A novel estrogen receptor-microRNA 190a-PAR-1-pathway regulates breast cancer progression, a finding initially suggested by genome-wide analysis of loci associated with lymph-node metastasis

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To identify microRNAs that are important in regulating breast cancer progression, the present study used data for the 199 961 single-nucleotide polymorphisms (SNPs) in 837 breast cancer patients genotyped in a recent genome-wide association study to identify loci associated with lymph node metastasis (LNM). SNPs tagging the 15q22.2 locus showed a significant association with LNM and miR-190a was found to be the only microRNA in this region. The role of miR-190a in LNM was supported by the findings that increased miR-190a expression inhibited cell migration and invasiveness and that the target of miR-190a was protease-activated-receptor 1 (PAR-1), which is a metastasis promoting protein in several cancers. In addition, the promoter region of miR-190a was defined and found to contain half of an estrogen response element, suggesting that miR-190a is regulated by estrogen receptor (ER) signaling. This was confirmed by the findings that miR-190a expression was activated by 17β-estradiol and that ERα bound directly to this promoter. The importance of this ERα-miR190a-PAR-1 link in breast tumorigenesis is suggested by the findings of (i) an association between genetic polymorphism of the miR-190a-containing region and LNM that is modified by SNPs of PAR-1 and is particularly significant in ERα-positive patients and (ii) a combined effect of ERα and miR-190a expression on tumor grade/cancer stage. More importantly, the level of miR-190a expression in primary breast carcinomas correlated with overall survival. These findings suggest a novel pathway in which ERα signaling regulates miR-190a expression, causing inhibition of PAR-1 expression, correlated with inhibition of cancer metastasis.

INTRODUCTION

The move from candidate gene association studies to genome-wide association studies (GWAS) has made it possible to explore the etiological contribution of genetic variants throughout the whole genome without relying on an a priori hypothesis. The Breast Cancer Association Consortium, the largest international consortium involved in breast cancer association studies with the aim of exploring genetic susceptibility to breast cancer, recently reported the results of a GWAS based on 10 052 women with incident breast cancer and 12 575

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controls that showed an association between increased risk of breast cancer and genotypic polymorphism at more than 40 genomic loci (1–3). In these publications, Asian women with breast cancer, including our own 837 patients, were found to have a similar at-risk single-nucleotide polymorphism (SNP) profile as western women, but population-specific loci were also detected (3). Furthermore, GWAS may be useful not only in predicting disease incidence, but also in exploring genetic susceptibility to disease progression or treatment efficacy. Therefore, the present study, aiming to identify microRNAs (miRNAs) that are important in regulating breast cancer progression (4), consisted of two phases, and, in the hypothesis-generating phase, used our genotyping data included in these GWASs to examine the loci associated with lymph node metastasis (LNM) in breast cancer. We were particularly interested in LNM because it is one of the earliest events during breast cancer metastasis (5), and a better understanding of how it occurs will help in the development of drug targets to prevent breast cancer mortality. The second phase, including a thorough examination of whether any known miRNAs were located within the same genetic region [i.e. linkage disequilibrium (LD) block] harboring these significant loci and functional exploration of these candidate miRNAs, yielded valuable information about the role of miRNAs in regulating breast cancer progression. Our findings suggest a novel pathway in which estrogen receptor α (ERα) regulates expression of miR-190a, an miRNA located adjacent to significant loci at 15q22.2, which can inhibit synthesis of the metastasis promoting protein, protease-activated-receptor 1 (PAR-1) and consequently inhibit breast cancer metastasis.

RESULTS

Genome-wide screening for single nucleotide polymorphisms associated with LNM suggests a role for miR-190a in regulating breast cancer metastasis

In order to identify miRNAs playing a critical role in regulating breast cancer metastasis, in the present study, we correlated the data for the 199 961 SNPs genotyped in each of our patients in a recent GWAS with the LNM status of the patients and found that 8364 SNPs were significantly associated with LNM (P < 0.05 for the comparison of the heterozygous/homozygous wild-type groups and homozygous variant group) (Fig. 1A and B). We examined the association based on a recessive model and an additive model and found that the two models gave consistent results. However, we considered that the subtle effects caused by variant alleles of SNPs would be more easily detected in the homozygous variants (recessive model) and therefore used this model, especially in the hypothesis-generating phase. We next examined whether any known miRNAs were located adjacent to these significant SNPs, as genetic variants at sequences near miRNAs might affect the transcription factor-promoter/enhancer interaction, resulting in altered miRNA expression. To do so, we used the bioinformatic tool GenePipe (6) and data generated in large collaborative initiatives, including the 1000 Genome Project and the International Cancer Genome Project. The region 50 kb 5’ to 50 kb 3’ of individual significant SNPs was checked and the results showed that, of the total of more than 1100 known miRNAs, 62 were located adjacent to 93 of the LNM-associated SNPs (Fig. 1A). Since the presence of LD provides better evidence for an association between putative LNM-associated miRNAs and their surrogate markers (i.e. LNM-associated SNPs), we examined whether the putative miRNAs and LNM-associated SNPs were located in the same LD blocks and found that 32 miRNAs were located in the same blocks as 41 LNM-associated SNPs (Fig. 1A). To verify these associations between polymorphism of miRNA-containing blocks and LNM status, an independent cohort of 280 genotyped patients was used to determine whether the SNPs tagging these miRNAs-containing blocks/regions were associated with the LNM status of the patients and 15 miRNAs represented by SNPs located in the same block were identified (Supplementary Material, Table S1), of which miR-146b (14) and miR-196a-1 (15) are known to be metastasis-regulating miRNAs in breast cancer. We focused on miR-190a at 15q22.2, as three of the SNPs tagging the miR-190a-containing block were all significantly associated with LNM, a finding consistent with that suggested by the initial screening, in which SNP rs4774553, tagging the miR-190a-containing block, was found to be significantly associated with LNM (Fig. 1C). It is notable that, based on Encyclopedia of DNA Elements (ENCODE) data (16), all four SNPs lie within the enhancer- and promoter-associated histone marks (H3K4Me1 and H3K27Ac), or deoxyribonuclelease I (DNase I) hypersensitivity sites (DHS) or are in complete LD with SNPs in the near-by H3K4Me1, H3K27Ac or DHS (Supplementary Material, Fig. S1), suggesting that the 15q22.2 identified is a transcriptionally active region (16). Importantly, miR-190a is the only gene in this region. To obtain mechanistic support for these associations, a cell-based assay was performed to measure the migration activity of the highly metastatic human breast cancer cell line MDA-MB-231 transiently transfected with individual putative miRNAs or control vector. As shown in Figure 1D, cells transfected with miR-103a-1, or miR-10b which are known to promote metastasis (7–10), showed significantly increased migration activity, whereas cells transfected with miR-124-1, which is known to suppress metastasis (11), displayed showed significantly reduced migration, supporting the validity of the screening. Therefore, it is of interest to observe that cells transfected with miR-190a, miR-320a, miR-597 or miR-635 showed significantly reduced migration activity. On the basis of the above clues, we hypothesized that a miR-190a-related pathway is important in regulating breast cancer metastasis.

Breast cancer cell lines stably transfected with miR-190a show decreased cell migration and invasion

To test the hypothesis that miR-190a regulates breast cancer metastasis, we used lentiviral induction of MDA-MB-231 cells to establish lines stably expressing miR-190a. Using quantitative RT-PCR, we confirmed that miR-190a was expressed in these lines, L190a-1 and L190a-2, at levels ~12-fold or 27-fold higher, respectively, than in control cells (Supplementary Material, Fig. S2). We then examined the functional consequences of overexpression of miR-190a in MDA-MB-231 cells on metastasis. First, we tested the effect on the expression of metastasis-related markers and cell morphology. Western blots showed that, in these two miR-190a-overexpressing cell lines, expression of the mesenchymal markers N-cadherin and vimentin...
was significantly lower than in control cells (Fig. 2A). Cell migration is regulated by actin polymerization, and F-actin, the polymerized form of G-actin, plays a critical role in filopodia formation and cell migration (17, 18). Immunofluorescence staining for G-actin and F-actin, using FITC-conjugated DNase I or rhodamine-conjugated phalloidin, respectively, showed that the miR-190a-overexpressing cells contained decreased amounts of F-actin and increased amounts of G-actin compared with control cells (Fig. 2B). miR-190a overexpression also resulted in a decrease in the number of filopodia (Fig. 2C).

We then used two methods, the wound healing assay and the Transwell assay, to study the migration of miR-190a-overexpressing cells. As shown in Figure 2D and E, L190a-1 and L190a-2 displayed reduced migration in both assays compared with the parent MDA-MB-231 cells, the effect being greater with the highly miR-190a-expressing L190a-2 cells. To test the invasive capabilities of the cells, we mimicked the extracellular matrix by Matrigel coating in a Matrigel Transwell invasion assay and found that miR-190a expression resulted in a significant reduction in invasiveness (Fig. 2F). These findings demonstrate that miR-190a can suppress metastasis-relevant events in breast cancer cells.

**Identification of the 3′ untranslated region of the metastasis promoter PAR-1 as a potential target of miR-190a**

To confirm that miR-190a is a metastasis suppressor, it was necessary to identify downstream targets suppressed by miR-190a. miRNAs repress protein expression as a result of their ‘seed region’ being complementary to the 3′ untranslated region (3′ UTR) of specific mRNAs. We therefore used sequence analysis to identify potential targets.
complementary (19) by the bioinformatics algorithm TargetScan (20) to search for possible target mRNAs for miR-190a. This analysis identified several metastasis-related genes as putative targets (data not shown) and PAR-1 was selected for further study because of its known critical role in initiating breast cancer metastasis (21). PAR-1 expression is directly linked to invasiveness of breast cancer cell line (22) and PAR-1 overexpression is involved in development of metastasis in breast cancer patients (23, 24) and in nude mice (25).

We first examined the hypothesis that specific miRNAs play a role in regulating PAR-1 expression. To do so, we prepared luciferase reporter gene (pGL-4.13) constructs containing the PAR-1 3′UTR in either the normal or reversed orientation and used the plasmids to transfect the MCF-7 breast cancer cell line. After 48 h, we then measured luciferase reporter gene activity and found that reporter gene activity in cells transfected with the construct containing the normally orientated PAR-1 3′UTR (3′UTR) was significantly lower than that in cells transfected with the reversed 3′UTR (r-3′UTR) or the control plasmid (pGL) (Fig. 3A). This suggests that this 3′UTR might be regulated by a miRNA-initiated mechanism in vivo. Then, we linked the association between miR-190a and the 3′UTR of PAR-1 which can be bound by miR-190a. To determine which site(s) of the 3′UTR was important for repression, we prepared and tested sequential deletions of the PAR-1 3′UTR and found that the site from nucleotides (nt) 2924–3162 of PAR-1 mRNA was responsible for this suppression effect (Fig. 3D). These results suggest that miR-190a regulates PAR-1 expression by binding to its 3′UTR.

To verify that the PAR-1 3′UTR was a direct target of miR-190a, we mutated four critical nucleotides (nt 3153–3156) in the miR-190a-targeting construct [nt 2924–3660 of PAR-1 3′UTR, pGL-PAR-1(30)] so as to disrupt base pairing between miR-190a and the PAR-1 3′UTR, generating the mutant PAR-1 3′UTR [pGL-PAR-1(m30)] (Fig. 3F). Furthermore, in order to confirm that the mutated region was the miR-190a binding site, we prepared a short sequence (23 base pairs) containing only the predicted miR-190a binding sequence (pGL-s190a) and mutated the same 4 bases as above to generate pGL-ms190a (Supplementary Material, Fig. S3). In both cases, the inhibitory effect of miR-190a was lost following mutation. These results show that miR-190a directly recognizes the PAR-1 3′UTR.

miR-190a regulates PAR-1 protein expression

To confirm the results obtained using the luciferase reporter, we transfected two PAR-1-expressing cell lines, MDA-MB-231 and 293T, with miR-190a and found that endogenous PAR-1 expression was suppressed (Fig. 4A). In addition, in 293T cells co-transfected with miR-190a and a vector containing for the coding sequence (CDS) of PAR-1 with or without the 3′UTR sequence, exogenous PAR-1 expression was inhibited in cells.
transfected with the 3′UTR-containing construct (CDS + UTR), but not in those containing the construct lacking the 3′UTR (CDS) (Fig. 4B). We also transfected MDA-MB-231 cells and 293T cells with the miR-190a inhibitor, LNA-si-miR-190a, to reduce mature miR-190a function and found that this increased endogenous PAR-1 expression in both cell types (Fig. 4C). However, PAR-1 mRNA levels were not affected by miR-190a overexpression (Fig. 4D). This result suggests that miR-190a downregulates PAR-1 expression through translational inhibition.

miR-190a expression is regulated by its independent promoter

To obtain a better understanding of the role in miR-190a in breast cancer metastasis, we next explored how miR-190a expression was regulated. miR-190a is an intragenic miRNA located on chromosome 15 in the intronic region of talin2 (TLN2) (Supplementary Material, Fig. S4A). If miR-190a were produced from the TLN2 transcript, mRNA levels of miR-190a and TLN2 would be expected to show a correlation. We therefore measured levels of TLN2 mRNA and mature miR-190a in several different cell lines by real-time PCR and ranked them from high to low. As shown in Supplementary Material, Figure S4B, there was no correlation between TLN2 and miR-190a levels, and this was verified using the Spearman correlation coefficient test, which gave a value of 0.238 (P = 0.57), indicating no correlation. We therefore tested whether miR-190a transcription was regulated by its own promoter by cloning several deletions of the region starting 5′-30 nt upstream of the pre-miR-190a locus into the promoter region of the luciferase reporter gene, pGL3-basic. We tested regions pGL3-(A) containing nt −5430 to −2431, pGL3-(B) containing nt −2431 to −780, pGL3-(C) containing nt −780 to −1, pGL3-(A + B) containing nt −5430 to −780 and pGL-(B + C) containing nt −2431 to −1 in MCF-7 cells, and found that pGL3-(B + C) and pGL3-(C) resulted in high luciferase activity (Fig. 5A), suggesting that region C contains the core promoter region of miR-190a. Consistent with this, a
ER-positive/human epidermal growth factor receptor 2-negative (31). In addition, high miR-190 expression has been observed in breast cancer, binds directly to the ERE or interacts with Sp1 and AP-1 and histone modification, has also suggested that the promoter region identified lies within a regulatory element (28). Taken together, these results suggest that miR-190a is regulated by its own independent promoter.

miR-190a expression is directly activated by the binding of ERα and activation is increased by estradiol treatment

One remaining intriguing question was why miR-190a is of particular importance in progression of breast cancer. To better understand the tissue specificity, given our finding that miR-190a expression is regulated by its own independent promoter, we examined the promoter sequence of miR-190a and, interestingly, found that region -73 to -69 was a half estrogen response element (ERE), a conserved palindrome sequence (5′-GGTCAnnnTGACC-3′) found in the promoters of ER-regulated genes. The ERE, a ligand-dependent transcription factor and a major prognostic indicator and therapeutic target in breast cancer, binds directly to the ERE or interacts with Sp1 and AP-1 to bind to DNA (29), and regulates gene expression (30). A previous genome-wide study to identify ERE sites by ChIP showed that 25% of these sites contain only a half ERE sequence (31). In addition, high miR-190 expression has been observed in ER-positive/human epidermal growth factor receptor 2-negative invasive ductal carcinoma (32). On the basis of these results, we hypothesized that miR-190a transcription is regulated by the ER. To test this, we first examined whether the ER could regulate the miR-190a promoter. To do so, we separated the C region (i.e. nt -1 to -780 identified in the previous section) into two parts, C-2 containing the ERE and C-1 lacking the ERE, and linked each to the reporter gene, then transfected the reporter plasmids into two cell lines with different ER expression, MCF-7 (ERα+) and MDA-MB-231 (ERα−), then measured luciferase activity with and without addition of the ER ligand 17β-estradiol. As shown in Figure 5B, the ERα+ MCF-7 cells transfected with the ERE-containing region C-2 showed high luciferase activity, which was further increased by addition of 17β-estradiol, while those transfected with C-1 showed no luciferase activity and estradiol had no effect. In contrast, no increased effect of estradiol addition was seen in C-2 transfected ERα- MDA-MB-231 cells. This result shows that the ERE represents a functional region of the promoter and that 17β-estradiol and ERα activate miR-190a expression.

To examine whether ERα binds directly to the half-ERE within the miR-190a promoter, we constructed a mutant clone in which the ERE sequence was mutated from TGACC to TTTTT and used it to transfect the ERα+ cell line MCF-7. As shown in Figure 5C, this mutant showed no increased luciferase expression following 17β-estradiol treatment. We then used the ChIP assay to immunoprecipitate ERα and any bound genomic DNA and used primers to amplify the C-2 region (nt -1 to -310) of the miR-190a promoter. The TFF1 promoter region, which is known to be activated by 17β-estradiol (33), was used as a positive control. As shown in the left panel of Figure 5D, ERα was able to bind directly to the miR-190a promoter and binding was increased on addition of 17β-estradiol. Real-time PCR showed that the
amount of miR-190a promoter bound by ERα was increased 
\(~5\)-fold in the presence of 100 nm 17β-estradiol compared with in its absence (Fig. 5D, right panel), suggesting that ERα binds directly to the ERE in the miR-190a promoter. To verify that the miR-190a promoter independently drives the generation of mature miR-190a from pre-miR-190a transcripts and that the production of mature miR-190a transcripts depends on its promoter, we constructed a plasmid containing the promoter and pre-miR-190a (C-2-miR-190a) (top panel of Supplementary Material, Fig. S5A), transfected it into MCF-7 cells and measured miR-190a expression using quantitative RT-PCR. As shown in Supplementary Material, Figure S5A, C-2-miR-190a-transfected cells showed increased mature miR-190a expression and this effect was enhanced by 17β-estradiol. We also measured endogenous miR-190a expression and found that mature miR-190a levels were increased by 100 nm 17β-estradiol, while TLN2 mRNA levels were unchanged (Supplementary Material, Fig. S5B). These data suggest that 17β-estradiol, acting through ERα, activates transcription of endogenous miR-190a.

The amount of miR-190a promoter bound by ERα was increased 
\(~5\)-fold in the presence of 100 nm 17β-estradiol compared with in its absence (Fig. 5D, right panel), suggesting that ERα binds directly to the ERE in the miR-190a promoter. To verify that the miR-190a promoter independently drives the generation of mature miR-190a from pre-miR-190a transcripts and that the production of mature miR-190a transcripts depends on its promoter, we constructed a plasmid containing the promoter and pre-miR-190a (C-2-miR-190a) (top panel of Supplementary Material, Fig. S5A), transfected it into MCF-7 cells and measured miR-190a expression using quantitative RT-PCR. As shown in Supplementary Material, Figure S5A, C-2-miR-190a-transfected cells showed increased mature miR-190a expression and this effect was enhanced by 17β-estradiol. We also measured endogenous miR-190a expression and found that mature miR-190a levels were increased by 100 nm 17β-estradiol, while TLN2 mRNA levels were unchanged (Supplementary Material, Fig. S5B). These data suggest that 17β-estradiol, acting through ERα, activates transcription of endogenous miR-190a.

To determine the role of ERα in the activation of miR-190a expression and how miR-190a targets PAR-1, endogenous PAR-1 levels in ERα-MDA-MB-231 cells and various transfectants were examined by western blotting. As shown in Figure 5E, PAR-1 expression was inhibited by either miR-190a alone or ERα plus miR-190a and was restored by addition of LNA-si-miR-190a. These data support the idea that ERα plays a role in miR-190a-regulated PAR-1 expression and show that PAR-1 expression is inhibited by ERα-miR-190a signaling.

**Genetic polymorphisms of the miR-190a-containing region correlate with LNM status and miR-190a levels correlate with survival**

This study started with a genome-wide screening of loci found to be significantly associated with LNM, and the subsequent mechanistic study led to the identification of an important novel ERα-miR-190a-PAR-1 pathway regulating breast cancer metastasis. It was therefore of interest to examine whether the findings obtained in the molecular and cell-based experiments were meaningful in breast cancer patients. We therefore examined the effect of ERα or PAR-1 on the association between the SNPs tagging the miR-190a-containing region and LNM status. Consistent with the finding that ERα, miR-190a and PAR-1 are functionally linked, the genetic association between miR-190a and LNM was strongly modified by genotypic polymorphism of the SNP (i.e. rs2262854) tagging the PAR-1-containing region, and a significant association between miR-190a SNPs and LNM was only seen in patients carrying a specific genotype (GG) of the PAR-1 SNP (Fig. 6A, upper panel). Furthermore, a significant association between
miR-190a SNPs and LNM was only seen in ERα-positive patients (Fig. 6A, lower panel), supporting the interaction between ERα and miR-190a.

We finally examined the clinical relevance of miR-190a expression in breast cancer progression. To do so, we measured miR-190a expression in specimens collected from 158 breast cancer patients at the start of a 10-year follow-up. We used laser capture microdissection to isolate tumor cells from the specimens to avoid normal tissue contamination and measured miR-190a expression by quantitative RT-PCR, normalizing it to that of U6 small nuclear RNA to allow for possible inter-tissue variation, and denoting expression status as ‘high’ or ‘low’ by comparing expression in the cancer tissue and adjacent non-cancerous tissue, expressed as the T/N value [miR-190a expression in the microdissected tumor tissue (T) divided by expression in the adjacent normal breast epithelium (N)] using a cutoff of 2-fold to define tumors with high or low expression (34). High miR-190a expression was seen in 70.7% of ERα-positive tumors compared with 57.3% of ERα-negative tumors, the difference reaching borderline significance (P = 0.08) (Fig. 6B). We then explored the effect of the interaction between ERα and miR-190a on breast cancer progression. When examined by logistic regression (Table 1), ERα-negative patients with low miR-190a expression had the highest risk of a worse clinical outcome, as indicated by a high tumor grade and late cancer stage, providing statistical evidence for the significance of a joint effect of ERα and miR-190a in determining these clinical features and for the importance of the functional interplay between ERα and miR-190a. More importantly, we compared the overall survival of breast cancer patients with different levels of miR-190a expression and, as shown in Figure 6C, found that patients with primary breast tumors with low miR-190a expression (T/N ratio ≤ 0.5) had a significantly worse overall survival than those with high miR-190a expression (T/N > 0.5).

DISCUSSION

In this study, using genotyping data obtained in a GWAS, we identified and verified the importance of a novel ERα-miR190a-PAR-1 pathway in regulating breast cancer metastasis. The purpose of this study was not to find loci associated with metastasis at the GWAS significance level for further fine mapping, but to use the GWAS data to generate a hypothesis for further exploration and functional confirmation. Genes and biologic pathways identified by an association study may suggest targets for interventions that are likely to have a greater impact than the identification of causal SNPs by fine mapping (35). The ~200 000 SNPs used in this GWAS included...
miR-190a expression was measured by quantitative real-time PCR.

The odds ratio and 95% confidence interval (95% CI) were estimated in a logistic regression model, in which a group of dummy variables was used to represent different combinations of ERα status and miR-190a expression.

(i) those purposely selected from significant loci identified by several GWAS by international consortia aimed at identifying susceptibility loci associated with breast, ovarian and prostate cancer, (ii) those selected for fine-mapping of known susceptibility, and (iii) those leading to functional change, thus possibly being more meaningful in terms of tumorigenic mechanisms (1). Since the genetic variants associated with cancer progression are not necessarily those associated with cancer development, some loci specifically related to cancer progression might have been missed in the initial phase of our study. However, the study started with a comprehensive genome-wide screening of loci significantly associated with LNM status, so the identified candidate miRNAs would not just be important ones, but the most important ones, in determining LNM during breast cancer metastasis. Based on this screening, in addition to miR-190a, several miRNAs known to play a role in regulating tumor metastasis were identified. These known metastasis-associated miRNAs included miR-103a-1, targeting Dicer for metastasis control (10), miR-10b and miR-125b, abnormality of which has been confirmed to initiate tumor invasion and metastasis (7–9, 12, 13), and miR-124-1, miR146b and miR-196a-1, being able to suppress breast cancer metastasis (11, 14, 15). These findings support both the validity of the methodology and the critical role of miR-190a and the ERα-miR-190a-PAR-1 pathway in regulating breast cancer metastasis. For almost all identified GWAS loci, the functional basis of the association between loci and disease remains unknown. Our study clearly demonstrates that the combined use of the GWAS loci and a growing number of publicly available genomic or epigenomic data and bioinformatic platforms is a very useful method for exploring the molecular mechanism of tumor progression. A specific major strength of our study was that our patients were all recruited from hospitals in which pathological and clinical examinations, treatment protocols and follow-up of disease progression had been carefully recorded and well standardized, with the result that possible bias due to intra-patient variation in detection and measurement would be minor, if any.

Interestingly, when we defined the promoter region of miR-190a, we found that it contained a half-ERE with a putative binding site for the important breast cancer indicator, the ER. In addition, ERα was shown to bind directly to this promoter region and activate miR-190a expression. These findings are consistent to previous observations that ERα is able to regulate miRNAs expression in the development and progression of breast cancer (36–38). It is well known that the sex steroid hormone estrogen regulates the growth, differentiation and function of the mammary gland and exerts these effects by binding to ERα. Thus, the identification of ERα as the upstream transcriptional regulator of miR-190a expression helps explain the issue of tissue specificity, i.e. why miR-190a is of particular importance in breast cancer progression. ERα expression in breast cancer is generally associated with a better clinical outcome (39), which is usually explained by the clinical intuition that a positive ERα status is possibility useful for applying endocrine therapy, but it also suggests ERα-associated mechanism in mediating tumor progression. For example, ERα-negative MDA-MB-231 breast cancer cells stably transfected with ERα and stimulated by estradiol showed decreased metastatic potential (40–43), and estrogen treatment was shown to result in increased desmosome formation to promote cell–cell adhesion (44) and to reduce breast cancer metastasis in a phase 2 trial (45). Moreover, in the absence of hormone, unliganded ERα inhibits breast cancer proliferation (46). The present study provides evidence for an additional mechanistic link, partially explaining why ERα-positive breast patients usually have a favorable progression. These results suggest that ERα is not only a therapeutic marker, but also plays a direct role in preventing metastasis.

miR-190a is located in the intronic region of the talin2 gene (TLN2) on chromosome 15. In a previous study in rat neurons (47), it was found that miR-190a expression relies on expression of its host gene TLN2 and that Yin Yang 1 (YY1) phosphorylation stimulates miR-190a expression by the binding of phosphorylated YY1 to the TLN2 promoter. However, in our study using human cell lines, we identified the promoter region of miR-190a and showed that ERα binds to this region and induces expression. Thus, in the human breast cancer cell lines studied, miR-190a expression is driven by an independent promoter. It is possible that different species or tissues do not use the same transcriptional regulation; furthermore, given the fact that miRNA expression is a tissue-specific phenomenon, different regulatory mechanisms may be involved in miR-190a expression in different tissues.

Our findings support the notion that miR-190a functions as a major ‘metastasis suppressor’. However, because metastasis

<table>
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<th>ERα status</th>
<th>miR-190a expression</th>
<th>No. of patients (%)</th>
<th>Odds ratio b (95% CI)</th>
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involves several genes and mechanisms, miR-190a might inhibit metastasis not only through the PAR-1 pathway, but also through other pathways. In our ongoing bioinformatic analysis, we have identified other metastasis-related genes that are putative targets for miR-190a. We cloned the predicted miR-190a-binding sites of five additional putative miR-190a target genes, PTCH1, DAG1, TP53INP1, HOXC5 and SAMD4A, into luciferase reporter constructs and found that the activity of the reporter gene was decreased when miR-190a levels increased (Supplementary Material, Fig. S6A). This effect of miR-190a on PTCH1 was confirmed at the protein level (Supplementary Material, Fig. S6B). These results suggest that miR-190a may inhibit metastasis through multiple pathways.

Breast cancer is one of the most common types of cancer in females (48). Compared with other tumors, it is relatively curable and has a good prognosis if diagnosed at an early stage; however, when tumor cells begin to metastasize, it has a poor prognosis, resulting in low survival (49). The ERα-miR-190a-PAR-1 pathway was shown in this study to mediate breast cancer metastasis. The novel information about the role of miR-190a as a ‘metastasis suppressor’, and the molecular mechanism by which ERα regulates miR-190a expression, is of clinical importance. miR-190a may be useful in the diagnosis and prevention of breast cancer progression.

MATERIALS AND METHODS

Breast cancer patients, genotyping, clinical features and follow-up

In the hypothesis-generating phase, the genome-wide genotyping data for 837 incident breast cancer patients were examined to identify loci associated with LNM. These 837 patients were included in the international Collaborative Gene-Environment Study (COGS) (1). Genotyping in the COGS was performed using a customized Illumina Infinium BeadChip (Illumina Inc., San Diego, CA, USA), which originally included 211,155 SNPs, and, after quality control exclusions, generated data for 199,961 SNPs. Two other independent groups of breast cancer patients were recruited, one (n = 280) of which was used to confirm the association between SNPs and LNM identified above, while the other (n = 158), from a 10-year follow-up study, was used to determine whether there was an association between miR-190a expression and survival. Blood specimens from the group of 280 patients were used to genotype SNPs tagging the miR-190a- and PAR-1-containing regions using a Sequenom iPLEX (Sequenom, Hamburg, Germany). Duplicate samples were used and positive and negative controls included on all plates, genotypes being autocalled by the software MassARRAY Typer version 3.4 (Sequenom) and confirmed by visual assessment of the data. Quality control of the assay has been described in our previous study (50). For the group of 158 patients, miR-190a expression was measured in breast cancer specimens and adjacent normal epithelium tissues collected at the start of a 10-year follow-up. To ensure that the tissue samples assayed consisted of pure breast tumor epithelial cells, laser capture microdissection was performed on routinely immunostained slides using a PixCell laser capture microscope (Arcturus Engineering, Mountain View, CA, USA) as described previously (51, 52). The characteristics of both sets of study participants have been described previously (50). Details of their clinical and pathological features were obtained from the tumor registries of our hospitals, the quality of which is well-recognized (53). Cause of death in the last group of patients was determined from death certificates.

Cell culture

All cells were purchased from the Taiwa Bioresource Collection and Research Center. MCF-7 cells (human breast cancer cell line) and 293T cells (human embryonic kidney cell line) were cultured in Dulbecco’s modified Eagle’s medium, while MDA-MB-231 cells (human breast cancer cell line) were cultured in RPMI-1640 medium (both media from Sigma-Aldrich, St Louis, MO, USA), both supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA).

Constructs, small interference RNA and transfection

cDNAs containing either the PAR-1 CDS plus the 3’UTR or only the CDS were cloned into the XhoI and XbaI sites of the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). Pre-miR-190a was amplified from genomic DNA by PCR and cloned into the EcoRI and XhoI sites of pcDNA3. Different lengths of the PAR-1 3’UTR were inserted into the 3’UTR region of the luciferase reporter gene in the pGL4.13 vector (Promega, Madison, WI, USA) at the XbaI site. Different lengths of the pre-miR-190a promoter were inserted into the promoter region of pGL3-basic (Promega) at the KpnI and NheI sites. Mutations were generated using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The sequence of the siRNA targeting PAR-1 was 5’-GAUCAUACGUG CGAUCAGA-3’ (Sigma-Aldrich). A LNA-modified antisense inhibitor for miR-190a (LNA-si-miR-190a) was purchased from Applied Biosystems (Foster City, CA, USA). Transfection of these plasmids or siRNAs was performed using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions.

Generation of stable clones overexpressing miR-190a

To overexpress miR-190a in the breast cancer cell line MDA-MB-231, pre-miR-190a was incorporated into the lentivirus-based expression vector pLKO-010 and the vector packaged in lentivirus particles (National RNAi Core Facility at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan). The pre-miR-190a-expressing plasmid was introduced into the cells by viral infection and infected cells were selected in the presence of 1 μg/ml of puromycin. miR-190a expression in these stable clones was confirmed by quantitative RT-PCR.

Cell lysis and immunoblotting

Preparation of whole cell extracts and western blotting was performed as described previously (54). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA). The antibodies used for immunoblotting were mouse monoclonal anti-PAR-1 antibody (ATAP2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-α-tubulin antibody (T6199; Sigma-Aldrich), rabbit monoclonal
anti-vimentin antibody (EPR3776; Genetex, San Antonio, TX, USA) and anti-N-cadherin antibody (EPR1792Y; Genetex), and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG reagents (Jackson Immunoresearch, West Grove, PA, USA), signals being detected using chemiluminescent reagents (Millipore, Temecula, CA, USA).

**Immunofluorescence staining**

MDA-MB-231 cells stably transfected with miR-190a or vector seeded on cover slips were fixed for 15 min at room temperature in 2% paraformaldehyde in phosphate-buffered saline (PBS), washed three times with PBS and permeabilized for 10 min at room temperature in PBS containing 0.1% Triton X-100. The cells were then labeled for 30 min at room temperature with rhodamine phalloidin, a probe for F-actin and Alexa Fluor 488 DNAseI, a probe for G-actin (both from Invitrogen). For nuclear staining, cells were treated with DAPI. The cover slips were then mounted on microscope slides with antifade reagent (Millipore, Temecula, CA, USA).

**Wound healing**

Culture inserts (Ibidi, Martinsried, Germany) were used for the wound healing assay. Cells were seeded at 5 × 10⁴/well and, after the formation of a confluent layer of cells, the culture inserts were removed, generating a ‘wound’, and the size of the remaining gap was measured at different time points.

**Cell migration and invasion assays**

For the migration assay, 1 × 10⁵ miR-190a-overexpressing MDA-MB-231 cells in 100 μl of serum-free RPMI-1640 medium were added to the top chamber of 8 μl pore-size 24-well Transwell plates (Costar, Cambridge, MA, USA) and 400 μl of RPMI-1640 medium containing 10% fetal bovine serum to the bottom chamber. After incubation for 24 h at 37°C, the insert was removed and the bottom of the insert washed two times with PBS, then invading cells were dissociated from the membrane using cell dissociation solution (Trevigen, Gaithersburg, MD, USA) and labeled with Calcein AM (Trevigen) in clean 24-well plates for 1 h, when calcein fluorescence was measured in a plate reader at 520 nm with excitation at 485 nm.

The Transwell invasion assay procedure was modified from the migration assay. The top chamber of 8 μl pore-size 24-well Transwell plates was coated with 50 μl of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), which is diluted in serum-free medium at a ratio of 1:5, then 5 × 10⁵ cells in 100 μl of serum-free RPMI-1640 medium were added and incubated for 24 h at 37°C.

The Transwell migration and invasion assays were performed at least three times.

**Luciferase reporter assay**

The luciferase constructs and pRL-tk, which encodes Renilla luciferase, were co-transfected into 1 × 10⁵ cells in 12-well plates. After 48 h, the cells were lysed by freeze-thawing in passive lysis buffer and the lysate centrifuged at 12 000g for 10 min at 4°C, and then luciferase activity in the supernatant was measured using Dual-Luciferase Reporter Assay System Kits (Promega).

**Real-time reverse transcription-PCR**

mRNAs were extracted using TRIZOL reagent (Invitrogen) and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was carried out using an SYBR green PCR kit (Applied Biosystems) and the results normalized to those for GAPDH. miR-190a levels were determined using the TaqMan miRNA assay (Applied Biosystems) and the results normalized to those for RNU6B and measured using the comparative CT method.

The PCR primer pairs used were 5'-GTGTACAGAGTGGAATAAAGCACAGAGCTTGTTACC-3' and 5'-CTAAGGCAGACTGACATGATTGATTCTGGTAC-3' for PAR-1, 5'-CAAGGAAGTCGCAAGACAGCACT-3' and 5'-TTGAGCCGCAACGCTGTCAGGT-3' for TLN2 and 5'-GAAGGTGAAGTCGCAGGT-3 and 5'-GAAGGTGAAGTCGCAGGT-3' for GAPDH.

**Chromatin immunoprecipitation**

ChIP was performed using EZ-Magna ChIP G kits (Millipore) following the manufacturer’s instructions and using anti-ERα antibody (CS200620; Millipore) for the precipitation stage. The immunoprecipitate was eluted with 50 μl of the supplied TE buffer, and 2 μl of DNA was used in the PCR reaction. The human TFF1 promoter, which contains an ERα binding site, was used as a positive control. The primer pairs used for the ChIP PCR were 5'-CCGGCCATCTCTCCTAATAGAA-3' and 5'-CCTTCCGCCAGGATTACAC-3' for TFF1, and 5'-CTTGTAGTTCTGGTATGAGGTG-3' and 5'-CTGCAGAAAAAGCAGCATCAGGTATGATTATCC-3' for miR-190a.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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