miR-187 Is an Independent Prognostic Factor in Breast Cancer and Confers Increased Invasive Potential In Vitro

Laoighse Mulrane1, Stephen F. Madden3, Donal J. Brennan1,2, Gabriela Gremel1, Sharon F. McGee1, Sara McNally1, Finian Martin2, John P. Crown4, Karin Jirström5, Desmond G. Higgins5, William M. Gallagher1, and Darran P. O’Connor1

Abstract

Purpose: Here, we describe an integrated bioinformatics, functional analysis, and translational pathology approach to identify novel miRNAs involved in breast cancer progression.

Experimental Design: Coinertia analysis (CIA) was used to combine a database of predicted miRNA target sites and gene expression data. Using two independent breast cancer cohorts, CIA was combined with correspondence analysis and between group analysis to produce a ranked list of miRNAs associated with disease progression. Ectopic expression studies were carried out in MCF7 cells and miRNA expression evaluated in two additional cohorts of patients with breast cancer by in situ hybridization on tissue microarrays.

Results: CIA identified miR-187 as a key miRNA associated with poor outcome in breast cancer. Ectopic expression of miR-187 in breast cancer cells resulted in a more aggressive phenotype. In a test cohort (n = 117), high expression of miR-187 was associated with a trend toward reduced breast cancer–specific survival (BCSS; P = 0.058), and a significant association with reduced BCSS in lymph node–positive patients (P = 0.036). In a validation cohort (n = 470), high miR-187 was significantly associated with reduced BCSS in the entire cohort (P = 0.021) and in lymph node–positive patients (P = 0.012). Multivariate Cox regression analysis revealed that miR-187 is an independent prognostic factor in both cohorts [cohort 1: HR, 7.37; 95% confidence interval (CI), 2.05–26.51; P = 0.002; cohort 2: HR, 2.80; 95% CI, 1.52–5.16; P = 0.001] and in lymph node–positive patients in both cohorts (cohort 1: HR, 13.74; 95% CI, 2.62–72.03; P = 0.002; cohort 2: HR, 2.77; 95% CI, 1.32–5.81; P = 0.007).

Conclusions: miR-187 expression in breast cancer leads to a more aggressive, invasive phenotype and acts as an independent predictor of outcome. Clin Cancer Res; 18(24); 6702–13. ©2012 AACR.

Introduction

Breast cancer is the most common female malignancy in the developed world, with more than 60,000 women in the European Union succumbing to the disease each year (1). However, as with most other malignancies, the predominant factor influencing mortality is not the primary tumor but metastases at distant sites. Despite this fact, relatively little is known about the key molecular mechanisms and determinants driving this terminal stage of the disease. Indeed, no doctrine akin to Hanahan and Weinberg’s “Hallmarks of Cancer” (2) has yet to be compiled exclusively for the metastatic process. Consequently, current diagnostic and treatment strategies for metastatic breast cancer are much less successful. As such, the identification of novel mediators of invasion and metastasis, in addition to novel biomarkers of breast cancer progression, is crucial if patient outcome is to improve.

miRNAs are small noncoding RNA molecules, 20 to 25 nucleotides in length, that negatively regulate target gene expression by either inhibiting translation or promoting degradation of target miRNAs (3). The latter being the predominant mode of action in mammalian systems (4). They are known to be aberrantly expressed in cancer and may function as either tumor suppressors or oncogenes, depending on which genes or pathways they regulate. As a
miR-187 and Breast Cancer

Translational Relevance

The identification of novel biomarkers and mediators of metastasis is essential to improve the outcome of patients with advanced disease. Within this context, miRNAs represent a new class of biomarkers and novel drug targets. Here, we describe a novel approach to identify and validate miRNAs involved in breast cancer progression using an in silico method in two independent breast cancer cohorts. We subsequently validated these findings using in situ hybridization on tissue microarrays in two additional patient cohorts and in an in vitro setting, showing that miR-187 is associated with poor prognosis in patients with breast cancer, specifically in those with 1 or more positive lymph nodes. These data suggest that miR-187 confers a more aggressive phenotype on breast cancer cells, resulting in increased invasion and migratory potential. We conclude that miR-187 is an independent marker of prognosis in lymph node-positive breast cancer and may play a role in promoting metastasis.

In the last decade, numerous miRNAs have been documented to influence the metastatic process (reviewed in ref. 5). In breast cancer, miR-10b (6), miR-21 (7), and miR-31 (8), among others, play key roles in tumor dissemination, eliciting their effects through the downregulation of mRNA targets involved in the metastatic process. Moreover, miR-31 and miR-21 have been associated with good and poor prognosis, (8, 9), indicating that miRNAs may be useful, not only as drug targets, but potentially as clinically relevant biomarkers.

Here, we used a novel bioinformatics approach, described in detail previously. They contained estrogen receptor (ER)-positive, predominantly postmenopausal patients, with advanced recurrent disease. Both groups received first-line tamoxifen for treatment of local and distal recurrences. Patients did not receive any systemic adjuvant treatment following surgery and/or radiotherapy, and tamoxifen was initiated as a first-line treatment of recurrent disease. For the purposes of this and previous studies (12, 13), recurrences within 6 months of initiation of tamoxifen were classified as having a poor outcome (Amsterdam dataset n = 21, Rotterdam dataset n = 18), whereas those that showed no recurrence within the same time frame were classified as having a good outcome (Amsterdam dataset n = 48, Rotterdam dataset n = 22).

The raw gene expression data (genepix.gpr files) were read, background corrected with the "normexp" option, and quantile normalized using the Bioconductor package limma (14) from the R statistical environment. Both datasets were analyzed independently and the intersections between the target prediction programs and the genes on the array are described later.

Cointertia analysis

For the Amsterdam (13) and Rotterdam (12) datasets, CIA and BGA were used to simultaneously analyze mRNA gene expression data and predicted miRNA target sites in the 3′-untranslated regions (UTR) of the same genes. This multivariate data integration technique uses the entire gene expression dataset, and therefore does not require any prefiltering of the transcriptomic data. CIA (15) was used to combine 2 linked datasets (gene expression data and predicted miRNA target information) for the same genes (10). CIA was first applied in an unsupervised manner for data exploration purposes. Supervised analysis was then conducted using BGA (16) to identify miRNAs associated with specific samples. The output from this analysis was 5 ranked lists of miRNAs associated with poor outcome, 1 for each of the 5 miRNA target prediction programs (see later). Lists were combined using consistency among the methods, as previously published (10). All calculations were carried out using the MADE4 library (17) of the open source R package. MADE4 can be downloaded freely from the Bioconductor website (18). All scripts used are available upon request from the authors.

miRNA target prediction

Target predictions of miRNAs were obtained from TargetScan (v4.1), TargetScanS (v4.1; refs. 19, 20), PicTarWay, PicTarWay (21), and miRanda (release 14; ref. 22). Each program exploits complementarity with the miRNA "seed" region and cross-species conservation in their prediction algorithms. The overlaps between the target prediction programs and the genes present on the array were as follows, TargetScan predicted 195 miRNAs across 4,513 genes, TargetScanS predicted 90 miRNAs across 2,902 genes, PicTarWay predicted 178 miRNAs across 4,840 genes, PicTarWay predicted 130 miRNAs across 1,767 genes, and miRanda predicted 470 miRNAs across 9,172 genes.

Materials and Methods

Gene expression profiling

Gene expression data were obtained from 2 cancer centers in Amsterdam (69 patients; ref. 13) and Rotterdam (40 patients; refs. 12, 13). Both datasets have been described in detail previously. They contained estrogen receptor (ER)-positive, predominantly postmenopausal patients, with advanced recurrent disease. Both groups received first-line tamoxifen for treatment of local and distal recurrences. Patients did not receive any systemic adjuvant treatment following surgery and/or radiotherapy, and tamoxifen was initiated as a first-line treatment of recurrent disease. For the purposes of this and previous studies (12, 13), recurrences within 6 months of initiation of tamoxifen were classified as having a poor outcome (Amsterdam dataset n = 21, Rotterdam dataset n = 18), whereas those that showed no recurrence within the same time frame were classified as having a good outcome (Amsterdam dataset n = 48, Rotterdam dataset n = 22).

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Cell lines

MCF7 breast cancer cells were received as a gift from Prof. Robert Clarke (Georgetown University, Washington, DC) and were cultured in phenol red–free Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen) supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin/streptomycin (50 U/mL; Gibco, Invitrogen), and 1% l-glutamine (2 mmol/L; Gibco, Invitrogen). SKBR3 cells were obtained from the European Collection of Cell Cultures and were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin/streptomycin (50 U/mL; Gibco, Invitrogen), and 1% l-glutamine (2 mmol/L; Gibco, Invitrogen). All cells were maintained at 37°C in humidified air with 5% CO2 and were confirmed to be free of contamination by *Mycoplasma* spp. Cell line authentication was carried out using microsatellite profiling.

Ectopic expression of miR-187

Stable, ectopic expression of miR-187 was achieved using the miR-Express lentiviral delivery system (Open Biosystems, now part of Thermo Fisher Scientific). Briefly, HEK293T cells were seeded at a confluency of 50% to 70% and transfected using a calcium phosphate transfection method with lentiviral expression constructs containing nontargeting control (NTC) sequences or sequences for miR-187 (Open Biosystems) along with pMD2.G and psPAX2 viral envelope and packaging vectors (courtesy of the Tetra Laboratory, Lausanne, Switzerland). Cells were incubated for a further 6 to 8 hours and the media was then changed. Viral particles were allowed to form over 48 hours before harvesting and filtration through 0.45-μm filters. Viral supernatant was diluted in fresh media and then used to infect target MCF7 or SKBR3 cells, seeded in a 6-well plate at a confluency of approximately 50% (7 × 10^4 cells/well). Infected cells were selected in media containing 2 μg/mL puromycin over a period of 3 to 5 days.

Patient tissue samples

Both patients cohorts consisted of patients with primary breast cancer. Cohort 1 consisted of 144 cases (117 in final analysis) of invasive breast cancer diagnosed at the Department of Pathology, Malmö University Hospital (Malmö, Sweden) between 2001 and 2002 and has been described previously (23). The median age at diagnosis was 64.5 years (range, 34–97), and the median follow-up period for breast cancer–specific survival (BCSS) was 6.6 years (range, 0.33–7.55). At last follow-up, 41 patients had died, 22 of whom were considered to be a direct result of breast cancer. Patients did not receive neo-adjuvant therapy and were treated with either modified radical mastectomy or wide local excision. Complete endocrine treatment data were available for 95 ER-positive patients, 67 of whom received adjuvant tamoxifen, 3 an aromatase inhibitor, and 25 a combination of tamoxifen and an aromatase inhibitor. Information on adjuvant chemotherapy was available for 143 patients, of whom 30 patients received treatment. Information on radiotherapy was available for 144 patients of whom 83 received adjuvant radiation.

Cohort 2 (previously described in ref. 24) consisted of 498 consecutive invasive breast cancer cases (470 in final analysis) diagnosed at the Department of Pathology, Malmö University Hospital, between 1987 and 1992. The median age at diagnosis was 65 years (range, 27–96) and the median follow-up time was 11 years (range, 0–17). Two hundred and sixty-three patients were dead at the last follow-up (December 2004), 90 of whom were considered to be as a direct result of breast cancer (breast cancer–specific death). Complete endocrine treatment data were available for 379 patients, 160 of whom received adjuvant tamoxifen. Information on adjuvant chemotherapy was available for 382 patients, of whom 23 patients received treatment. Clinicopathologic characteristics of patients included in this study from cohorts 1 (n = 117) and 2 (n = 470) are described in Supplementary Table S1. The studies were approved by the ethical committee at Lund University (Lund, Sweden).

Tissue microarrays and in situ hybridization

Tissue microarrays (TMA) were constructed as previously described (25). In brief, TMA sections were baked at 60°C for 60 minutes, dewaxed in xylene, and rehydrated. Tissue was permeabilized by treatment with 10-μg/mL proteinase K for 5 minutes at 37°C. Slides were washed with diethyl pyrocarbonate (DEPC)–treated PBS and refixed with 4% paraformaldehyde. Tissue was acetylated using a solution of triethanolamine (TEA) and acetic anhydride (2.1 mL TEA in 125 mL DEPC-treated water, 125 μL acetic anhydride added just before use), and then washed with DEPC-treated PBS. Digoxigenin-labeled locked nucleic acid (LNA) miRCURY probes (labeled at both the 3’ and 5’ ends; miR-187 probe sequence: CCGGGTGCAACACAAGACCA; Scramble probe sequence: GTTGAACAGCTCTAGCTAGCC-CA; Exiqon) were diluted to a concentration of 20 nmol/L in hybridization solution [50% formamide, 5× saline-sodium citrate (SSC), 0.1% Tween, 9.2 mmol/L citric acid, 50 mmol/L MgCl2, pH 9.5] for 10 minutes at room temperature. Sections were washed twice in buffer 1 and once in buffer 2 (100 mmol/L Tris–HCl, 1 M NaCl, pH 7.5) at room temperature. Sections were blocked (0.1% Triton-X100, 1% normal goat serum in buffer 1) for 30 minutes before applying 200 μL antidigoxigenin alkaline phosphatase Fab fragments (1:1,000 dilution in blocking buffer, Roche Applied Science). Sections were washed twice in buffer 1 and once in buffer 2 (100 mmol/L Tris–HCl, 100 mmol/L NaCl, 30 mmol/L MgCl2, pH 9.5) for 10 minutes each. Nitroblue tetrazolium/5-bromo-4-chloro-3-indoly phosphate (NBT/BCIP) tablets (Roche Applied Science) were dissolved in water and levamisole added to a concentration of 1 mmol/L. After the desired development time (in this case, 4 days as optimized on full-face sections), the
colorimetric reaction was stopped by incubating slides in stop solution (100 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.1). Any additional NBT/BCIP solution was washed away with water, and slides were mounted using an aqueous mounting medium (50% glycerol and 50% water).

TMA cores were scored manually using a semiquantitative scoring system comprising negative, low, and high-staining intensity by 2 independent observers.

**Image acquisition and management**

Digital images were captured using the Aperio ScanScope XT Slide Scanner (Aperio) with a ×20 objective as previously described (26). TMA images were managed using Spectrum and snapshots taken using ImageScope software (Aperio).

**Statistics**

Spearman rho correlation was used to estimate the relationship between duplicate cores from individual tumors, between quantitative real-time PCR (qRT-PCR) signal and ISH signal (manual scores). Kaplan–Meier, univariate, and multivariate Cox regression analyses were used to illustrate differences between recurrence-free survival (RFS) and BCSS according to miR-187 expression. Pearson χ² test was used to evaluate associations between miR-187 expression and clinicopathologic characteristics. All survival calculations were conducted using SPSS version 17.0 (SPSS Inc., now part of IBM). A 2-tailed Student t test was used to determine statistical significance over 3 independent repeats of in vitro experiments using Microsoft Excel 2007 (Microsoft Corporation). In all cases, a P value less than 0.05 was considered statistically significant.

**Results**

**Cointertia analysis identifies miRNAs linked to poor outcome in breast cancer**

For each independent dataset [Amsterdam (69 patients; ref. 13) and Rotterdam (40 patients; ref. 12)], a combination of unsupervised CIA (using correspondence analysis) and supervised CIA (using BGA) was used to simultaneously analyze mRNA-expression levels and miRNA target-prediction information in the 3′-UTRs of the same genes. This allowed the identification of miRNAs associated with poor outcome. Five such analyses were conducted, 1 for each target prediction program (10). Figure 1 shows an example of unsupervised analysis of the Amsterdam dataset using the TargetScan target prediction program.
program. The plot is in 2 parts, Fig. 1A depicts a correspondence analysis plot of patient samples and Fig. 1B depicts miRNAs associated with these samples. The 2 plots are interrelated; samples in part (A) are associated with miRNAs in part (B) in the same orientation relative to the origin. In Fig. 1A, there is a trend along the vertical axis separating the patients with a poor outcome from those who remained disease free for 6 months. In Fig. 1B, miR-187 is in the same orientation from the origin as the poor outcome tumors in Fig. 1A, suggesting that this miRNA is associated with this trend in the data.

To systematically identify miRNAs specifically associated with poor outcome, a supervised analysis was conducted combining CIA and BGA. miRNAs associated with poor prognosis for each of the datasets are listed in Supplementary Table S2. miR-187 had the highest average rank in both datasets.

Ectopic expression of miR-187 in breast cancer cells results in increased migratory and invasive potential

MCF7 and SKBR3 cells were engineered to ectopically express miR-187 and expression validated using qRT-PCR (Supplementary Figs. S1A and S3A, respectively). miR-187 expression could not be detected in either parental MCF7 or NTC vector-transduced cells. As miR-187 was originally identified in a progression-related dataset in which patients had received tamoxifen treatment, the cells were assessed for resistance to both tamoxifen and the pure antiestrogen fulvestrant. However, ectopic expression of miR-187 had no observable effect on response to either agent ($p = 0.407$ for tamoxifen, $p = 0.425$ for fulvestrant; Fig. 2A and B).

Cells were then assessed using standard assays to model various aspects of tumor progression. In a scratch wound migration assay, a significant increase in wound closure was noted in miR-187 expressing MCF7 cells compared with...
cells expressing a NTC (P = 0.005; Fig. 2C). Similarly, miR-187 expressing MCF7 cells showed an increased ability to grow in anchorage-independent conditions (P = 0.005 compared with NTC cells, P = 0.006 relative to parental MCF7 cells; Fig. 2D), but no increase in overall growth rate was observed (Supplementary Fig. S1B). This increase in migration and soft-agar colony formation was mimicked in SKBR3 cells engineered to ectopically express miR-187 (Supplementary Fig. S3B and S3C). In addition, in a 3-dimensional spheroid invasion assay, miR-187 expressing MCF7 cells showed a higher degree of invasion into surrounding collagen compared with NTC cells (P = 0.004) and parental MCF7 cells (P = 0.002), which were minimally invasive even after 14 days (Fig. 2E, quantification of invasion can be seen in Supplementary Fig. S1C). Furthermore, ectopic expression of miR-187 resulted in a significant increase in expression of collagenase-3 [matrix metalloproteinase 13 (MMP13; Fig. 2F)], but no changes in other matrix metalloproteases (MMP3, MMP11 and MMP21; Supplementary Fig. S2A) or common markers of epithelial–mesenchymal transition (EMT) were noted (Supplementary Fig. S2B).

**In situ** hybridization reveals that high levels of miR-187 breast cancer correlate with decreased survival in breast cancer patients

Having identified miR-187 as associated with poor outcome in recurrent breast cancer and having shown an association between miR-187 expression and an aggressive phenotype in **vitro**, we proceeded to assess the prognostic value of miR-187 in primary breast cancers using 2 independent cohorts. Staining of miR-187 by ISH was manually scored by cytoplasmic intensity (Fig. 3A and B). No staining was observed when using a scrambled probe (Fig. 3A). A high level of concordance was also observed between duplicate cores (Spearman rho analysis, r = 0.800, P < 0.001) and between independent observers (Spearman rho analysis, r = 0.898, P < 0.001). Although there were originally 144 patients on the TMA for cohort 1 and 498 in cohort 2, due to core loss during sectioning and hybridization, 117 and 470 patients, respectively, were suitable for analysis. To ensure that this did not introduce selection bias, Pearson χ² analysis was used to estimate the association between missing patients and clinicopathologic characteristics. No significant association was noted in either cohort (data not shown).

To confirm that ISH-staining intensity correlated to relative expression levels of miR-187, RNA was extracted from 8 representative tumors (4 ISH-negative and 4 ISH-positive) and TaqMan-based qRT-PCR was conducted. A high degree of correlation was seen between ISH scores and relative expression of the miRNA (Spearman rho analysis, r = 0.823, P = 0.012; Fig. 3C).

Kaplan–Meier analysis of cohort 1 (n = 117) revealed a nonsignificant stepwise association between miR-187 expression and BCSS (Fig. 4A). On the basis of these findings, data were dichotomized, whereby tumors in the high-miR-187 group were compared with those in negative/low miR-187 groups. This revealed a trend toward reduced BCSS in the high-miR-187 group (P = 0.058; Fig. 4B). Subset analysis based on lymph node status was conducted and it showed that high expression of miR-187 was associated with reduced BCSS in lymph node–positive (P = 0.036; Fig. 4D) but not in lymph node–negative patients (P = 0.715; Fig. 4C). Multivariate Cox regression analysis correcting for age, tumor size, lymph node status, grade, ER, progesterone receptor (PR), and HER2 status showed that miR-187 was an independent prognostic marker in the entire cohort 1 and particularly in lymph node–positive patients (HR, 13.74; 95% confidence interval (CI), 2.62–72.03; P = 0.002; Table 1).

In cohort 2 (n = 470), a similar trend was noted, whereby tumors expressing high levels of miR-187 were associated with a reduced BCSS (Fig. 5A) and RFS (Supplementary Fig. S4A). Using the same threshold for dichotomization of data as cohort 1, high expression of miR-187 was associated with a reduced BCSS (P = 0.021) and RFS (P = 0.013) in the entire cohort (Fig. 5B and Supplementary Fig. S4B, respectively). Subset analysis based on lymph node status again showed that high-miR-187 expression was associated with a reduced BCSS (P = 0.012) and RFS (P = 0.037) in lymph node–positive patients but not in the lymph node–negative subgroup (P = 0.248 for BCSS and P = 0.162 for RFS; Fig. 5 and Supplementary Fig. S4C and S4D). Multivariate Cox regression analysis using the model described earlier again showed that miR-187 was an independent prognostic marker for both BCSS (HR, 2.8; 95% CI, 1.52–5.16; P = 0.001) and RFS (HR, 1.99; 95% CI, 1.21–3.28; P = 0.007) in the entire cohort and particularly in the lymph node–positive group (BCSS (HR, 2.77; 95% CI, 1.32–5.81; P = 0.007); RFS (HR, 2.21; 95% CI, 1.18–4.14; P = 0.014; Table 1). High miR-187 expression showed a borderline-significant association with tumor size in cohort 1 (P = 0.048), but did not correlate with any other clinicopathologic factors in either TMA cohort (Supplementary Tables S3 and S4). In addition, we further examined the prognostic potential of miR-187 within the context of ER expression in both cohort 1 and cohort 2. Kaplan–Meier survival analysis of cohort 1 stratified according to ER status showed that high expression of miR-187 was associated with reduced BCSS in ER-positive patients (n = 102, P = 0.007). This effect was not evident in ER-negative patients in this cohort (n = 15, P = 0.355; Supplementary Fig. S5A and S5B).

In cohort 2, miR-187 expression was not associated with overall survival (OS) or BCSS when stratification was conducted on the basis of ER status. We did observe a trend toward an association between high-miR-187 expression and reduced RFS in this cohort, although this trend did not reach statistical significance (n = 372, P = 0.055; Supplementary Fig. S5C and S5D).

**Discussion**

Using a comprehensive bioinformatics approach, we have discovered that miR-187 is associated with poor outcome in 2 independent breast cancer cohorts. **In vitro** data from MCF7 cells stably expressing miR-187 indicated that
while the cells did not show any alterations in growth rate or drug sensitivity, they displayed significantly increased invasive and migratory capacity along with an enhanced ability to grow in anchorage-independent conditions (also shown in SKBR3 cells). In addition, using LNA-ISH to determine miR-187 expression in tumor tissue, we have shown that miR-187 is an independent marker of poor prognosis in 2 further breast cancer cohorts, particularly in lymph node-positive patients.

There have been few studies using computational methods to predict miRNA activity from gene expression datasets (27, 28) with most bioinformatic tools concentrated on predicting miRNA targets (19, 22) or predicting the presence of a miRNA within genomic sequences (29) or in high-throughput sequencing analyses (30, 31). The approach applied in this article (10) makes use of publicly available target prediction programs to infer miRNA activity in patient samples associated with certain characteristics or trends of interest, adding a wealth of new information to already existing transcriptomic datasets. In addition, the method used requires no preprocessing of the gene expression data, such as the generation of clusters or gene lists and facilitates data visualization and exploration. For a dysregulated miRNA to be detected using this approach, it must act through mRNA degradation rather than translational inhibition, which may not always be the case. However, Guo and colleagues recently characterized miRNA degradation as the primary mode of miRNA action (4) and that decreased mRNA expression accounted for approximately 80% of the reduced protein production.

This technique was applied to 2 progression-related breast cancer gene expression datasets, and miR-187 was
associated with poor outcome in both cases. Supporting this, ectopic expression of miR-187 in MCF7 cells resulted in a more aggressive phenotype in vitro (characterized by an increased ability to grow in anchorage-independent conditions and an increased invasive and migratory capacity). These phenotypic effects were also observed in an independent breast cancer cell line, SKBR3. The expression of collagenase-3 (MMP13), a matrix remodeling enzyme known to be involved in the progression of many cancers (32, 33), was also found to be increased in MCF7 cells expressing miR-187, consistent with the observed increase in invasion into a collagen matrix. The cells retain an epithelial morphology and do not show any alterations in common markers of EMT, suggesting that matrix remodeling is the mechanism of increased invasion. Furthermore, when assessed in 2 further breast cancer cohorts, high levels of miR-187 were found to correlate with poor outcome independent of other clinicopathologic factors, further validating the in silico and in vitro results. In particular, the association between miR-187 and poor prognosis in lymph node–positive subgroups in both cohorts provides further evidence for the role of miR-187 in the progression of the disease.

miR-187 has been previously identified as being abberantly expressed in various cancers including nasopharyngeal (34), renal (35), pancreatic (36), thyroid (37), gastric (38), and esophageal cancers (39), as well as neuroblastoma (40), although the changes in expression vary greatly, indicating that it may be involved in carcinogenesis in a tissue-specific manner. In a recent study, miR-187 was shown to be

Figure 4. High levels of miR-187 correlate with decreased BCSS in patients with lymph node–positive breast cancer in cohort 1 (n = 117). Kaplan–Meier survival analysis of miR-187 expression levels in (A) all patients (negative, low, and high-miR-187 expression; n = 117), (B) all patients (dichotomized score: negative/low miR-187 vs. high miR-187; n = 117), (C) lymph node–negative (n = 62), and (D) lymph node–positive (n = 46) patients.
instrumental in the regulation of ovarian cancer progression through the targeting of disabled homolog 2 (Dab2), and was significantly associated with increased OS and RFS in a cohort of patients with the disease \( n = 176; \) ref. 41. In breast cancer, Blenkiron and colleagues have observed that miR-187 is expressed at higher levels in ER-negative versus positive tumors and seems to be over-represented in HER2-positive cancers (42), whereas Hui and colleagues note that the expression of the miRNA is more than 2.6-fold increased in breast cancer versus normal tissue (43). Interestingly, no specific association with ER or HER2 status was noted in this study, although the numbers of ER-negative and HER2-positive patients in both TMA cohorts were too low to make a full assessment in this regard. More recently, Rodríguez-González and colleagues report that miR-187 was significantly differentially expressed in ER-positive lymph node-positive patients who did not respond to tamoxifen therapy versus those that did \( n = 38 \) using qRT-PCR (44). However, they failed to see a similar effect in a subsequent screening of 246 patients.

The mechanism underlying the relationship between miR-187 and disease progression remains to be elucidated. However, we postulate that miR-187 negatively regulates genes either directly or indirectly involved in the expression of MMP13 and potentially other regulators of migration and metastasis. Interestingly, MMP13 itself has been shown to be a poor prognostic marker in breast cancer, with high levels in tumor cells correlating with decreased OS (33). Further studies are needed to fully elucidate the relationship between miR-187 and MMP13 in breast cancer and to ascertain if the increase in expression seen in vitro is replicated in the in vivo setting.

To date, there has been 1 publication on the effect of miR-187 on migration in ovarian cancer, reporting that miR-187 decreased migration in cell lines through the targeting of Dab2, which caused an inhibition of EMT (42). However, no changes in classical markers of EMT (vimentin, N-cadherin, E-cadherin, β-catenin) were noted in this study. As such, miR-187 does not seem to act as a modulator of EMT in breast cancer cells. Furthermore, in vitro data have yet to be published on the function of miR-187 in breast cancer, with functional studies in other cancers mainly focused on growth or apoptosis. However, the available data in this regard are conflicting. In 1 study, antisense inhibition of miR-187 led to decreased cellular growth in HeLa cells (45), whereas another study reported that overexpression of miR-187 in HeLa cells caused a decrease in cell viability and an induction of apoptosis (46). In addition, overexpression of miR-187 in human ovarian granulosa cells, reduced expression of proapoptotic protein, BAX, and the proliferative marker proliferating cell nuclear antigen (PCNA; ref. 47). Hence, it seems that miR-187 may act in a cell or organ-specific manner, altering target expression and thus phenotype depending on the gene expression profile of that cell line.

Although miRNAs are not currently used as cancer therapeutics, their successful application in in vivo studies strongly supports their development as novel drugs for the treatment of the various different stages of carcinogenesis. Indeed, the first report of the use of miRNAs in vivo related to restoration of the tumor-suppressive activities of let-7 in the

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<th>Table 1. Univariate and multivariate Cox regression analysis of miR-187 in cohorts 1 and 2</th>
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<td><strong>Univariate</strong></td>
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<td><strong>Cohort I</strong></td>
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<td>All patients ( n = 117 )</td>
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<td>Lymph node–positive ( n = 46 )</td>
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<td>Lymph node–negative ( n = 62 )</td>
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<td><strong>Cohort II</strong></td>
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<td>All patients ( n = 470 )</td>
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<td>Lymph node–positive ( n = 156 )</td>
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<td>Lymph node–negative ( n = 263 )</td>
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<td>All patients ( n = 462 )</td>
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<td>Lymph node–positive ( n = 155 )</td>
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</table>

NOTE: Significant contributing factors in multivariate Cox regression analysis were: <sup>a</sup>age and HER2; <sup>b</sup>tumor size, grade, PR status, nodal status; <sup>c</sup>tumor size, grade, PR status; <sup>d</sup>Nottingham Histological Grade (NHG), ER status; <sup>e</sup>grade, PR status, nodal status; <sup>f</sup>grade, PR status; <sup>g</sup>NHG, HER2 status. <sup>h</sup>Corrected for age, tumor size, NHG, lymph node status, ER, PR, and HER2 status.
prevention and treatment of lung tumors in mice (48). Successful in vivo studies relating to miR-31 and miR-10b have also been conducted in breast cancer (49, 50) although none have yet progressed to clinical trials. That said, the first phase II trial of a miRNA inhibitor targeting the liver-specific miR-122 for the treatment of hepatitis C virus (HCV) concluded recently with promising results (51).

In conclusion, we report the use of a novel bioinformatic screen to identify miRNAs associated with poor outcome in breast cancer gene expression cohorts and their validation in independent patient cohorts. In addition, we hypothesize that miR-187 may play a role in the overall progression of breast cancer and in the induction of a more aggressive and invasive phenotype. In this regard, miR-187 may function as not only an independent marker of poor prognosis but may serve as a novel drug target in patients with breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.F. Madden, D.J. Brennan, J.P. Crown, W.M. Gallagher, D.P. O’Connor
Development of methodology: S.F. Madden, G. Gremel, D.P. O’Connor
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Mulrane, D.J. Brennan, S.F. McGee, K. Jirström, W.M. Gallagher, D.P. O’Connor
Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): L. Mulrane, S.F. Madden, D.J. Brennan, S.F. McGee, D.G. Higgins, W.M. Gallagher, D.P. O’Connor
Writing, review, and/or revision of the manuscript: L. Mulrane, S.F. Madden, D.J. Brennan, F. Martin, J.P. Crown, W.M. Gallagher, D.P. O’Connor

Figure 5. High levels of miR-187 correlate with decreased BCSS in patients with breast cancer in cohort 2 (n = 470). Kaplan–Meier survival analysis of miR-187 expression levels in (A) all patients (negative, low, and high-miR-187 expression; n = 470), (B) all patients (dichotomized score: negative/low miR-187 vs. high miR-187; n = 470), (C) lymph node-negative (n = 263), and (D) lymph node-positive (n = 156) patients.
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References

37. Nikiforova MN, Tseng GC, Steward D, Diorio D, Nikiforov YE. Micro-
RNA expression profiling of thyroid tumors: biological significance and
38. Lo SS, Hung PS, Chen JH, Tu HF, Fang WL, Chen CY, et al. Over-
expression of miR-370 and downregulation of its novel target
TGFbeta-RII contribute to the progression of gastric carcinoma. Oncog-
MZ. MicroRNA profiling of Barrett’s oesophagus and oesophageal
40. Chen Y, Stallings RL. Differential patterns of microRNA expression in
neuroblastoma are correlated with prognosis, differentiation, and
41. Chao A, Lin CY, Lee YS, Tsai CL, Wei PC, Hsueh S, et al. Regulation of
ovarian cancer progression by microRNA-187 through targeting dis-
42. Blenkiron C, Goldstein LD, Thorne NP, Spreter I, Chin SF, Dunning MJ,
et al. MicroRNA expression profiling of human breast cancer identifies
global micro-RNA profiling with formalin-fixed paraffin-embedded
44. Rodriguez-Gonzalez FG, Siewerts AM, Smid M, Look MP, Meijer-van
Gelder ME, de Weerd V, et al. MicroRNA-30c expression level is an
independent predictor of clinical benefit of endocrine therapy in advanced estrogen receptor positive breast cancer. Breast Cancer
45. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of
human miRNAs and indications for an involvement of miRNA in cell
46. Park SY, Lee JH, Ha M, Nam JW, Kim VN. miR-29 miRNAs activate p53
47. Sirotkin AV, Laukova M, Ovcharenko D, Brenaut P, Miyneck M.
Identification of microRNAs controlling human ovarian cell proliferation
L, et al. The let-7 microRNA reduces tumor growth in mouse models of
50. Valastyan S, Chang A, Benaich N, Reinhardt F, Weinberg RA. Activ-
aion of miR-31 function in already-established metastases elicits
51. Reesink HW, Janssen HLA, Zeuzem E, Lawitz S, Rodriguez-Torres M,
Patel K, et al. Final results: randomized, double-blind, placebo-con-
trolled safety, anti-viral proof-of-concept study of miravirsen, an oli-
gonucleotide targeting miR-122, in treatment-naïve patients with
genotype 1 chronic HCV infection. 47th International Liver Congress
(EASL); 2012; Barcelona, Spain. J Hepatol 2012;56:pS1–S614.
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Laoighse Mulrane, Stephen F. Madden, Donal J. Brennan, et al.


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