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WAVE3, an actin remodeling protein, is regulated by the metastasis suppressor microRNA, miR-31, during the invasion-metastasis cascade

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Abstract

WAVE3, an actin cytoskeleton remodeling protein, is highly expressed in advanced stages of breast cancer and influences tumor cell invasion. Loss of miR-31 has been associated with cancer progression and metastasis. Here we show that the activity of WAVE3 to promote cancer cell invasion is regulated by miR-31. An inverse correlation was demonstrated between expression levels of WAVE3 and miR-31 in invasive *versus* non-invasive breast cancer cell lines. miR-31 directly targeted the 3'-UTR of the *WAVE3* mRNA and inhibited its expression in the invasive cancer cells; i.e., miR-31-mediated down-regulation of *WAVE3* resulted in a significant reduction in the invasive phenotype of cancer cells. This relationship was specific to the loss of *WAVE3* expression since re-expression of a miR-31-resistant form of *WAVE3* reversed miR-31-mediated inhibition of cancer cell invasion. Furthermore, expression of miR-31 correlates inversely with breast cancer progression in humans, where an increase in expression of miR-31 target genes was observed as the tumors progressed to more aggressive forms. In conclusion, a novel mechanism for the regulation of *WAVE3* expression in cancer cells has been identified, which controls the invasive properties of cancer cells. The study also identifies a critical role for *WAVE3*, downstream of miR-31, in the invasion-metastasis cascade.

Keywords

WAVE3; miR-31; breast cancer; invasion-metastasis cascade

Introduction

Cancer metastasis is a complex process, requiring tumor cells to escape from a primary site, circulate in the blood or lymphatic systems, and then re-establish new tumors at favored niches. The mechanisms that control the invasion-metastasis cascade and ultimately leads to spread of cells to distant sites remain poorly understood. We have shown that the *WAVE3* protein, whose activity is critical for the regulation of actin cytoskeleton dynamics through activation of the Arp2/3 complex, is integrally involved in cancer cell motility, invasion and

metastasis (1–5). microRNAs have been established as master modulators of gene expression, and as such, are able to regulate diverse and complex cellular pathways ranging from embryonic development and wound healing to cancer cell invasion and metastasis (6–8). In fact, a single microRNA, miR-31, has recently been shown to concurrently modulate the expression of multiple target genes, and as a result, affect several aspects of the invasion-metastasis cascade including motility, invasion and resistance to anoikis (9;10).

In a previously published study, we reported a highly significant correlation between the expression levels of WAVE3 and advanced stages of breast cancer (4), clearly supporting the function of WAVE3 as a metastasis promoter protein (11–13). However, mechanisms for regulation of WAVE3 expression levels during tumor progression are unresolved. In our studies to dissect the role of WAVE3 in cancer cell invasion and metastasis, we established a possible role of WAVE3 during the epithelial to mesenchymal transition (EMT) (14), an early prerequisite step during the invasion-metastasis cascade (15;16). Regulation of WAVE3 during EMT was mediated by the miR-200 family of microRNAs (8;17–19). In the present report, we show that the function of WAVE3 is also regulated during the late stages of the invasion-metastasis cascade by microRNA miR-31. An inverse correlation between WAVE3 and miR-31 microRNA expression levels is observed in invasive versus non-invasive breast cancer cell lines, and this inverse correlation extends to human primary breast tumors. Our data support a new model in which regulation of WAVE3 expression by two different microRNAs, miR-200b and miR-31, plays a key role in controlling the invasion-metastasis cascade of cancer cells.

Materials and Methods

Materials

The antibodies used in this study were: rabbit anti-WAVE3 from New England Peptide, Inc.; rabbit anti-Integrin $\alpha 5$, rabbit anti-RhoA (67B9) and rabbit anti-Radixin (C4G7) from Cell Signaling Technology; mouse anti-E-Cadherin and mouse anti-Vimentin from BD Biosciences; rabbit anti-GFP from Clontech Laboratories; goat HRP-conjugated anti-mouse IgG and goat HRP-conjugated anti-rabbit IgG from Calbiochem. We used the On-Targetplus SMARTpool (Thermo Scientific) siRNA L-1012301-00-0010 to knockdown the expression of WAVE3. Pre-miRNA miR-31 precursors, anti-miR-31 inhibitor and negative control miRNA were from Life Technologies and were used as per the manufacturer's instructions.

Human breast cancer specimens

Primary tumors and matched normal tissues were collected and processed under an approved IRB protocol from the Cleveland Clinic. A total of 8 grade I and 11 grade III breast invasive ductal carcinomas and their respective matched normal tissues were collected. When redundant tissue was available from resection specimens, tumor and distant adjacent normal tissue were identified grossly, snap frozen and stored at -80°C . Before using these samples, quality assurance was performed by embedding them individually in OCT mounting media and preparing a frozen section hematoxylin and eosin (H&E) stained slide. Tumors were graded using the standard Bloom-Richardson grading system. For the tumor block, the frozen section slides were examined and if non-tumor tissue was present in addition to the neoplasm, it was trimmed to obtain the highest percent tumor cellularity within a given block. A summary of other clinical characteristics of these tumors is presented in supplemental Table S1. Normal tissue slides were also reviewed to ensure that only non-neoplastic breast tissue was present. The frozen tissue block was then sectioned on the cryostat to obtain 15 ten micron sections.

Cell Culture, transfections and immunoblotting

The established human cancer cell lines were obtained from American Type Culture Collection and cultured as previously described (14). Transient transfections and immunoblotting were performed as described previously (2–4;14).

Semi-quantitative and Real-time quantitative–PCR

Total RNA was extracted from established cancer cell lines and frozen tissue sections using TRIzol reagent (Invitrogen), following to the manufacturer's instructions. cDNA was generated and used as a template for semi-quantitative RT-PCR performed as previously described (3;4;14;20). Expression levels of microRNAs were quantified by real-time quantitative RT-PCR using the human TaqMan MicroRNA Assays Kits (Applied Biosystems). The reverse transcription reaction was carried out with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Quantification of WAVE3, RDX, ITGA5, RhoA, E-Cadherin and GAPDH expression levels was performed using the respective gene-specific primers (Table S2) and the RT² SYBR Green/Fluorescein qPCR Master Mix (SABiosciences) following the manufacturer's instructions. Quantitative PCR was performed on the BioRad iCycler PCR system where the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The cycle threshold (Ct) values were calculated with SDS 1.4 software (Bio-Rad). The expression levels of each transcript were normalized using the $2^{-\Delta\Delta Ct}$ method (21;22) relative to GAPDH. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the transcript of interest. The $\Delta\Delta Ct$ was then calculated by subtracting ΔCt of the matching normal human breast tissue from the ΔCt of cancer tissues, or the ΔCt of MCF10A cell line for the established cancer cell lines. Fold change in the gene was calculated according to the equation $2^{-\Delta\Delta Ct}$.

Plasmid Construction, Site-directed Mutagenesis and WAVE3 3'UTR reporter analysis

The GFP-WAVE3 fusion expression vector construction was previously described (20). The 3' untranslated region (3'UTR) of WAVE3 was PCR-amplified using WAVE3 cDNA IMAGE clone 4838122 as a template, and subcloned into pmirGlo vector downstream of the firefly luciferase expression cassette (Promega). The correct sequence and orientation were verified by sequencing. The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate the WAVE3 3'UTR mutant where the seed sequence recognized by miR-31 was deleted. pmirGlo reporter plasmids (1 µg total plasmid) were transfected with Lipofectamine 2000 (Invitrogen) into the specified cancer cells then seeded in 12-well plates (3×10^4 cells per well). Cells were collected after 48 h for assay using the Dual-Luciferase reporter assay system (Promega). For cotransfection experiments, synthetic microRNA mimics or miR Negative Control (5 nM), were added to the transfection mix. All experiments were performed in triplicate with data averaged from at least three independent experiments.

Matrigel Invasion Assay

The invasive potential of the parental and transfected cells was assessed using the Matrigel invasion chambers from BD Biosciences as described (3;4;14).

Oligonucleotide sequences

Sequences of the oligonucleotide primers used for genomic PCR, RT-PCR, to amplify the entire WAVE3 3'UTR or the individual seed sequences, as well as the primers used for mutagenesis were from IDT (San Diego, CA) and are listed in supplementary Table S2.

Statistical analysis

The data are presented as the mean $\pm$ standard error of the mean of at least three independent experiments. The results were tested for significance using the unpaired Student's *t* test as well. A two way ANOVA test was applied to the Matrigel invasion data from re-expression of WAVE3 in the miR-31-expressing cells.

Results

WAVE3 expression is inversely correlated to miR-31 overexpression in invasive versus non-invasive cancer cell lines

We had previously performed *in silico* analysis (23–25) to search for potential binding sites for microRNAs in WAVE3 (14) and noted that miR-31 has a single target site in the 3'UTR of WAVE3 (Fig. 1A), which is a perfect match to the seed sequence of miR-31 (Fig. 1B). The potential relationship between miR-31 and WAVE3 became particularly noteworthy in view of the recent reports of the major role of miR-31 in cancer metastasis (9;10) and the role of WAVE3 in cancer progression and metastasis (3;4;12–14). To investigate whether miR-31 has a role in the regulation of WAVE3 activity in cancer cell invasion and metastasis, we first determined whether there is a correlation between the expression levels of WAVE3 and miR-31. Quantitative real-time RT-PCR was used to determine the expression levels of WAVE3 and miR-31 in breast cancer cell lines that have been extensively characterized in terms of invasive, tumorigenic and metastatic properties. The ratio of WAVE3 to miR-31 expression levels is displayed in Figure 1C. A clear and significant inverse correlation in expression levels of miR-31 and WAVE3 in invasive versus non-invasive breast cancer cell lines was observed. In the invasive and metastatic MDA-MB-231, MDA-MB-435 and BT549 breast cancer cell lines, which express high levels of WAVE3, only low levels of miR-31 could be detected. In contrast, in non-invasive T-47D, MCF7 cell lines and the normal breast MCF10A cells, miR-31 was expressed at high levels, while WAVE3 could be detected at low levels. We also analyzed the WAVE3/miR-31 ratio in the invasive LNCaP prostate cancer cells where miR-31 levels were also low.

To directly test the relationship between the miR-31 target site in the 3'UTR of WAVE3 and posttranscriptional repression of WAVE3, we introduced miR-31 into MDA-MB-231 and LNCaP cells and assayed for WAVE3 expression levels. Overexpression of synthetic precursors of miR-31 in either MDA-MB-231 (Fig. 1D and 1F) or LNCaP cells (Fig. 1E and 1G) resulted in a significant decrease in WAVE3 expression compared to control cells, whereas no difference in expression levels of GAPDH were detected between the control cells and miR-31-transfected cells. The effect of miR-31 on WAVE3 expression was also confirmed at the protein level by Western blots (Fig. 1H and 1I). These results therefore verify the posttranscriptional repression of WAVE3 by miR-31.

WAVE3 was reported to be present in a protein complex that also contains Abi1, NCKAP1 and CYFIP1 (26). The presence of WAVE3 in such multi-complex was suggested to keep WAVE3 in an auto-inhibited state in resting cells (26;27). WAVE1 and WAVE2, two other members of the WAVE/WASP family, were also suggested to be associated to this protein complex (28;29). We therefore investigated whether the regulation of WAVE3 expression downstream of miR-31 alters the expression levels of other members of the WAVE complex, which might in turn affect the activity of entire complex. Quantitative real-time RT-PCR of RNAs from MDA-MB-231 and LNCaP cells that were transiently transfected with miR-31, showed a ~50% decrease in WAVE3 expression in the miR-31-overexpressing cells compared to the control cells while no change in expression levels was observed in any of the other members of the WAVE complex analyzed (Fig. S1).

miR-31 directly targets the 3'UTR of WAVE3 and represses its expression

We used the Firefly-Renilla dual luciferase reporter gene assay to demonstrate that the posttranscriptional repression of the WAVE3 transcript is caused by direct binding of miR-31 to its target seed sequence within the 3'UTR of WAVE3. The entire 3'UTR of WAVE3 was subcloned within the 3'UTR of the luciferase gene into the pmirGlo vector, and luciferase activity was measured in MDA-MB-231 breast cancer cells which express very low or no levels of miR-31. We found no difference in luciferase activity between MDA-MB-231 cells transfected with control vector alone or control vector cotransfected with either miR-31 or control microRNA (Fig. 2A), clearly demonstrating the lack of miR-31 effect in the absence of its specific target sequence. In contrast, overexpression of miR-31 resulted in a ~60% reduction in luciferase activity in MDA-MB-231 cells transfected with the WAVE3 3'UTR-containing vector compared to the cells transfected with the same vector in the absence of miR-31 or cotransfected with control microRNA (Fig. 2B). As an independent approach to confirm that miR-31 specifically targets and binds to its seed sequence in the 3'UTR of WAVE3 and inhibits its expression, we deleted the seed sequence of miR-31 in the 3'UTR of WAVE3. This maneuver abrogated the effect of the exogenous miR-31 in MDA-MB-231 cells (Fig. 2C). Similar results were obtained when these experiments were repeated in LNCaP prostate cancer cells (Fig. S2), demonstrating that the effect of miR-31 is not restricted to a single cell line but appears to extend to other cancers. Together, these results clearly show that miR-31 specifically targets and represses WAVE3 expression.

miR-31-mediated downregulation of WAVE3 expression inhibits cancer cell invasion

We investigated the effects of miR-31-mediated suppression of WAVE3 on the invasive phenotype of tumor cells using a Matrigel assay. A ~75% reduction in the number of cells invading the Matrigel-covered membrane was observed for both MDA-MB-231 (Fig. 3A) and LNCaP cells (Fig. 3B) treated with siRNA against WAVE3, compared to the control cells. These results are consistent with our previously published studies for MDA-MB-231 cells (3;4;14). Similar numbers (~65% reduction in invasion) were obtained with overexpression of miR-31 in these two cell types (Fig. 3A and 3B); clearly demonstrating that suppression of WAVE3 levels using siRNA or miR-31 reduces the invasive capability of cancer cells.

In Fig. 1, we showed that MCF7, a non-invasive breast cancer cell line, expresses high levels of miR-31 and low levels of WAVE3. We next investigated the effect of loss of endogenous miR-31, by transient overexpression of anti-miR-31 in MCF7 cells, on the expression levels of WAVE3 and the invasion potential of these cells. Loss of endogenous miR-31 resulted in more than a ten-fold increase in the number of invasive MCF7 cells compared to mock-transfected or control cells (Fig. 3C & 3D). The increase invasion of the anti-miR31-expressing cells was also associated with a ~2-fold increase in expression levels of WAVE3 mRNA (Fig. 3E & 3F). Thus, the acquisition of an invasive phenotype of the otherwise non-invasive MCF7 cells might be the result, at least in part, of lifting the inhibition of WAVE3 expression imposed by miR-31.

Re-expression of miR-31-resistant WAVE3 overcomes the miR-31 inhibition of invasion

microRNAs can regulate expression of several genes simultaneously, a characteristic that also applies to miR-31, which was reported to concomitantly inhibit expression of several key genes involved in cancer invasion and metastasis (9;10). Therefore, we investigated whether the inhibition of cancer cell invasion is the result of the specific downregulation of WAVE3 and not a generalized effect of miR-31. Accordingly, we determined whether inhibition of cancer cell invasion by miR-31 could be rescued by restoring WAVE3 expression. MDA-MB-231 cells were transfected with a WAVE3 expression construct

which does not contain the target seed sequence for miR-31, and is therefore refractory to miR-31. As a control, the WAVE3 expression construct was also transfected into siWAVE3-expressing MDA-MB-231 cells. As expected, WAVE3 expression levels were significantly reduced in the siWAVE3-expressing cells, whereas no significant changes were observed in the miR-31-expressing cells or in the cells with the control transfections (Fig. 4A, 4B and Fig. S3). Also as expected, MDA-MB-231 cells expressing either endogenous or exogenous WAVE3 showed a significant reduction in their invasive activity in the presence of siRNA against WAVE3, reflective of the siWAVE3-mediated downregulation of WAVE3 expression. Invasion of MDA-MB-231 cells was also inhibited in cells when endogenous WAVE3 was targeted by miR-31. The invasion defect associated with miR-31 expression was, however, completely rescued by re-expression of WAVE3 (Fig. 4C and 4D). These results were also reproduced in the LNCaP prostate cancer cells (Fig. S4).

Expression of miR-31 correlates inversely with human breast cancer progression

Since established cell lines cannot fully capture the clinical characteristics of tumors, we extended our analyses to human breast cancer specimens. Valastyan and colleagues compared the expression levels of miR-31 between primary breast tumors and distant metastases and reported that miR-31 expression correlates inversely with metastasis (9). No information was provided on how miR-31 expression fluctuates during the progression of primary tumors from low to high grade. We therefore investigated the changes in expression levels of miR-31 and its target WAVE3 in eight grade I (Fig. 5A) and 11 grade III (Fig. 5B) tumors using real-time quantitative RT-PCR. Expression of miR-31 was reduced by more than 80% in the more advanced grade III tumors (Fig. 5C). The ER, PR and HER2 status in breast cancer patients is critical for predicting the outcome of the disease, where the triple-negative tumors are often associated with poor prognosis and worst disease outcome (30). Among the 11 grade III tumors analyzed, five were triple-negative (Table S1). Interestingly, these tumors expressed significantly low levels of miR-31 compared to their counterparts expressing ER and PR, and no amplification of HER2 (data not shown). On the other hand, loss of miR-31 expression in the grade III tumors was associated with a ~5-fold increase in WAVE3 expression (Fig. 5D). We also found a significant reduction in the expression levels of miR-200b in the more advanced tumors, as an indication of the extensive EMT that takes place in the grade III tumors (Fig. 5E). We used miR-16 as a control for our quantifications (Fig. 5F) since no link between the expression levels of miR-16 and breast cancer has been reported.

Expression of other breast cancer metastasis-relevant genes also correlates with breast cancer progression

miR-31 was also reported to target RhoA, RDX and ITGA5, a set of breast cancer metastasis related genes (9;10). First, we confirmed the miR-31-mediated suppression of these genes. When miR-31 was introduced into MDA-MB-231 and LNCaP cells, neither of which expresses miR-31, expression levels of RDX, ITGA5 and WAVE3 were significantly reduced in both cell lines (Fig. 6A and 6B). On the other hand, knockdown of WAVE3 expression in both MDA-MB-231 and LNCaP cells, using siRNA specifically targeting WAVE3, had no effect on the expression levels of the other miR-31 target genes. In the breast cancer specimens, expression levels of ITGA5 (Fig. 6C) and RhoA (Fig. 6D) were also found to correlate with breast cancer progression where a two-fold increase in the levels of both genes was seen in grade III tumors. We did not detect any significant changes in the expression levels of RDX between grade I and grade III tumors (Fig. 6E), which might not be detected in our small cohort. On the other hand, E-Cadherin, which is lost during the mesenchymal to epithelial transition and is required for acquisition of the invasive phenotype of cancer cells (17;19;31;32), was significantly reduced as tumors progressed from the lower to higher grade (Fig. 6F).

Discussion

Metastasis, the main cause of death in cancer patients, is a multi-step process requiring a sub-population of cancer cells from the primary tumor to acquire invasive properties by first undergoing a profound morphological change, the epithelial-to-mesenchymal transition. These cancer cells must then enter and survive in the blood and lymphatic systems until reaching their target organ where they extravasate and proliferate. For a tumor to successfully metastasize, orchestrated changes in the expression of several genes are required, and such coordination may be achieved by microRNAs which function as master regulators of gene expression. microRNAs have the unique ability to regulate gene networks both in normal physiological and pathological conditions, including during cancer metastasis (7–10;16;33;34). In the present study, we identify miR-31, a powerful regulator of the invasion-metastasis cascade (9;10), as being directly involved in WAVE3 regulation. Several signaling pathways are known to be activated during the multiple steps of the invasion-metastasis cascade, including those regulating the remodeling of actin cytoskeleton, cell motility, migration and cancer cell invasion (35), processes in which WAVE3 has been implicated.

Based on these relationships, we hypothesized that WAVE3 might be regulated by miR-31 to promote cancer progression and metastasis, and we used multiple functional assays to test this hypothesis. First, semi-quantitative RT-PCR, real-time quantitative RT-PCR and western blotting showed a clear inverse correlation between the expression levels of WAVE3 and miR-31 in invasive *versus* non-invasive breast cancer cell lines. We also confirmed this inverse correlation in low-grade versus high-grade human primary breast tumors. Second, overexpression of miR-31 mimics in aggressive cancer cells resulted in a significant downregulation of WAVE3 expression levels, concomitant with a loss of the invasive potential of these cells. Third, deletion of the seed sequence for miR-31 in the 3'UTR of the WAVE3 cDNA abrogated the miR-31-mediated inhibition of reporter gene activity, clearly demonstrating that miR-31 directly targets the 3'UTR of WAVE3 and represses its expression. Fourth, we showed that the loss of the invasive phenotype associated with the forced expression of miR-31 was completely rescued by re-expression of exogenous miR-31-resistant WAVE3. Hence, inhibition of cancer cell invasion is a result of the specific loss of WAVE3, independent of other genes targeted by miR-31. Finally, we showed that loss of expression of miR-31, which exerts an inhibitory effect on cancer progression target genes, takes place early as the cancer advances from lower to higher grade.

Several studies have determined that the expression levels of miR-31 are reduced in human breast cancers, and the entire miR-31 locus is deleted in other tumors. From these studies, a clear correlation has emerged between the loss of miR-31 expression and tumor aggressiveness (36–38). miR-31 was recently found to specifically modulate the expression levels of a specific set of genes that enhance the metastatic properties of tumor cells (9;10). Conversely, a key role for WAVE3 in cancer progression and metastasis has also been established (3;4;11–14). For example, WAVE3 expression levels were increased more than two-fold in a subpopulation of invasive cancer cells derived from the bulk of primary breast cancer tumor (13), and stable knockdown of WAVE3 significantly impeded the metastatic potential of aggressive breast cancer cells in experimental and spontaneous metastasis models in mice (4). Consequently, upon identifying a conserved seed sequence for miR-31 in the 3'UTR of WAVE3 (14) we speculated that miR-31 and WAVE3 expression may be interrelated. In the present study, we show that WAVE3 is indeed specifically targeted and repressed by miR-31, which in turn leads to suppression of WAVE3-mediated cancer cell invasion.

Members of the WAVE protein family that includes WAVE1, WAVE2 and WAVE3, were shown to be present in a protein complex that also contains Abi1, NCKAP1 and CYFIP1 (26;28;29;39;40). The sequestering of the WAVE proteins in such complex was suggested as a mechanism to maintain the entire WAVE complex in an inactive state (26;27). Silva and colleagues have recently reported that reduced expression of CYFIP1, a member of the WAVE complex, was associated with a poor prognosis for epithelial tumors (41), and proposed that CYFIP1 functions as an invasion suppressor gene by affecting the activity of the entire complex. This observation is not in conflict with the role of WAVE3 as a metastasis promoter gene (3;4;12;14;20). Silva et al. used MCF10A normal mammary cells as a model and these cells express very low levels of WAVE3 (Fig. 1 and ref (3;4;14) compared to WAVE1 and WAVE2 and, therefore, may have no or very little contribution to the overall activity of the entire complex in these cells. We have also shown that knockdown of WAVE3 expression downstream of miR-31 has no effect on the expression levels of the remaining members of the WAVE complex (Fig 1S), clearly supporting the specific contribution of WAVE3 to the invasion phenotype independent of the other members of the WAVE complex. Furthermore, there are several reports in the literature describing different functions of the each member of the WAVE complex even when they are present in the same cell type (39;40;42–47), thus suggesting that individual members of the WAVE complex may have specific functions independent of the complex.

miR-31 has also been found to target genes other than WAVE3 with relevance to cancer cell invasion and metastasis (9;10), including RhoA, RDX and ITGA5, which are highly expressed in aggressive forms of human cancers (48–50). Valastyan and colleagues showed that re-expression of individual miR-31 target genes in miR-31 over-expressing cancer cells can, to variable degrees, reverse the phenotype imposed by miR-31 over-expression (9;10). Our results show that expression of a miR-31 resistant form of WAVE3 in miR-31 over-expressing aggressive cancer cells results in a complete rescue of the invasive phenotype of these cells, indicating that WAVE3 function is required for the regulation of cancer cell invasion independent of the other miR-31 effectors genes.

We investigated the changes in expression levels of miR-31 and WAVE3 during the progression of the primary human breast tumors from a low to a high and more aggressive grade and found a very significant correlation between WAVE3 mRNA levels and tumor grade. As shown in the present study, the opposite scenario applies to miR-31: a significant inverse correlation in grade I versus grade III tumors was found. Previous studies compared the expression levels of miR-31 between primary and matched breast cancer metastases and reported an inverse correlation (9). Our findings show that changes in miR-31 expression levels take place early during the progression of the primary tumor to a more aggressive and a metastasis-prone tumor. Loss of miR-31 and consequently, an increase in WAVE3 levels set the stage for a subpopulation of cells from the primary tumor to acquire the invasive properties needed to complete the various steps within the invasion-metastasis cascade. The results from the present study, together with our published data on the regulation of WAVE3 downstream of miR200, support a dual role of WAVE3 during cancer progression and metastasis (Fig. 7). Our previous studies have shown that WAVE3 is also repressed by other microRNAs, the miR-200 family (14). The members of this family of microRNAs are major regulators of the EMT process, which is a required event during the early steps of the invasion-metastasis cascade (14). The present study supports a possible implication of WAVE3 in the late steps of the invasion-metastasis cascade when miR-31 expression is suppressed (see Fig. 7). Because of its role in regulation of the actin cytoskeleton, it is not surprising that WAVE3 is involved in both early and late steps of the invasion-metastasis cascade. During the EMT process, loss of the miR-200 microRNAs results in downregulation of E-Cadherin at cell junctions with a concomitant increase in cell motility that is attributable to the increase in activity of the actin polymerization and cytoskeleton

remodeling proteins such as WAVE3. However, later during the invasion-metastasis cascade process, a significant increase in invasion capability is required for the cancer cells to invade the tissue surrounding the primary tumor and successfully accomplish the critical steps of the invasion-metastasis cascade, thus establishing new tumors in the target sites.

To further dissect the role of WAVE3 during the invasion-metastasis cascade, downstream of both miR-200b and miR-31, in vivo analyses and further studies of human tissue samples are warranted to follow up to these studies. Understanding the regulatory mechanisms of WAVE3 as a metastasis-promoter gene is a critical step towards the elucidation of its role in cancer invasion and metastasis. This information is also of potential clinical significance in that it provides clues on how to inhibit the abnormally elevated WAVE3 expression levels in cancer in order to prevent metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. WAVE3 expression is elevated in cell lines with low levels of miR-31 microRNA. (A) Domain structure of the WAVE3 transcript showing the location of the seed sequence of miR-31 within the 3'UTR. (B) Alignment of the mature sequence of miR-31 shows a perfect match to a target sequence in the 3'UTR of WAVE3 cDNA, accession number NM_006646. (C). Inverse correlation between miR-31 and WAVE3 in invasive versus non-invasive cancer cell lines as determined by quantitative real-time RT-PCR. (D and E) Semi-quantitative RT-PCR analysis, (F and G) quantitative real-time RT-PCR analysis and (H and I) western blotting analysis of WAVE3 expression in LNCaP and MDA-MB-231 cells, respectively, transfected with miR-31 or siRNA against WAVE3. WAVE3 expression levels were markedly reduced in the miR-31- and siWAVE3-transfected cells. GAPDH was used as a control. *, $p < 0.01$ compared to untransfected or control-transfected cells.

Figure 2. microRNA miR-31 directly targets the 3'UTR of WAVE3 and represses its expression. (A). Control experiment where firefly luciferase reporter plasmid pmirGlo control, was transiently transfected into MDA-MB-231 cells in the presence or absence of miR-31 or the control microRNA, and luciferase activities were measured after 48 h. (B) pmirGlo vector containing the entire 3'UTR of WAVE3 (W3-3'UTR), transiently transfected into MDA-MB-231 cells, in the presence or absence of miR-31 or the control microRNA, and luciferase activities were measured after 48 h. (C) pmirGlo vector containing WAVE3-3'UTR with deleted miR-31 seed sequence (W3-3'UTRd31), transiently transfected into MDA-MB-231 cells, in the presence or absence of miR-31 or the control microRNA, and luciferase activities were measured after 48 h. For all luciferase assays, Renilla luciferase activity was used for normalization. The data are the mean $\pm$ s. d. of at least 3 independent transfections. *, $p < 0.01$ compared to untransfected or control-transfected cells.

Figure 3. miR-31-mediated downregulation of WAVE3 expression inhibits cancer cell invasion in invasive cancer cells, while downregulation miR-31 increases invasion in non-invasive cancer cells. Quantification of invasive cells using a Matrigel invasion assay of MDA-MB-231 (A) and LNCaP (B) cells, transfected with either siRNA to WAVE3 or miR-31 precursor. (C) real-time quantitative RT-PCR and (D) Semi-quantitative RT-PCR quantification of WAVE3 in MCF7 cells transfected with either control microRNA or miR-31. (E) Quantification of invasive MCF7 cells transfected with either control microRNA or miR-31. Representative micrographs of invasive cells are shown in (F). At least 6 different fields were counted from each experiment. The data are the mean $\pm$ s. d. of at least 3 independent assays. *, $p < 0.01$ compared to untransfected or control-transfected cells.

Figure 4. Re-expression of miR200-resistant WAVE3 reverses miR31-mediated inhibition of LNCaP cell invasion. **(A)** Immunoblotting (IB) with the indicated antisera of MDA-MB-231 cells over-expressing GFP or GFP-WAVE3 fusion protein, and co-transfected with either siWAVE3 or miR-31. **(B)** IB with the indicated antisera of MDA-MB-231 cells over-expressing GFP or GFP-WAVE3 fusion protein, and co-transfected with either control siWAVE3 or control miR. **(C and D)** Quantification of invasive cells for the indicated treatment. At least 6 different fields were counted from each experiment. The data are the mean $\pm$ s. d. of at least 3 independent assays. *, $p < 0.01$ compared to untransfected or control-transfected cells.

Figure 5.

Expression of miR-31 correlates inversely with human breast cancer progression. (A) H&E staining of a representative Bloom-Richardson grade I invasive ductal carcinoma. At 1.5x, greater than 75% tubule formation is shown (150x original magnification). At 20x, the malignant glands are composed of uniform nuclei with fine chromatin (200x original magnification). At 40x, higher power detail of the uniform grade I nuclei that compose the carcinoma. The nuclei have fine chromatin, smooth nuclear membranes, and inconspicuous nucleoli. Mitotic figures are not readily identifiable (400x original magnification). (B) H&E staining of a representative Bloom-Richardson grade III invasive ductal carcinoma. At 1.5x, illustration of less than 10 tubules formed within this haphazard arrangement of malignant epithelium in a desmoplastic stroma (150x original magnification). At 20x, malignant cells display nuclear variability in size and shape and identifiable nucleoli (200x original magnification). At 40x, higher power detail of the grade III nuclei with marked variability in nuclear size and shape, nuclear membrane irregularities and readily identifiable nucleoli. Multiple mitotic figures are easily seen within this high-power field (400x original magnification). (C, D, E and F) Quantitative Real-time RT-PCR of miR-31, WAVE3,

miR-200b and miR-16, respectively, in human primary Bloom-Richardson grade I and grade III invasive ductal carcinoma. The expression levels of each gene were normalized to GAPDH and plotted as fold change between the tumor and the corresponding normal tissue.

Figure 6. Expression of other breast cancer metastasis-relevant genes also correlates with breast cancer progression. (A and B) Western blotting analysis of WAVE3, RDX and ITGA5 in MDA-MB-231 (A), and LNCaP (B) with the indicated transient treatments. β -Actin was used as internal control for loading consistency. (C, D, E, and F) Quantitative Real-time RT-PCR of ITGA5, RhoA, RDX and E-Cad, respectively, in human primary Bloom-Richardson grade I and grade III invasive ductal carcinoma. The expression levels of each gene were normalized to GAPDH and plotted as fold change between the tumor and the corresponding normal tissue.

Figure 7. Mechanisms of WAVE3 regulation in cancer metastasis. Model illustrating the regulation of WAVE3 during the invasion-metastasis cascade. WAVE3 is regulated at the posttranscriptional level during the EMT process by miR-200, which results in increased cell motility, and, during the late stage of the invasion metastasis cascade, by miR-31 which results in increase in the invasive phenotype that is critical for tumor metastasis.