion and metastasis.

We next ectopically expressed HOXC8 transgene (only HOXC8 coding region and thus not miR-196-targeted) in MDA-MB-231 cells that lentivirally expressed miR-196a2- or miR-196b. Transwell migration assay showed that enforced HOXC8 transgene expression greatly restored migration of miR-196-transduced MDA-MB-231 cells (Fig.5A). Similarly, HOXC8 transgene also largely restored *in vitro* invasion in these cells (Fig.5B). Identical results were also obtained with miR-196-transduced MDA-MB-436 cells (Fig.S5A and Fig.S5B). Moreover, we found that enforced HOXC8 transgene expression largely restored the ability of miR-196a2- or miR-196b-transduced MDA-MB-231 cells for spontaneous lung metastasis (metastatic lesions were detected on lungs of 60% of mice receiving cells with HOXC8 transgene expression) (Fig.5C and Table S1). These results support the functional link between miR-196 and HOXC8.

Ratio of miR-196s to HOXC8 mRNA correlates to migration of established breast cancer cell lines

To further characterize the importance of HOXC8 in cell migration/metastasis, we performed immunoblotting to examine HOXC8 protein levels in various breast cancer cell lines that were either migratory or poorly migratory (Fig.6A). The levels of HOXC8 protein were generally higher in migratory lines (Fig.6A); however, the levels of miR-196s (miR-196a and miR-196b together) or HOXC8 mRNA were not associated with migration of these lines (Fig.6B). Interestingly, we detected an excellent correlation between cell migration and the ratio of miR-196s to HOXC8 mRNA ($P = 0.012$) in these cell lines: low ratio was seen in the migratory cell lines and high ratio with poorly migratory ones (Fig.6B). These results thus show that the ratio of miR-196s to HOXC8 mRNA, rather than the levels of miR-196s or HOXC8 mRNA, reflects the migration status of a particular cell line.

Ratio of miR-196s to HOXC8 mRNA correlates to metastasis status of clinical breast tumor specimens

As laboratory study may not always recapitulate clinical cancer metastasis, we extended our study into 25 clinical human breast tumor specimens and 4 normal breast tissues. The set of

tumors samples consisted of primary tumors resected from 8 patients with ultimate metastatic recurrence and 17 patients without suffering metastatic relapse (Table S2). Immunohistochemistry with anti-HOXC8 mAb showed that none of the normal breast tissues was positive for HOXC8 staining (Fig.6C and Table S1). HOXC8 expression was detected in 7 out of 8 metastatic tumor specimens (87.5%) but in only 5 out of 17 metastasis-free tumor samples (30%; $P < 0.02$ *vs* metastatic tumors) (Fig.6C and Table S2), indicating that HOXC8 level is elevated in metastatic breast tumors. Similar to what we observed in established cell lines, qRT-PCR showed that the levels of HOXC8 mRNA or miR196s (miR-196a and miR-196b together) were not correlated with metastatic status of these samples (Fig.6D); instead, the ratio of miR-196s to HOXC8 mRNA was significantly lower in metastatic tissues than that of metastasis-free specimens or normal breast tissues ($P = 0.0022$ and 0.0055 respectively; Fig.6D). Further analysis also showed that the ratio of miR-196s to HOXC8 mRNA was not associated with ER, PR or HER2 status of these clinical samples ($p = 0.943$, 0.957 and 0.110 respectively; Table S2). These results suggest that the ratio of miR-196s to HOXC8 mRNA may be specifically correlated with the metastasis status of breast tumors.

DISCUSSION

Majority of cancer-related death is caused by metastasis and recent studies have demonstrated that miRNAs play an important role in the regulation of metastasis process (18). Mainly through comparing the difference in miRNA levels or expression patterns between metastatic and non-metastatic tumor specimens, particular miRNAs have been found to be functionally important for cancer metastasis. For example, miR-10b, miR-373 and miR-520c promote tumor metastasis (6,7) while miR-31, miR-126 and miR-335 suppress tumor metastasis (8,9). However, the technical limitations of used technologies may hinder the broader use of such strategy as some functionally important miRNAs could be present at the levels under the detection capacity. In this study, we sought to identify migration-pertinent miRNAs through a functional screening. Our screening identified previously reported miR-31, miR-126 and miR-335 (Fig.1), thus validating the effectiveness of our approach. In addition, we identified another 11 migration-inhibitory miRNAs that include all three members of miR-196 family (miR-196a1, miR-196a2 and miR-196b) (Fig. 1). Further studies also show that members of miR-196 family can potently suppress *in vitro* invasion and *in vivo* metastasis (Fig.2). We believe that our functional screening strategy can complement with the current profiling analysis for identifying disease-associated miRNAs.

MicroRNAs can suppress metastasis by targeting genes essential for metastasis at various steps including adhesion/migration/invasion, apoptosis, angiogenesis and colonization (19). For example, Let-7 suppresses metastasis through the inhibition of apoptosis, angiogenesis and colonization by targeting Ras and HMGA2 (20,21). Anti-metastasis capability of miR-335 is mediated through the suppression of cell migration by downregulating SOX4, TNC, PTPRN2 and MERTK expression (9). Moreover, miR-31 also blocks breast cancer metastasis through the suppression of cell migration and is functionally linked to multiple migration-relevant genes including Fzd3, ITGA5, RDX, or RhoA (8). In this study, we show that miR-196s suppress metastasis at the step of cell migration (Fig.1). Although miR-196s may target multiple genes potentially pertinent to cell migration, anti-metastatic role of miR-196s is apparently mainly associated with HOXC8 because miR-196s significantly inhibit HOXC8 expression (Fig.3) and ectopical expression of HOXC8 transgene reverses miR-196-caused inhibition in cell migration, *in vitro* invasion and metastasis (Fig.5). Our results thus add another miRNA into the expanding group of metastasis-associated miRNAs.

HOXC8 is a member of homeobox gene family (22) and has been shown to play a role in the acquisition of the invasive and metastatic phenotype in prostate cancer (23). We show

that HOXC8 is critically involved in breast cancer metastasis as knockdown of HOXC8 suppresses cell migration, invasion and metastasis (Fig.4). The importance of HOXC8 in metastasis is further supported by the observation that HOXC8 protein level is elevated in migratory breast cancer cells and metastatic breast tumor specimens (Fig.6). Though it is currently unknown how HOXC8 facilitates cell migration/metastasis, HOXC8 can regulate the expression of many migration-associated genes including cadherin 11, embigin, transgelin and calponin (24), in which cadherin 11 and embigin promote cell migration (25,26) while transgelin and calponin inhibit cell migration (27,28). It is of interest to determine whether the miR-196-HOXC8 circuit is involved in cell migration/metastasis by regulating the expression of these genes.

Almost all currently-identified metastasis-suppressive miRNAs exhibit reduced levels of expression in metastatic tumor specimens (18). Interestingly, we showed that the levels of miR-196s did not correlate to metastasis status of clinical breast tumor specimens (Fig.6). Instead, we observed an excellent correlation between the ratio of miR-196s to HOXC8 mRNA and metastasis status in these clinical sample. Lower ratio was found in metastatic specimens and *vice versa* (Fig.6). Our findings indicate that the ratio of miR-196s to HOXC8 mRNA may be a reliable indicator for breast cancer metastasis.

In summary, we show that member of miR-196 family can suppress breast cancer cell migration and metastasis by inhibiting HOXC8 expression. Distinct from those previously identified metastasis-associated miRNAs in which their levels correlate to metastasis status of clinical specimens, the ratio of miR-196s to HOXC8 mRNA, rather than miR-196 levels, correlates to breast cancer metastasis. Our study implicates that, in addition to solely relying on miRNA levels, the ratio of miRNA level to its target transcript should be taken into serious consideration for precise prediction of miRNA function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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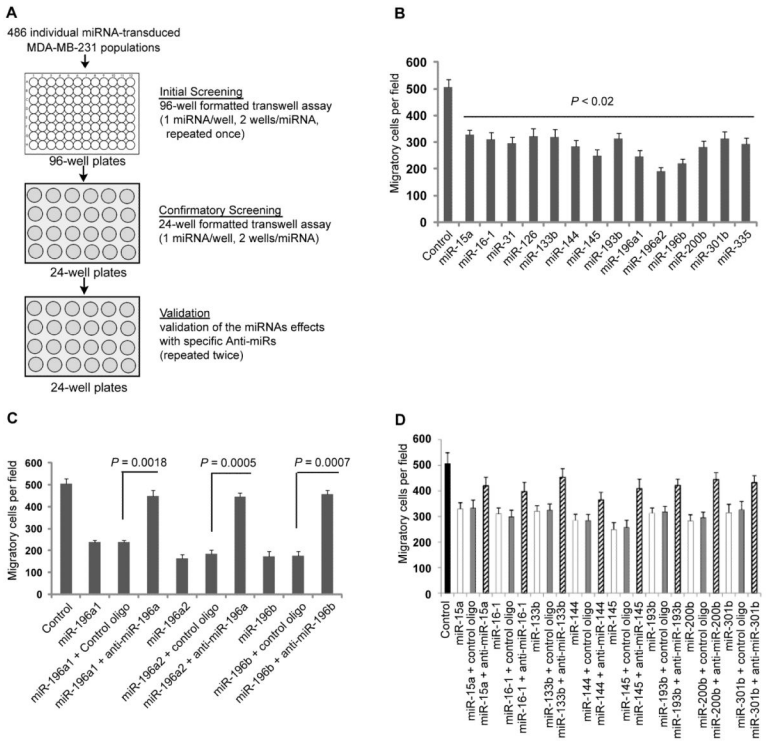

Figure 1. Functional screening to identify miRNAs that can inhibit MDA-MB-231 cell migration

A. Flow chart of identifying miRNAs that can affect cell migration. ***B.*** MDA-MB-231 cells were transduced with empty lentiviral vector (control) or lentiviral vector containing miR-15a, miR-16-1, miR-31, miR-126, miR-133b, miR-144, miR-145, miR-193b, miR-196a1, miR-196a2, miR-196b, miR-200b, miR-301b or miR-335 and the transduced cells were analyzed for cell migration using Transwells as described in Materials and Methods. Values are means ± SEM. ***C.*** MDA-MB-231 cells transduced with control, miR-196a1, miR-196a2 or miR-196b-containing lentivirus were treated with control Anti-miR, Anti-miR-196a or Anti-miR-196b for 3 days, then detached with 10mM EDTA and analyzed for cell migration. Values are means ± SEM. ***D.*** MDA-MB-231 cells transduced with miR-15a, miR-16-1, miR-133b, miR-144, miR-145, miR-193b, miR-200b or miR-301b-containing lentivirus were transfected with 0.2μM control or their respective Anti-miRs for 1 day and then replenished with fresh medium. After another 2-day culture, cells were detached with 10mM EDTA and analyzed for cells migration. Values are means ± SEM.

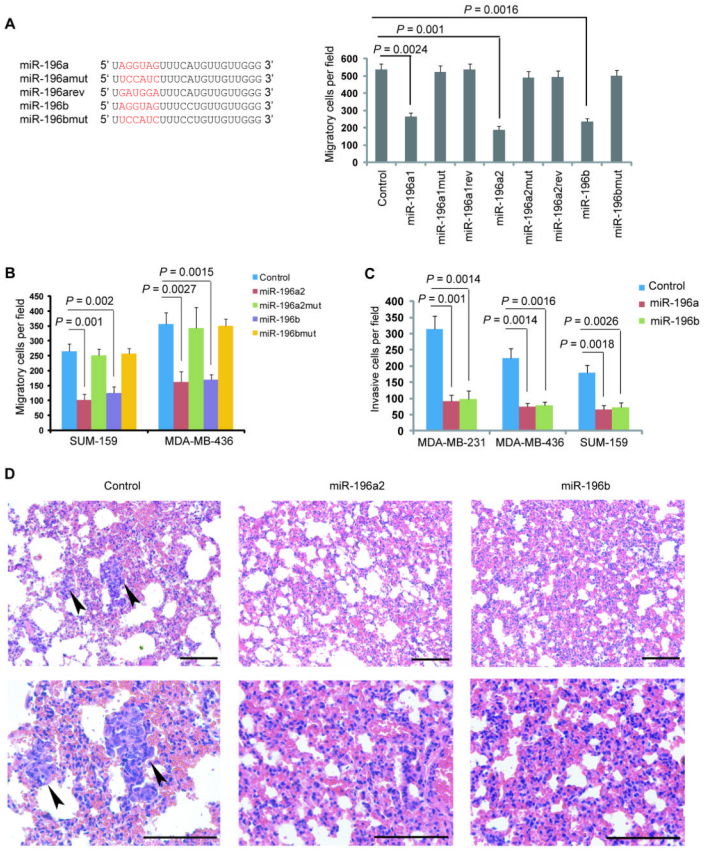

Figure 2. Members of miR-196 family suppress breast cancer cell migration, *in vitro* invasion and metastasis

A. MDA-MB-231 cells were lentivirally transduced with empty vector or vector encoding miR-196a1, miR-196a1mut, miR-196a1rev, miR-196a2, miR-196a2mut, miR-196a2rev, miR-196b or miR-196bmut and then analyzed for cell migration using Transwells as described in Materials and Methods. Values are means ± SEM. Wild-type and mutant miR-196a and miR-196b sequences are shown on the left (Red letters are wild-type and mutated seed sequences). ***B.*** SUM-159 and MDA-MB-436 cells are lentivirally transduced with empty vector or vector encoding miR-196a2, miR-196a2mut, miR-196b or miR-196bmut and then analyzed for cell migration. Values are means ± SEM. ***C.*** MDA-MB-231, MDA-MB-436 and SUM-159 cells were lentivirally transduced with empty vector or vector encoding miR-196a2 or miR-196b and then analyzed for *in vitro* invasion using Matrigel invasion chambers as described in Materials and Methods. Values are means ± SEM. ***D.*** The lungs from mice receiving control, miR-196a2 or miR-196b-transduced MDA-MB-231 cells for 7 weeks were fixed and sectioned. The sections were subjected to H&E staining and visualized under a light microscope. Arrows indicate metastatic lesions. Top panels: lower magnifications; bottom panels: higher magnifications; scale bar: 100 μm.

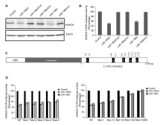

Figure 3. Members of miR-196 family inhibit HOXC8 expression

A. MDA-MB-231 cells were lentivirally transduced with empty vector (control) or vector encoding miR-196a2, miR-196a2mut, miR-196a2rev, miR-196b or miR-196bmut, then lysed and cell lysates subjected to immunoblotting to detect HOXC8 protein with HOXC8 mAb. Membrane was stripped and reprobed with βactin mAb. ***B.*** HOXC8 3′-UTR reporter gene plasmid was co-transfected into MDA-MB-231 cells with empty vector (control) or vector encoding miR-196a2, miR-196a2mut, miR-196a2rev, miR-196b or miR-196bmut for 2 days. Cells were lysed and cell lysates analyzed for luciferase activities as described in Materials and Methods. Values are means ± SEM. ***C.*** Diagram of HOXC8 3′-UTR reporter construct. Potential miR-196 target sites in HOXC8 3′-UTR are shown in the black boxes. Numeric numbers indicate the nucleotide positions of each potential target site. ***D.*** HOXC8 3′-UTR luciferase reporter gene plasmid containing mutation in potential miR-196 target site(s) was co-transfected into MDA-MB-231 cells with empty vector (control) or vector encoding miR-196a2 or miR-196b for 2 days. Cells were lysed and cell lysates analyzed for luciferase activities. Values are means ± SEM. Left: mutation in a single miR-196 target site; right: mutation in multiple miR-196 target sites; WT: wild-type HOXC8 3′-UTR.

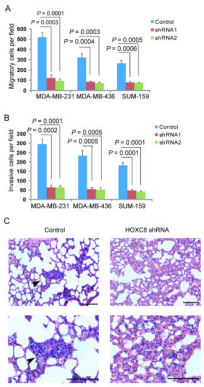

Figure 4. Knockdown of HOXC8 suppresses breast cancer cell migration, *in vitro* invasion and metastasis

A. MDA-MB-231, MDA-MB-436 and SUM-159 cells were lentivirally transduced with HOXC8 shRNA or luciferase shRNA (control), and then analyzed for cell migration using Transwells as described in Materials and Methods. Values are means ± SEM. ***B.*** MDA-MB-231, MDA-MB-436 and SUM-159 cells were transduced with HOXC8 shRNA or luciferase shRNA (control), and then analyzed for *In vitro* invasion using Matrigel invasion chambers as described in Materials and Methods. Values are means ± SEM. ***C.*** The lungs from mice receiving control or HOXC8-knockdown MDA-MB-231 cells for 7 weeks were fixed and sectioned. The sections were subjected to H&E staining and visualized under a light microscope. Arrows indicate metastatic lesions. Top panels: lower magnifications; bottom panels: higher magnifications; scale bar: 100 μm.

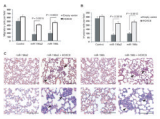

Figure 5. Metastasis-suppressive role of miR-196s is functionally linked to HOXC8

HOXC8 transgene was forced to be expressed in MDA-MB-231 cells that had been lentivirally trasduced with empty vector (control) or vector containing miR-196a2 or miR-196b. ***A.*** Cells were analyzed for cell migration using Transwells as described in Materials and Methods. Values are means ± SEM. ***B.*** Cells were analyzed for *in vitro* invasion using Matrigel invasion chamber. ***C.*** H&E staining of lung sections from mice euthanized 7-weeks after orthotopical injection of miR-196a2 or miR-196b-transduced MDA-MB-231 with or without enforced HOXC8 transgene expression. Arrows indicate metastatic lesions. Top panels: lower magnifications; bottom panels: higher magnifications; scale bar: 100 μm.

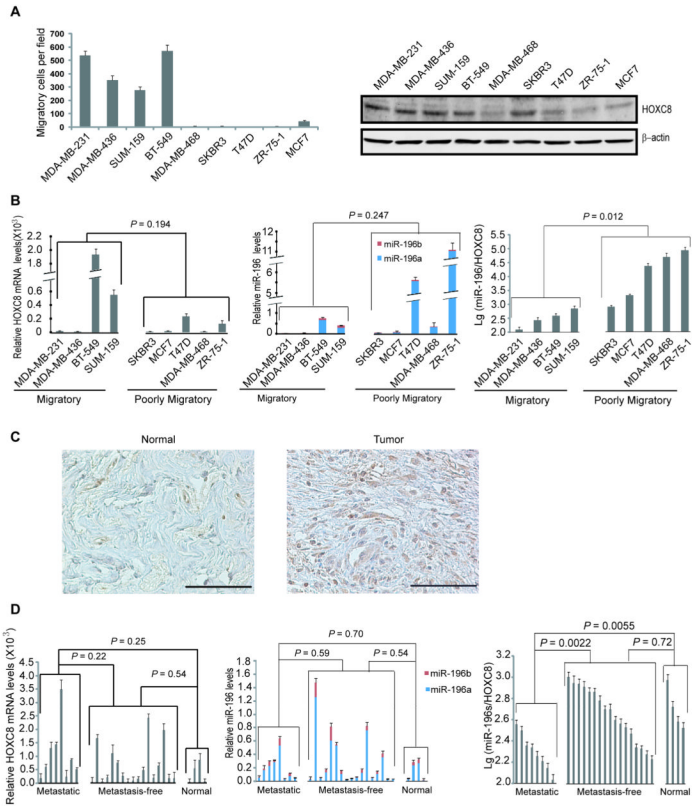

Figure 6. Ratio of miR-196s to HOXC8 mRNA correlates to migration of established breast cancer cell lines and metastatic status of clinic breast tumor specimens

A. Portions of overnight-cultured MDA-MB-231, MDA-MB-436, SUM-159, BT549, MDA-MB-468, SK-BR3, T47D, ZR-75-1 and MCF7 cells were detached with 10mM EDTA, resuspended in serum-free medium and analyzed for cell migration using Transwells as described in Materials and Methods. Values are means ± SEM. The remaining cells were lysed and cell lysates subjected to immunoblotting to detect HOXC8 and βactin proteins with the respective antibodies. ***B.*** Total RNA was isolated from these cell lines and then subjected to qRT-PCR to measure the levels of miR-196s (miR-196a + miR-196b) and HOXC8 mRNA. Data were normalized to the levels of GAPDH mRNA. Values are means ± SEM. The ratio of miR-196s to HOXC8 mRNA was calculated using formula Lg(miR-196s/HOXC8). ***C.*** Immunohistochemistry of HOXC8 in normal breast and breast tumor tissues (two different magnifications; scale bar: 100μm). **D.** Total RNA was isolated from frozen normal breast tissue and primary breast tumor specimens and then subjected to qRT-PCR to measure the levels of miR-196s (miR-196a + miR-196b) and HOXC8 mRNA. Data were normalized to the levels of GAPDH mRNA. Values are means ± SEM. The ratio of miR-196s to HOXC8 mRNA was calculated using formula Lg(miR-196s/HOXC8).