The Oncogenic Role of miR-155 in Breast Cancer

Sam Mattiske*, Rachel J Suetani, Paul M Neilsen, David F Callen

Running title: miR-155 and breast cancer

Funding

Funding was provided from the National Health and Medical Research Council Australia. SM is supported by a University of Adelaide Faculty of Health Sciences Divisional Scholarship.

Conflict of Interest

The authors have no conflict of interest to disclose.

*Corresponding Author:

Address: Hanson Institute, University of Adelaide, Frome Road, SA 5005 AUSTRALIA

Phone: +61 8 8222 3450

Email: samuel.mattiske@adelaide.edu.au

Word count: 3429

Figures/tables: 1 table, 2 figures, 2 supplementary tables
Abstract

miR-155 is an oncogenic microRNA with well described roles in leukemia. However, additional roles of miR-155 in breast cancer progression have recently been described. A thorough literature search was conducted to review all published data to date examining the role of miR-155 in breast cancer. Data on all validated miR-155 target genes was collated to identify biological pathways relevant to miR-155 and breast cancer progression. Publications describing the clinical relevance, functional characterisation, and regulation of expression of miR-155 in the context of breast cancer are reviewed. 147 validated miR-155 target genes were identified from the literature. Pathway analysis of these genes identified likely roles in apoptosis, differentiation, angiogenesis, proliferation and EMT. The large number of validated miR-155 targets presented here provide many avenues of interest as to the clinical potential of miR-155. Further investigation of these target genes will be required to elucidate the specific mechanisms and functions of miR-155 in breast cancer. This is the first review examining the role of miR-155 in breast cancer progression. The collated data of target genes and biological pathways of miR-155 identified in this review suggest new avenues of research for this oncogenic microRNA.
Introduction

MicroRNAs (miRNAs) are small noncoding RNAs which control expression of target genes by either inhibiting protein translation or directly targeting mRNA transcripts of target genes for degradation (1). Each miRNA has a specific seed sequence 7-8 nucleotides long, which directly binds to complementary sequences in regulatory regions of target genes. These binding regions are often in the 3’ UTR of target genes, but increasingly are being reported in other non-coding regions such as promoter or intronic regions (2). The short length of the seed sequence facilitates the targeting of many transcripts by a single miRNA (3). Some estimates suggest that 30% of all eukaryotic genes are regulated by miRNAs (4; 5). miR-155, a miRNA widely reported to be involved in lymphoma, is also now emerging to have a role in the progression of solid cancers (6). This review will focus on the microRNA miR-155, and its role in breast cancer.

miRNAs were discovered in 1993 when the *C.elegans* lin-4 gene, which is transcribed but not translated, was found to regulate levels of LIN-14 protein (7; 8). Since this discovery there have been over 500 miRNAs described, regulating a wide range of genes and cellular processes, although the total predicted number of unique miRNAs encoded by the human genome is estimated to be over 1000 (9). Many of these miRNAs are organised as gene clusters and transcribed as multicistronic messages – for example, the *MIRH1* gene encodes 6 different miRNAs (10). The transcription and processing of miRNAs has been well characterised, and is depicted in Figure 1 using miR-155 as an example. miRNAs originate from a ~70 nucleotide RNA hairpin pre-miRNA processed from the RNA transcript of the host gene (11) (in the case of miR-155, the host gene *BIC*). The pre-miRNA is typically cleaved by the Drosha and
Dicer exonucleases into a ~22 nucleotide RNA duplex. One strand of the duplex becomes the mature miRNA and is usually the functional, regulatory unit (12; 13) while the other is designated miR* and is usually degraded. The mature miRNA is loaded into Argonaute proteins, forming the RNA Induced Silencing Complex (RISC). The mature miRNA may then bind to its target by partial complementarity of target gene mRNA and either inhibit translation or cause degradation of the mRNA.

The miR-155 host gene, *BIC*, was first described in 1989 and postulated to be involved in the progression of lymphoma (14). In 2002, Lagos-Quintana *et al* identified miR-155 as a regulatory RNA (15). Subsequently, studies have focussed on the roles of miR-155 in lymphoma (16-19), and also in viral infection, cardiovascular disease and solid cancers (6; 20-22). miR-155 has over 400 predicted gene targets (23) and more than 100 confirmed *bona fide* targets. There is now an emerging role of miR-155 in breast cancer progression (20; 21; 24) which is the focus of this review.

**Clinical Relevance of miR-155 in Breast Cancer**

Studies show the expression level of miR-155 is upregulated in breast cancer with high levels of miR-155 associated with clinicopathological markers, tumour subtype and poor survival rates, summarised in Table 1 (21, 25-34). Of 29 miRNAs found to be dysregulated in breast cancer, the majority were downregulated, with only miR-155 and miR-21 significantly upregulated (25). Expression levels of 15 of these dysregulated miRNAs independently predict the invasive potential of breast tissue samples (25). A small microarray study of 8 fresh breast tumour samples found miR-155 was upregulated in the breast tumours compared to normal adjacent tissue (34). In a larger study, 62 breast carcinomas were analysed to determine miR-155 levels.
Out of 17 non-invasive tumours, only 2 (12%) exhibited a high level of miR-155 expression. Conversely, 41 of the 45 invasive tumours (91%) displayed miR-155 upregulation (32). In a further study expression levels of FOXO3A, a miR-155 target gene, was determined in 77 primary breast tumours, 38 recurrent tumours and 11 normal tissue samples. Results showed miR-155 was upregulated and FOXO3A downregulated in a majority of primary tumours, and also that high miR-155 and low FOXO3A expression was associated with recurrent tumours after radiotherapy or chemotherapy (21). These studies linked miR-155 expression to both invasiveness and recurrence of breast tumours, and demonstrated that expression levels of miR-155 and its specific target genes are of potential clinical prognostic value.

In a robust study of lung, stomach, prostate, colon, pancreatic tumours and 363 breast tumours, Volinia et al. globally compared miRNA expression levels in multiple tumour and pooled normal tissue samples to identify dysregulated miRNAs in tumour samples. Comparisons of normal and tumour tissue derived from each individual tissue showed that miR-155 expression was upregulated in breast, colon and lung cancers. Interestingly, miR-155 was one of only two miRNAs (the other being the miR-200 family) found to be upregulated in both breast and lung cancer, implying that these microRNAs may be part of a common mechanism in the development of cancer in these organs (26).

miR-155 expression levels have been shown to be associated with metastasis events and invasive properties of breast cancer. In one study, increased miR-155 expression was associated with high tumour grade, advanced stage and lymph node metastasis (31). Disease free and overall survival were also negatively correlated with miR-155
levels, further showing the potential of miR-155 as a miRNA of clinical interest.

These findings were further supported by two studies involving microarray analyses of FFPE breast cancer samples, which found that miR-155 expression was upregulated in metastases (28, 33).

Since miR-155 is associated with poor prognosis and/or metastasis, a correlation of miR-155 levels with breast cancer clinicopathological markers would be expected.

Analysis of 93 breast cancers for both miRNA levels alongside mRNA levels, to classify tumour subtypes, showed miR-155 levels were significantly upregulated in basal-like tumours and in estrogen receptor negative (ER-) tumours (27). The correlation with basal-like tumours has particular clinical relevance due to the poor prognosis of this tumour subtype.

Studies have investigated whether serum samples could be used to identify aberrant miRNA expression levels in breast cancer patients. In a small study of 21 patients Zhu et al. found that multiple miRNAs could be detected in sera and the miRNA levels correlated with the levels in tissue samples (29). The expression of miR-155 was higher in the serum of PR+ breast cancer patients than in the serum of PR- patients (29). Further studies confirmed these findings, with a significant correlation ($R^2=0.853$) between miRNA levels in fresh breast cancer tissue and matched serum samples (30). They confirmed that miR-155 was upregulated in breast cancer, and also that high miR-155 was associated with grade II and III tumours and ER- and PR- tumours (30). The detection of miR-155 expression levels in serum is a potential clinical prognostic indicator of tumour grade and hormone receptor status. The relationship of PR status and miR-155 expression is unresolved with two studies
reporting contradictory results (27, 29). The topic of serum miRNAs is also somewhat controversial, with some studies suggesting that serum miRNA levels are robust (35; 36), and others claiming that the miRNAs often used as normalisation controls are highly variable in sera samples, and thus miRNA quantification in sera is not reproducible (37). This suggests analysis of serum alone is not sufficient to determine whether miR-155 is differentially expressed. Since the number of samples in these studies is generally low, resolution requires a more robust study.

Taken together, these studies show miR-155 expression is upregulated in breast cancer, consistent with its status as an oncomiR, and is associated with more aggressive breast tumours. However, the relationships between miR-155 and clinicopathological markers, such as ER and PR status and tumour subtype, is inconsistent, probably due to small sample sizes and methodological aspects. For example, the upregulation of miR-155 expression in PR+ tumours was only identified in one study of a small number of samples (29). Further studies are required to confirm, and elucidate the basis, of the relationship between miR-155 and hormone receptor status.

**Functional characterisation of miR-155 oncogenic activities in breast cancer**

An important step in determining the clinical significance of miR-155, is to determine whether high expression levels are causally related to the development of breast cancer. *In vitro* effects of altering miR-155 expression levels were assessed in a panel of breast cancer cell lines (21). miR-155 expression was inhibited by anti-miR in HS578T cells. An anti-miR is a 2′-O-methyl oligoribonucleotide that inhibits the action of a miRNA. One proposed mechanism for anti-miR action is antisense binding
to the mature miRNA positioned in the RISC (38). The HS578T cell line expresses high levels of endogenous miR-155, and anti-miR-155 application resulted in cell cycle arrest and induction of apoptosis, implicating miR-155 in these processes (21). Conversely, ectopic overexpression of miR-155 in BT474 cells, which express very low levels of endogenous miR-155, promoted cell proliferation and survival and also improved chemoresistance (21). Taken together, these findings demonstrate that miR-155 has a role in cell proliferation and apoptosis, two cellular processes frequently aberrant in cancer. Similar results have also been reported in breast cancer cell lines MDA-MB-231 and MCF-7 where ectopic miR-155 overexpression increased proliferation, while inhibition of miR-155 expression by a specific anti-miR inhibits proliferation and increases radio-sensitivity of cells in vitro (20; 31).

Xenografted human breast cancer cells in immunodeficient mice provide in vivo confirmation of miR-155 as an oncomiR. Xenografts of MDA-MB-231 cells showed reduced tumour volumes compared to control xenografts when anti-miR-155 is expressed, while overexpression of miR-155 accelerated tumour growth (20). Similar, a xenograft of MDA-MB-468 cells, with low endogenous miR-155 expression, showed accelerated tumour growth when miR-155 was overexpressed (24). In the same study, knockdown of miR-155 in an orthotopically transplantated mouse tumour cell line inhibited tumour growth (24). Contrary to this, a recent study using the 4T1 mouse mammary model showed that miR-155 had no effect on growth of the primary tumour (39).

Although numerous studies show miR-155 is upregulated in human breast cancer, the cause of aberrant miR-155 levels is not well characterised. TGFβ treatment of NMuMG cells results in significant upregulation of miR-155 and an epithelial to
mesenchymal transition (EMT) (32). TGFβ is known to drive EMT, where immobile epithelial cells alter their morphology to become motile mesenchymal cells to promote invasion (40) and consequently cancer progression (41; 42). In NMuMG cells, Smad4, a key signalling molecule in the TGFβ pathway, can bind to the BIC promoter and enrich miR-155 expression levels, thereby augmenting the TGFβ EMT process (32). Knockdown of miR-155 in NMuMG cells by anti-miR suppressed, and ectopic overexpression of miR-155 enhanced TGFβ-mediated EMT (32). Furthermore, a key molecule in EMT, RhoA, is a target of miR-155, and expression of RhoA is reduced when miR-155 is ectopically expressed. When RhoA was expressed without its 3’UTR (containing the miR-155 seed sequence) the EMT phenotype caused by miR-155 was abrogated (32). The ability to reverse a severe phenotypical change by reexpressing just one of the targets of miR-155 alludes to a potential therapeutic approach. Many miRNAs are known to have a role in metastasis and EMT (43), so in light of these findings it is plausible the basis of miR-155 in promoting breast cancer, in particular the higher grade invasive breast cancers, is from the promotion of EMT. However, the findings from the 4T1 mouse model (39) contradict the findings in the NMuMG cell line (32). Unfortunately, both of the cell lines are of mouse origin. A miR-155 target gene in a mouse model will not necessarily be a target gene in humans, as the 3’UTR region of transcripts is a common location for miRNA seed sequences, and is not highly conserved between mice and humans. These conflicting results call into question the suitability of using a mouse-specific model for a miRNA study.

Regulation of miR-155 expression
Perhaps the most remarkable recent finding in relation to the role of miR-155 in breast cancer is the involvement with BRCA1. BRCA1, the breast cancer susceptibility gene, is involved in DNA damage repair and cell cycle progression. Mutations of BRCA1 are associated with a high risk of developing breast cancer (24). In a recent study, mouse embryonic stem cells expressing the R1699Q BRCA1 underwent spontaneous differentiation. The mutant cells displayed high levels of miR-155, and overexpression of miR-155 in BRCA1 wild type cells gave a similar phenotype to the mutant, indicating that BRCA1 was acting through miR-155 (24). In mice, a loss of functional BRCA1 results in miR-155 upregulation. These results were recapitulated in human cell lines, where deficient BRCA1 cells have 50-fold higher miR-155 levels compared to those with functional BRCA1 (24). Furthermore, the transient overexpression of BRCA1 reduces expression of miR-155. In clinical samples it was found that miR-155 levels were two to six fold higher in BRCA1 mutant tumours (24). The mechanism of BRCA1 regulation of miR-155 was through direct binding of BRCA1 protein to the miR-155 promoter. This in turn recruits histone deacetylase (HDAC) to repress the expression of BIC and thus miR-155 (24). This close association with the breast cancer susceptibility gene reinforces the importance of miR-155 in breast cancer.

**Target genes of miR-155**

The function of microRNAs are limited to inhibition of their target mRNA and consequent effects on cellular processes. miR-155 clearly has a role in breast cancer, and understanding this role requires the identification of critical miR-155 target genes.
Targets can be an *in silico* prediction software commonly used to identify putative target genes of particular miRNAs by alignment of the 7 or 8 nucleotide seed sequence with the 3’ UTR of 30,858 human transcripts based on conservation between human and mouse sequences (23). Targets can version 6 predicts 440 miR-155 targets (23; 44) based on sequence homology and conservation. Confirmation of these potential targets requires validation *in vitro*. To this end, we conducted literature search to identify published validated miR-155 target genes. A target was defined as validated when there was a specific luciferase 3’UTR reporter assay, which defines if miR-155 directly targets the transcript, together with at least one other quantitative method, such as qRT-PCR or Western blot analysis, to assess the repression of the expression levels of the endogenous target gene.

Supplementary Table 1 displays a comprehensive list of 147 validated target genes identified in a wide range of miR-155 studies (45-87), and their prediction status by Targets can. 103 target genes (including 11 target genes validated in other studies) were identified in a single high throughput next generation sequencing (NGS) study and validated by luciferase reporter assay (50). The remaining 44 target genes and their method of validation are displayed in Supplementary Table 2. Of the validated miR-155 target genes, approximately half (48%) were predicted by Targets can software (23; 44). This highlights the drawbacks in relying on *in silico* prediction tools to investigate potential miRNA targets. The discrepancy between predicted and observed miR-155 binding sites is affected by miR-155 targeting non-conserved sites in target genes, as Targets can by default searches for seed sequences conserved between human and mouse. Performing a Targets can search irrespective of site conservation predicts 2390 potential miR-155 targets, and encompasses all but 9
validated target genes. This is the first comprehensive collation of all known miR-155 target genes, and will be a valuable resource for future reference and research.

Although only a fraction of validated miR-155 target genes have a confirmed role in breast cancer, a number of the targets are involved in cancer-related pathways such as apoptosis, proliferation and EMT (20; 21; 32; 88; 89), as shown in Figure 2. The presence of validated miR-155 targets in these pathways highlight the importance of miR-155 in cancer progression.

**Conclusion**

As an oncomiR, expression levels of miR-155 are consistently upregulated in breast tumour samples. Studies have defined the clinical significance of miR-155 in breast cancer with an association with clinical markers, more aggressive tumours and decreased survival. However, there are some contradictory findings reported, for instance the varied association of miR-155 with hormone receptor status. It is also unclear as to whether miR-155 functions initiate cancer, or predominantly promotes tumour progression. In a mouse model, miR-155 has been shown to transform B-cells (90) but in breast cells has only been shown to enhance cancerous properties of tumour cells. More investigation is required to fully understand the significance of aberrantly high levels of miR-155 in breast cancer.

Exploration of the function of miR-155 in breast cancer cell lines and xenograft models shows that miR-155 enhances tumour growth, promotes cell proliferation, inhibits apoptosis and acts as a mediator of TGFβ-driven EMT. In particular, the role of miR-155 in EMT has promising therapeutic potential, given that miR-155 levels
have been shown to be elevated in invasive tumours and in breast tumour metastases. The large number of validated miR-155 targets presented in Supplementary Table 1 provide many avenues of further investigation as to the clinical potential of miR-155. The further investigation of these targets will be required to confirm the mechanistic and regulatory actions of miR-155, and their contribution to breast cancer.
References


Table 1 – Summary studies examining miR-155 expression in breast cancer

<table>
<thead>
<tr>
<th>miR-155</th>
<th>Tissue type</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ in breast cancer</td>
<td>76 Breast tumour</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td>10 Normal breast</td>
<td></td>
</tr>
<tr>
<td>↑ in breast cancer</td>
<td>363 Breast tumour</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>177 Normal breast</td>
<td></td>
</tr>
<tr>
<td>↑ in ER- tumours</td>
<td>93 Breast tumour</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>5 Normal breast</td>
<td></td>
</tr>
<tr>
<td>↑ in malignant breast tissue</td>
<td>34 Breast tumour</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>6 Normal Breast</td>
<td></td>
</tr>
<tr>
<td>↑ in PR+ tumours</td>
<td>Serum – 13 breast cancer patients</td>
<td>(29)</td>
</tr>
<tr>
<td>↑ in grade II and III tumours</td>
<td>Tumour, normal adjacent tissue and serum from 68 breast cancer patients</td>
<td>(30)</td>
</tr>
<tr>
<td>↑ in ER- PR- tumours</td>
<td>Tissue and serum from 40 healthy patients</td>
<td></td>
</tr>
<tr>
<td>Associated with higher tumour grade, advanced tumour stage, lymph node metastasis</td>
<td>92 Breast tumour and normal adjacent tissue</td>
<td>(31)</td>
</tr>
<tr>
<td>↑ in 41 of 45 invasive</td>
<td>45 Invasive breast tumour</td>
<td>(32)</td>
</tr>
<tr>
<td>↑ 2 of 17 noninvasive tumours</td>
<td>17 Noninvasive breast tumour</td>
<td></td>
</tr>
<tr>
<td>↑ in 55 breast tumours</td>
<td>77 breast tumour</td>
<td>(21)</td>
</tr>
<tr>
<td>↑ 31 recurrent tumours</td>
<td>11 Normal breast</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38 Recurrent breast tumour</td>
<td></td>
</tr>
<tr>
<td>↑ in breast metastases</td>
<td>13 Breast tumour and paired metastasis</td>
<td>(33)</td>
</tr>
<tr>
<td>↑ in tumours</td>
<td>8 Breast tumour and normal adjacent tissue</td>
<td>(34)</td>
</tr>
</tbody>
</table>

Tables
Figure Legends

Figure 1: Cellular processing and downstream effects of miR-155 in breast cancer.

The pri-miR-155 RNA hairpin transcript is processed from the RNA transcript of the BIC gene. Transcription of BIC is promoted by Smad4, and inhibited by BRCA1. After processing by Drosha, Pasha, Exportin-5 and Dicer, the mature miR-155 forms a complex with Argonaute proteins called the RNA Induced Silencing Complex (RISC), in order to inhibit the translation of miR-155 target mRNAs, such as RhoA, FOXO3A and SOCS1. The inhibition of target genes by miR-155 in breast cancer can cause such effects as an increase in EMT, cell plasticity, cell survival, growth, chemoresistance and radioresistance.

Figure 2 - miR-155 target genes involved in cancer-related pathways.

Validated miR-155 target genes are present in multiple pathways associated with cancer and cancer progression, including but not limited to: EMT, proliferation, block of differentiation, apoptosis, sustained angiogenesis. Pathway analysis was completed using DAVID bioinformatics resource (v 6.7).
Figure 1: Cellular processing and downstream effects of miR-155 in breast cancer.

- BIC gene
  - Smad4
  - BRCA1
- BIC RNA transcript
  - Nucleus
  - Cytoplasm
  - Exportin-5
  - Drosha
  - Pasha
- pri-miR-155
  - Dicer
  - pre-miR-155 (62nt)
  - miR-155 duplex (22nt)
  - mature miR-155 (22nt)
  - RISC
  - Degradation
  - miR-155* (+ Argonaute proteins)
- Degradation/Inhibition of translation of key breast cancer targets
  - eg FOXO3A, RhoA, SOCS1
- Cell survival
- Cell growth
- Chemoresistance
- Radioresistance
- Cell plasticity
- EMT
Figure 2—miR-155 target genes involved in cancer-related pathways
The Oncogenic Role of miR-155 in Breast Cancer

Sam Mattiske, Rachel J Suetani, Paul M Neilsen, et al.

Cancer Epidemiol Biomarkers Prev Published OnlineFirst June 26, 2012.