miR-187 is an independent prognostic factor in breast cancer and confers increased invasive potential in vitro


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Additional Footnotes: *Shared senior authorship.

Keywords: microRNA, breast cancer, prognosis.

Running Title: miR-187 and breast cancer

Conflict of interest statement
The Authors declare no conflict of interest
Statement of 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 hybridisation on tissue microarrays in two additional patient cohorts and in an in vitro setting, demonstrating that miR-187 is associated with poor prognosis in breast cancer patients, specifically in those with one 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.
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

Co-inertia analysis 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 cohorts of breast cancer patients by in situ hybridisation 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 towards 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%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.
Introduction

Breast cancer is the most common female malignancy in the developed world, with over 60,000 women in the EU succumbing to the disease each year (1). However, as with most other malignancies, the predominant factor influencing mortality is not the primary tumour, 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.

MicroRNAs (miRNAs) are small non-coding RNA molecules, 20-25 nucleotides in length, that negatively regulate target gene expression by either inhibiting translation or promoting degradation of target mRNAs (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 tumour suppressors or oncogenes, depending on which genes or pathways they regulate. As a consequence, miRNAs have been implicated in many areas of cancer progression including tumour development, drug resistance and metastasis.

In the last decade, numerous miRNAs have been documented to influence the metastatic process (reviewed in 5). In breast cancer, miR-10b (6), miR-21 (7) and miR-31 (8), among others, play key roles in tumour 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 employed a novel bioinformatics approach, described in detail in (10), to identify miRNAs associated with the progression of breast cancer. This method integrates gene expression data with a database of predicted miRNA targets, using correspondence analysis (11), between group analysis (BGA) and co-inertia analysis (CIA) and produces a ranked list
of miRNAs associated with a specific gene signature and phenotype. The technique is designed to detect miRNAs that act through mRNA target degradation (12). We used gene expression data from two independent breast cancer patient cohorts to identify miRNAs involved in the progression of breast cancer (comprised of 40 (13) and 69 (14) patients, respectively) and then validated these results in vitro and in vivo by in situ hybridisation in two independent patient cohorts.
Materials and Methods

Gene expression profiling

Gene expression data were obtained from two cancer centres in Amsterdam (69 patients, (14)) and Rotterdam (40 patients (13, 14)). Both datasets have been described in detail previously. They contained oestrogen receptor (ER)-positive, predominantly post-menopausal 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 (13, 14) recurrences within six months of initiation of tamoxifen were classified as having a poor outcome (Amsterdam dataset n=21, Rotterdam dataset n=18), while those that demonstrated 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 normalised using the Bioconductor package limma (15) from the R statistical environment. Both datasets were analysed independently and the intersections between the target prediction programs and the genes on the array are described below.

Co-inertia analysis

For the Amsterdam (14) and Rotterdam (13) datasets, co-inertia analysis (CIA) and between group analysis (BGA) was used to simultaneously analyse mRNA gene expression data and predicted miRNA target sites in the 3’ UTRs of the same genes. This multivariate data integration technique uses the entire gene expression dataset and therefore does not require any pre-filtering of the transcriptomic data. CIA (16) was used to combine two 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 performed using BGA (17) to identify miRNAs associated with specific samples. The output from this analysis was five ranked lists of miRNAs associated
with poor outcome, one for each of the five miRNA target prediction programs (see below). Lists were combined using consistency among the methods, as previously published (10). All calculations were carried out using the MADE4 library (18) of the open source R package. MADE4 can be downloaded freely from the Bioconductor web site (19). 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) (20, 21), PicTar4way, PicTar5way (22) and miRanda (release 14) (23). 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, PicTar4way predicted 178 miRNAs across 4,840 genes, PicTar5way predicted 130 miRNAs across 1,767 genes and miRanda predicted 470 miRNAs across 9,172 genes.

**Cell lines**

MCF7 breast cancer cells were received as a gift from Professor Robert Clarke (Georgetown University, USA) and were cultured in phenol red-free Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA), 1% penicillin/streptomycin (50 units/ml) (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% L-glutamine (2 mM) (Gibco, Invitrogen, Carlsbad, CA, USA). SKBR3 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK) and were cultured in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich, St Louis, MO, USA), 1% penicillin/streptomycin (50 units/ml) (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% L-glutamine (2 mM) (Gibco, Invitrogen, Carlsbad, CA, USA). 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, Waltham, MA, USA). Briefly, HEK293T cells were seeded at a confluency of 50-70% and transfected using a calcium phosphate transfection method with lentiviral expression constructs containing non-targeting control (NTC) sequences or sequences for miR-187 (Open Biosystems) along with pMD2.G and psPAX2 viral envelope and packaging vectors (courtesy of the Trono Laboratory, Lausanne, Switzerland). Cells were incubated for a further 6-8 hours and the media was then changed. Viral particles were allowed to form over 48 hours before harvesting and filtration through 0.45 \( \mu \)m 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 X 10^4 cells per well). Infected cells were selected in media containing 2 \( \mu \)g/ml puromycin over a period of 3-5 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 (28) 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 which 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 was 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 which 30 patients received treatment. Information on radiotherapy was available for 144 patients of which 83 received adjuvant radiation.

Cohort 2 (previously described in (29)) consisted of 498 consecutive invasive breast cancer cases (470 in final analysis) diagnosed at the Department of Pathology, Malmö University Hospital.
Hospital, Malmo, Sweden, 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). 263 patients were dead at the last follow-up (December 2004), 90 of which were considered to be as a direct result of breast cancer (breast cancer-specific death). Complete endocrine treatment data was available for 379 patients, 160 of whom received adjuvant tamoxifen. Information on adjuvant chemotherapy was available for 382 patients, of which 23 patients received treatment. Clinicopathological 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.

**Tissue microarrays and in situ hybridization**

Tissue microarrays (TMAs) were constructed as previously described (30). In brief, TMA sections were baked at 60°C for 60 minutes, de-waxed in xylene and rehydrated. Tissue was permeabilised by treatment with 10 μg/ml proteinase K for 5 minutes at 37°C. Slides were washed with diethylpyrocarbonate-treated phosphate-buffered saline (DEPC-treated PBS) and re-fixed 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-labelled locked nucleic acid (LNA) miRCURY probes (labelled at both the 3’ and 5’ ends; miR-187 probe sequence: CCGGCTGCAACACAAGACGA; Scramble probe sequence: GTGTAACACGTCTATACGCCCA) (Exiqon, Vedbaek, Denmark) were diluted to a concentration of 20 nM in hybridisation solution (50% formamide, 5X SSC, 0.1% Tween, 9.2 mM citric acid, 50 μg/ml heparin, 500 μg/ml yeast RNA). Hybridisation was carried out overnight at 60°C in a humidified chamber.

Slides were then washed in a series of dilutions of saline sodium-citrate buffer (20X SSC; Sigma-Aldrich, St Louis, MO, USA) heated to 60°C (twice for 30 minutes in 2X SSC, twice for 15 minutes in 1X SSC and twice for 15 minutes in 0.1X SSC) and then incubated in Buffer 1 (100 mM Tris-HCl, 150 mM 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 anti-
DIG alkaline phosphatase fab fragments (1:1000 dilution in blocking buffer, Roche Applied Science, Penzberg, Germany). Sections were washed twice in Buffer 1 and once in Buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) for 10 minutes each. NBT/BCIP tablets (Roche Applied Science, Penzberg, Germany) were dissolved in water and levamisole added to a concentration of 1 mM. After the desired development time (in this case, 4 days as optimised on full face sections), the colourimetric reaction was stopped by incubating slides in stop solution (100 mM Tris-HCl, 1 mM 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, 50% water).

TMA cores were scored manually using a semi-quantitative scoring system comprised of negative, low and high staining intensity by two independent observers.

**Image acquisition and management**

Digital images were captured using the Aperio ScanScope XT Slide Scanner (Aperio, Vista, CA, USA) with a 20X objective as previously described (31). TMA images were managed using Spectrum and snapshots taken using ImageScope software (Aperio, Vista, CA, USA).

**Statistics**

Spearman’s Rho correlation was used to estimate the relationship between duplicate cores from individual tumours, between 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 breast cancer-specific survival (BCSS) according to miR-187 expression. Pearson’s chi-squared test was used to evaluate associations between miR-187 expression and clinicopathological characteristics. All survival calculations were performed using SPSS version 17.0 (SPSS Inc., now part of IBM, Armonk, NY, USA). A 2-tailed student’s t-test was used to determine statistical significance over three independent repeats of *in vitro* experiments using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). In all cases, a p value < 0.05 was considered statistically significant.
Results

Co-intertia analysis identifies miRNAs linked to poor outcome in breast cancer

For each independent dataset [Amsterdam (69 patients) (14) and Rotterdam (40 patients) (13)], a combination of unsupervised CIA (using correspondence analysis) and supervised CIA (using BGA) was used to simultaneously analyse 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 performed, one for each target prediction program (10). Figure 1 shows an example of unsupervised analysis of the Amsterdam dataset using the TargetScan target prediction program. The plot is in two parts, Figure 1 (A) depicts a correspondence analysis plot of patient samples and Figure 1 (B) depicts miRNAs associated with these samples. The two plots are interrelated; samples in part (A) are associated with miRNAs in part (B) in the same orientation relative to the origin. In Figure 1 (A), there is a trend along the vertical axis separating the patients with a poor outcome from those who remained disease free at 6 months. In Figure 1 (B), miR-433-5p and miR-187 are in the same orientation from the origin as the poor outcome tumours in Figure 1 (A), suggesting that these miRNAs are associated with this trend in the data.

In order to systematically identify miRNAs specifically associated with poor outcome, a supervised analysis was performed 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 cells were engineered to ectopically express miR-187 and expression validated using qRT-PCR [Supplementary Figure S1 (A) and S3 (A), respectively]. miR-187 expression could not be detected in either parental MCF7 or non-targeting control (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 anti-oestrogen 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) [Figure 2 (A) & (B)].

Cells were then assessed using standard assays to model various aspects of tumour progression. In a scratch wound migration assay, a significant increase in wound closure was noted in miR-187 expressing MCF7 cells compared to cells expressing a non-targeting control (p=0.005) [Figure 2 (C)]. Similarly, miR-187 expressing MCF7 cells demonstrated an increased ability to grow in anchorage-independent conditions (p=0.005 compared to NTC cells, p=0.006 relative to parental MCF7 cells) [Figure 2 (D)], but no increase in overall growth rate was observed [Supplementary Figure S1 (B)]. This increase in migration and soft-agar colony formation was mimicked in SKBR3 cells engineered to ectopically express miR-187 [Supplementary Figure S3 (B) and (C)]. Additionally, in a three-dimensional spheroid invasion assay, miR-187 expressing MCF7 cells demonstrated a higher degree of invasion into surrounding collagen compared to NTC cells (p=0.004) and parental MCF7 cells (p=0.002), which were minimally invasive even after 14 days [Figure 2 (E), quantification of invasion can be seen in Supplementary Figure S1 (C)]. Furthermore, ectopic expression of miR-187 resulted in a significant increase in expression of collagenase-3 (MMP13) [Figure 2 (F)] but no changes in other matrix metalloproteases (MMP3, MMP11 and MMP21) [Supplementary Figure S2 (A)] or common markers of epithelial-mesenchymal transition (EMT) were noted [Supplementary Figure S2 (B)].

**In situ hybridisation 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 demonstrated 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 two independent cohorts. Staining of miR-187 by *in situ* hybridisation (ISH) was manually scored by cytoplasmic intensity [Figure 3 (A) & (B)]. No staining was observed when using a scrambled probe [Figure 3 (A)]. A high level of concordance was also observed between duplicate cores (Spearman’s Rho analysis, r=0.800, p<0.001) and between independent observers (Spearman’s 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 hybridisation, 117 and 470 patients respectively, were suitable for analysis. To ensure that this did not introduce selection bias, Pearson’s chi-squared analysis was used to estimate the association between missing patients and clinicopathological 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 tumours (4 ISH negative and 4 ISH positive) and TaqMan-based qRT-PCR was performed. A high degree of correlation was seen between ISH scores and relative expression of the miRNA (Spearman’s Rho analysis, r=0.823, p=0.012) [Figure 3 (C)].

Kaplan Meier analysis of Cohort 1 (n=117) revealed a non-significant stepwise association between miR-187 expression and breast cancer-specific survival (BCSS) [Figure 4 (A)]. Based on these findings, data were dichotomised whereby tumours in the high miR-187 group were compared to those in negative/low miR-187 groups. This revealed a trend towards reduced BCSS in the high miR-187 group (p=0.058) [Figure 4 (B)]. Subset analysis based on lymph-node status was performed and demonstrated that high expression of miR-187 was associated with reduced BCSS in lymph node positive (LN-positive) (p=0.036) [Figure 4 (D)] but not in lymph node-negative (LN-negative) patients (p=0.715) [Figure 4 (C)]. Multivariate Cox regression analysis correcting for age, tumour size, lymph node status, grade, ER, PR and HER2 status demonstrated that miR-187 was an independent prognostic marker in the entire Cohort 1 and particularly in lymph node positive patients (HR 13.74, 95% CI 2.62-72.03, p=0.002) (Table 1).

In Cohort 2 (n=470), a similar trend was noted whereby tumours expressing high levels of miR-187 were associated with a reduced BCSS [Figure 5 (A)] and recurrence-free survival (RFS) [Figure S4 (A)]. Using the same threshold for dichotomisation 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 [Figure 5 (B) and S4 (B), respectively]. Subset analysis based on lymph node status again demonstrated that high miR-187 expression was associated with a reduced BCSS (p=0.012) and RFS (p=0.037) in LN-positive patients but not in the LN-negative
subgroup \((p=0.248\) for BCSS and \(p=0.162\) for RFS) [Figures 5 and S4 (C) & (D)]. Multivariate Cox regression analysis using the model described above again demonstrated 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 LN 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 demonstrated a borderline significant association with tumour size in Cohort 1 \((p=0.048)\) but did not correlate with any other clinicopathological factors in either TMA cohort [Supplementary Tables S3 & S4]. Additionally, 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 demonstrated 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 Figure S5 (A) & (B)).

In Cohort 2, miR-187 expression was not associated with OS or BCSS when stratification was performed based on ER status. We did observe a trend towards 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 Figure S5 (C) & (D)).
Discussion

Using a comprehensive bioinformatics approach we have discovered that miR-187 is associated with poor outcome in two 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 demonstrated in SKBR3 cells). Additionally, using LNA-ISH to determine miR-187 expression in tumour tissue, we have demonstrated that miR-187 is an independent marker of poor prognosis in two 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 (32, 33) with most bioinformatic tools concentrated on predicting miRNA targets (20, 23) or predicting the presence of a miRNA within genomic sequences (34) or in high-throughput sequencing analyses (35, 36). The approach applied in this paper (10) makes use of publicly available target prediction programmes 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 pre-processing of the gene expression data, such as the generation of clusters or gene lists and facilitates data visualisation 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 et al. recently characterised miRNA degradation as the primary mode of miRNA action (12) and that decreased mRNA expression accounted for ~80% of the reduced protein production.

This technique was applied to two 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 (characterised 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 remodelling enzyme known to be involved in the progression of many cancers (37, 38), 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 demonstrate any alterations in common markers of EMT, suggesting that matrix re-modelling is the mechanism of increased invasion. Furthermore, when assessed in two further breast cancer cohorts, high levels of miR-187 were found to correlate with poor outcome independent of other clinicopathological factors, further validating the in silico and in vitro results. In particular, the association between miR-187 and poor prognosis in LN-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 (39), renal (40), pancreatic (41), thyroid (42), gastric (43) and oesophageal cancers (44), as well as neuroblastoma (45), 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 instrumental in the regulation of ovarian cancer progression through the targeting of Dab2, and was significantly associated with increased OS and RFS in a cohort of patients with the disease (n=176) (46). In breast cancer, Blenkiron et al. have observed that miR-187 is expressed at higher levels in ER-negative versus -positive tumours and appears to be over-represented in HER2-positive cancers (47), while Hui et al. note that the expression of the miRNA is >2.6 fold increased in breast cancer versus normal tissue (48). 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 et al. report that miR-187 was significantly differentially expressed in ER-positive LN-positive patients who did not respond to tamoxifen therapy versus those that did (n=38) using qRT-PCR (49). 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 tumour cells correlating with decreased overall survival (38). 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 one 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 Disabled homologue 2 (Dab2), which caused an inhibition of EMT (47). 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 appear to act as a modulator of EMT in breast cancer cells. Furthermore, in vitro data has 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 is conflicting. In one study, antisense inhibition of miR-187 led to decreased cellular growth in HeLa cells (50), while another study reported that overexpression of miR-187 in HeLa cells caused a decrease in cell viability and an induction of apoptosis (51). Additionally, overexpression of miR-187 in human ovarian granulosa cells reduced expression of pro-apoptotic protein, BAX, and the proliferative marker PCNA (52). Hence, it appears 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 tumour-suppressive activities of let-7 in the prevention and treatment of lung tumours in mice (53). Successful in vivo studies relating to miR-31 and miR-10b have also been conducted in breast cancer (54, 55) although none have yet progressed to clinical trials. That said, the first Phase II trial of a microRNA inhibitor targeting the liver-specific miR-122 for the treatment of Hepatitis C (HCV) concluded recently with promising results (56).
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. Additionally, we hypothesise 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 breast cancer patients.
Acknowledgements

The authors would like to thank Prof Rene Bernards, Netherlands Cancer Institute, Amsterdam for assistance with the in silico datasets.

Grant Support

Funding is acknowledged from the Irish Research Council for Science, Engineering and Technology (IRCSET) (Laoighse Mulrane), the Health Research Board of Ireland (award RP/2007/251) (Stephen Madden, Darran P O'Connor, Desmond G Higgins, William M Gallagher), and Science Foundation Ireland through the Molecular Therapeutics for Cancer, Ireland Strategic Research Cluster (award 08/SRC/81410) (Darran P O'Connor, Laoighse Mulrane, John P Crown, William M Gallagher).
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**Tables**

**Table 1.** Univariate and Multivariate cox regression analysis of miR-187 in Cohorts 1 and 2

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<tr>
<td><strong>Cohort I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Patients (n=117)</td>
<td>2.44</td>
<td>0.94-6.33</td>
</tr>
<tr>
<td>Lymph Node Positive (n=62)</td>
<td>3.20</td>
<td>1.01-10.10</td>
</tr>
<tr>
<td>Lymph Node Negative (n=46)</td>
<td>1.56</td>
<td>0.14-17.18</td>
</tr>
<tr>
<td><strong>Cohort II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Patients (n=470)</td>
<td>1.71</td>
<td>1.08-2.72</td>
</tr>
<tr>
<td>Lymph Node Positive (n=156)</td>
<td>2.09</td>
<td>1.16-3.74</td>
</tr>
<tr>
<td>Lymph Node Negative (n=263)</td>
<td>1.68</td>
<td>0.69-4.08</td>
</tr>
<tr>
<td><strong>RFS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Patients (n=462)</td>
<td>1.61</td>
<td>1.10-2.35</td>
</tr>
<tr>
<td>Lymph Node Positive (n=155)</td>
<td>1.70</td>
<td>1.03-2.83</td>
</tr>
<tr>
<td>Lymph Node Negative (n=260)</td>
<td>1.56</td>
<td>0.83-2.91</td>
</tr>
</tbody>
</table>

*corrected for for age, tumour size, NHG, lymph node status, ER, PR and HER2 status.

Significant contributing factors in multivariate cox regression analysis were aAge and HER2; bTumour size, grade, PR status, Nodal status; cTumour size, grade, PR status; dNHG, ER status; eGrade, PR status, Nodal status; fGrade, PR status; gNHG, HER2 status.
Figure Legends

Figure 1. Visualisation of the horizontal (axis 3) and vertical (axis 4) axes of unsupervised CIA for the Amsterdam dataset using TargetScan miRNA target predictions.
(A) NSC plot of breast cancer samples with poor outcome (red) and good outcome (blue). The vertical axis splits the data between poor and good prognosis samples. (B) NSC plot of miRNAs. miRNAs that are in the same orientation relative to the origin as the breast cancer samples with poorer outcome [represented in red in (A)] are associated with those samples. Circled in red is miR-187, which is the most highly ranked miRNA in the supervised analyses.

Figure 2. Ectopic expression of miR-187 in MCF7 cells results in increased migration, invasion and anchorage-independent colony formation.
(A) and (B) MTT cell viability assay demonstrating response of miR-187 expressing MCF7 cells to anti-oestrogens. Graph is representative of one of three independent repeats (+/- SD). No statistically significant change in response to either drug was noted (p=0.407 for tamoxifen and p=0.425 for fulvestrant); (C) Scratch wound assay demonstrating that MCF7 cells expressing miR-187 display greater migratory potential than parental MCF7 cells or those infected with a non-targeting control vector for one of three independent repeats +/- standard deviation (SD); p=0.005 relative to NTC cells, Student’s t-test based on the slope of each line; (D) Soft agar colony formation assay demonstrating that expression of miR-187 in MCF7 cells increases anchorage-independent growth compared to control cell lines for one of three independent repeats, +/- SD; (E) Spheroid invasion assay demonstrating that spheroids formed by MCF7 cells expressing miR-187 show increased invasion into collagen compared to control cell lines; (F) Western blot of MMP13 in cell lysates with β-actin as a loading control;

Figure 3. In situ hybridisation demonstrates differential miR-187 expression in breast cancer and correlates with relative qRT-PCR levels.
In situ hybridisation (ISH) staining of tissue cores from invasive breast cancer patients. (A) TMA cores displaying different levels of miR-187, scored manually as negative, low miR-187 expression or high expression along with the same TMA cores hybridised with a scramble LNA probe showing no staining; (B) High magnification (20X) images from the TMA cores in
(A) and (C) Correlation of manual ISH scores in 8 sample breast tumours with relative miR-187 levels determined by qRT-PCR demonstrating a high degree of correlation ($r=0.823$, $p=0.012$).

**Figure 4. High levels of miR-187 correlate with decreased breast cancer-specific survival in lymph node positive breast cancer patients 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 (dichotomised 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.

**Figure 5. High levels of miR-187 correlate with decreased breast cancer-specific survival in breast cancer patients 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 (dichotomised 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.
Figure 2

Panel A: Graph showing cell viability against Tamoxifen concentration. The p-value is 0.407.

Panel B: Graph showing cell viability against Fulvestrant concentration. The p-value is 0.425.

Panel C: Graph showing wound closure over time. The p-value is 0.005.

Panel D: Bar graph showing increase in colony formation. The p-values are 0.005 and 0.006.

Panel E: Images showing cell growth over days 0, 4, and 14 for MCF7 miR-187, MCF7 NTC Vector, and MCF7 Parental.

Panel F: Western blot analysis showing protein expression of MMP13 and β-actin for MCF7 Parental, MCF7 NTC Vector, and MCF7 miR-187 (70kDa, 55kDa, 35kDa).
miR-187 negative

Low miR-187

High miR-187

Scramble miR-187 negative

Scramble low miR-187

Scramble high miR-187

miR-187 positive

miR-187 negative

Scramble miR-187 negative

Scramble low miR-187

Scramble high miR-187

Figure 3

r=0.823, p=0.012

miR-187 relative expression (fold change)

miR-187 ISH
Cohort 1: Breast cancer-specific survival

All patients (n=117)

- miR-187 negative: Low, n=11
- miR-187 positive: Low, n=72
- miR-187 negative: High, n=34
- miR-187 positive: High, n=34

$p=0.099$

Lymph node-negative (n=62)

- miR-187 negative: Low, n=47
- miR-187 positive: Low, n=15
- miR-187 negative: High, n=15
- miR-187 positive: High, n=15

$p=0.715$

Lymph node-positive (n=46)

- miR-187 negative: Low, n=29
- miR-187 positive: Low, n=17
- miR-187 negative: High, n=17
- miR-187 positive: High, n=17

$p=0.036$

All patients: high vs. low miR-187

- Low miR-187: n=83
- High miR-187: n=34

$p=0.058$
Cohort 2: Breast cancer-specific survival

**All patients (n=470)**

- **Cohort 2: Breast cancer-specific survival**
- **A** All patients (n=470)
- **p=0.043**

**Lymph node-negative (n=263)**

- **B** Lymph node-negative (n=263)
- **p=0.021**

**Lymph node-positive (n=156)**

- **C** Lymph node-positive (n=156)
- **p=0.248**

- **D** Lymph node-positive (n=156)
- **p=0.012**

**Figure 5**

Research.
miR-187 is an independent prognostic factor in breast cancer and confers increased invasive potential in vitro

Laoighse Mulrane, Stephen F Madden, Donal J Brennan, et al.

*Clin Cancer Res* Published OnlineFirst October 11, 2012.

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