MicroRNA-190b Targets RFWD3 in ER Positive Breast Cancer

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Research Article

Keywords: Breast cancer, microRNA-190b, estrogen receptor, RFWD3, TCGA, Prognosis

Posted Date: March 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1369734/v1

Abstract

Background: In the year 2020 breast cancer was the most common form of cancer. Roughly 70% of breast cancers are estrogen receptor positive. MicroRNA-190b has previously been reported to be up-regulated in estrogen receptor positive breast cancers. Our group has previously demonstrated that microRNA-190b is hypomethylated in ER+ breast cancers, potentially leading to its upregulation.

Results: In this study, using data from the Cancer Genome Atlas, we confirm that microRNA-190b is overexpressed in breast cancer via differential expression analysis and show that high expression of microRNA-190b results in more favorable outcomes in Luminal A patients (HR=0.29, 95% CI 0.12-0.71, P value=0.0063). MicroRNA190b target analysis, using immunoprecipitation of biotin labelled microRNA-190b, followed by RNA sequencing, identified RFWD3 as one of microRNA-190b's regulatory targets in estrogen receptor positive breast cancer. Survival analysis of RFWD3 showed that elevated levels result in poorer overall survival in Luminal A breast cancer patients (HR = 2.22, 95% CI 1.33-3.71, P = 0.002). Gene ontology analysis of our sequencing results indicate that miR-190b may have a role in breast cancer development and/or tumorigenesis and that it may be a suitable tool in characterization between the estrogen receptor positive subtypes, Luminal A and Luminal B.

Conclusions: We show that miR-190b targets RFWD3 in ER+ breast cancers leading to lower RFWD3 protein expression. Low levels of RFWD3 are associated with better outcomes in Luminal A breast cancer patients but not in Luminal B patients.

Background

In 2020 breast cancer was the top diagnosed cancer around the world with approximately 2.3 million new occurrences, representing 11.7% of total cancer cases. Leading to approximately 685.000 deaths, it was the 5th leading cause of global cancer related mortality and accounted for 1 in 6 cancer deaths in women[1]. Breast cancer is commonly divided into five clinical subtypes based on genomic profiling and/or histopathological parameters [2]. Patient prognosis, therapeutic response and strategy varies with each subtype, which are thought to be a consequence of different oncogenic drivers and evolutionary paths [3].

The majority of breast cancers, or approximately 70%, are estrogen receptor-positive (ER+) and are divided into the subtypes LuminalA (LumA) and LuminalB (LumB) [4]. These cancers are most commonly treated by inhibiting the estrogen receptor or by the inhibition of estrogen itself [5]. Luminal breast cancers have a relatively good prognosis, however, a subgroup remains resistant to therapy. This is especially seen in patients with LumB breast cancers which are younger at diagnosis, have faster growing tumors and have generally worse prognosis in comparison to LumA patients [6]. Further characterization between the two luminal subtypes, as for all subtypes, is thus vital for understanding the oncogenic drive of these cancers and to improve patient outcomes [4].

MicroRNAs (miRNA) are small non-coding RNAs which play a role in gene silencing by post-transcriptionally binding to the 3'UTR of mRNA, resulting in inhibition of protein translation. Their expression is tissue specific and leads to fine tuning of gene expression [7]. They are known to have a widespread phenotypic impact, have the ability to bind to multiple genes and are known to have a role in cancer and carcinogenesis [8, 9, 10, 11].

It has been previously noted that microRNA-190b (miR-190b) is overexpressed in ER + tumors [12]. In addition, we have previously shown that loss of DNA methylation at the promoter of miR-190b leads to its overexpression in ER + breast cancers and that breast cancer specific survival is more favorable in patients with miR-190b hypo-methylated LumA breast tumors [13]. Though miR-190b's impact on some cancers has been researched, such as impairing insulin signaling and gluconeogenesis through targeting IGF-1 in hepatocellular carcinoma [14], and mediating radio-sensitivity in gastric cancer by targeting Bcl-2 [15], miR-190b's role in breast cancer remains unclear, particularly in the context of ER status.

In this study we aimed at investigating miR-190b in breast cancer, correcting for ER status to account for transcriptional variability between the two groups. We analyzed RNA-seq data from The Cancer Genome Atlas (TCGA) and show miR-190b expression results that are consistent with what we had already seen for DNA methylation [13] where miR-190b is overexpressed in ER + tumors and overall patient survival is more favorable in patients with miR-190b overexpression in LumA cancers. Furthermore, differential expression analysis in miRNA-seq from TCGA confirmed miR-190b to be differentially expressed between normal breast tissue and tumors. We performed a biotin-miR-190b pulldown followed by RNA-seq in the ER + breast cancer cell line T-47d for target discovery and found RFWD3, which plays a role in genome maintenance [15], to be a clinically relevant target of miR-190b.

Results

MiR-190b is overexpressed in ER + tumors

Using RNA-seq data from the TCGA database we confirm what we have previously seen in the Icelandic cohort [13]. miR-190b was significantly overexpressed in primary tumors overall (n = 1090) compared to normal breast tissue (n = 104) while there was no significant difference between primary and metastatic (n = 7) tumors nor between normal tissue and metastatic tumors (Kruskal-Wallis, P value = 2.2^{-16} followed by Dunn's multiple comparison, Median values: normal tissue = 2.51, metastatic tumors = 5.07, primary tumors = 5.70) (Fig. 1A). MiR-190b was significantly higher expressed in ER + tumors (n = 810) compared to both ER- tumors (n = 237) and normal tissue (n = 104), while there was no significant difference between ER- tumors and normal tissue (Kruskal-Wallis, P value = 2.2^{-16} followed by Dunn's multiple comparison, Median values: normal tissue = 2.51, ER-=2.33, ER + = 6.14) (Fig. 1B). Finally, miR-190b was overexpressed in both LumA and LumB compared to other subtypes which is in harmony with our previously published methylation data (Kruskal-Wallis P value = 2.2^{-16} followed by Dunn's multiple comparison, Median values: Basal = 1.97 (n = 188), HER2 = 3.84 (n = 81), Normal-like = 3.45 (n = 40), LumA = 6.15 (n = 564), LumB = 6.31 (n = 203) (Fig. 1C).

MiR-190b is significantly overexpressed in differential expression analysis.

Differential miRNA-expression analysis of primary tumors compared to solid normal tissue from TCGA resulted in 353 (22%) up-regulated and 227 (14%) down-regulated miRNAs (FDR < 0.05) out of 1626 nonzero total read counts (Supplementary data 1). 152 (9.3%) of the miRNAs had a log2-fold change ± 1. MiR-190b had a log2-fold change of + 3.37 (P-adjusted = 1.50^{-13}). Figure 2 shows the top 30 differentially expressed miRNAs in primary breast cancers compared to normal tissue in the TCGA cohort. We see no clear clustering of LumA or LumB. The most distinct expression patterns can be seen for miR-184, miR-196a1/2, miR-190b and miR-210. MiR-190b's transcriptional pattern shows a clear clustering of normal tissue and Basal tumors.

Clinical outcome by miR-190b

Table 1 outlines the pathological and clinical characteristics of the cohort. [Insert table one] We pursued what was briefly introduced in our previous publication where Cox proportional hazards regression over time was used to study overall survival in the breast cancer patients based on miR-190b expression levels [13]. Median follow up was 2.21 years while maximum follow up was 19.35 years. A significant difference in survival was found, overall, in the patients in miR-190b high vs low expression (HR = 0.55, 95% CI 0.32–0.95, P value = 0.033). Upon looking into patients with ER + tumors, we did not see a significant difference in survival based on miR-190b expression. When looking into specific subtypes we saw, similarly to our previously published results, significantly better survival in patients with high expression of miR-190b in LumA tumors (HR = 0.29, 95% CI 0.12–0.71, P value = 0.0063). No difference was seen in patients with LumB nor ER- tumors in general.

Table 1
The pathological and clinical characteristics of the TCGA cohort.

		Overall
n		1097
Sample Type (%)	Primary Tumor	1090 (99.4)
	Metastatic	7 (0.6)
Subtype (PAM50) (%)	Basal	188 (17.1)
	Her2	81 (7.4)
	LumA	564 (51.4)
	LumB	203 (18.5)
	NA	2 (0.2)
	Normal	40 (3.6)
	NA	19 (1.7)
Gender (%)	FEMALE	1085 (98.9)
	MALE	12 (1.1)
Age at diagnosis (median [IQR])		58.00 [49.00, 67.00]
Year of diagnosis (%)	2016	1097 (100.0)
Race (%)	AMERICAN INDIAN OR ALASKA NATIVE	1 (0.1)
	ASIAN	61 (5.6)
	BLACK OR AFRICAN AMERICAN	183 (16.7)
	WHITE	764 (69.6)
	NA	88 (8.0)
Icd 10 (%)	C50.2	2 (0.2)
	C50.3	3 (0.3)
	C50.4	3 (0.3)
	C50.5	1 (0.1)
	C50.8	2 (0.2)

		Overall
	C50.9	1085 (98.9)
	C50.919	1 (0.1)
Lymph node presentation (%)	NO	28 (2.6)
	YES	704 (64.2)
	NA	365 (33.3)
Histology (%)	Infiltrating Carcinoma NOS	1 (0.1)
	Infiltrating Ductal Carcinoma	782 (71.3)
	Infiltrating Lobular Carcinoma	204 (18.6)
	Medullary Carcinoma	6 (0.5)
	Metaplastic Carcinoma	10 (0.9)
	Mixed Histology (please specify)	29 (2.6)
	Mucinous Carcinoma	17 (1.5)
	Other, specify	47 (4.3)
	NA	1 (0.1)
Menopause status (%)	Indeterminate (neither Pre or Postmenopausal)	33 (3.0)
	Peri (6–12 months since last menstrual period)	41 (3.7)
	Post (prior bilateral ovariectomy OR > 12 mo since LMP with no prior hysterectomy)	705 (64.3)
	Pre (< 6 months since LMP AND no prior bilateral ovariectomy AND not on estrogen replacement)	226 (20.6)
	NA	92 (8.4)
Progesterone receptor level (%)	< 10%	139 (12.7)
	10–19%	31 (2.8)
	20–29%	14 (1.3)
	30–39%	17 (1.5)
	40–49%	19 (1.7)
	50–59%	17 (1.5)

		Overall
	60–69%	19 (1.7)
	70–79%	43 (3.9)
	80–89%	30 (2.7)
	90–99%	106 (9.7)
	NA	662 (60.3)
Estrogen receptor status (%)	Indeterminate	2 (0.2)
	Negative	237 (21.6)
	Positive	810 (73.8)
	NA	48 (4.4)
MiR-190b expression (median [IQR])	5.70 [3.86, 6.66]	

mRNA differential expression analysis based on miR-190b expression.

To examine mRNA transcription relative to miR-190b expression, we performed differential expression analysis on tumor mRNA using miR-190b expression as a continuous variable correcting for ER-status. Of 58222 nonzero total read counts, 5294 read counts (9.1%) were upregulated and 11440 (20%) were downregulated (FDR < 0.05) (Supplementary data 2). Gene ontology enrichment analysis resulted in 100 significant pathways (Supplementary data 3) (Supplementary Fig. 1).

As miRNA activity is known to be highly variable based on transcriptional patterns, we assume there may be an interaction between miR-190b and ER status, leading us to specifically investigate this. In other words, we sought to define genes that are differentially expressed depending on the combination of miR-190b and ER status rather than simply correcting for ER. There were 9775 (17%) upregulated and 5229 (9%) downregulated (FDR < 0.05) read counts (Supplementary data 4). Gene ontology term enrichment resulted in 233 significant pathways (Supplementary Fig. 2).

MiR-190b target discovery by bio-miR-190b pulldown-seq

To analyze potential breast cancer specific miR-190b targets in vivo, we did bio-mir-190b pulldowns in the ER + breast cancer cell line T-47d followed by RNA sequencing. This resulted in 134 (0.38%) positively enriched reads out of 35437 nonzero read counts (Supplementary data 5). Figure 3 shows a heatmap of the 30 most significant positively enriched genes.

Gene ontology term enrichment analysis results in 70 significant pathways (Fig. 4) (Supplementary data 6).

Clinical impact of miR-190b‘s associated targets.

Of the 133 positively enriched targets from the bio-miR190b pulldown 77 were significantly correlated with miR-190b expression in the TCGA data and had a slope greater than ± 0.1 (P < 0.05, Spearman’s rho < -0.1 and > 0.1). Of the enriched targets 30 were significantly relevant to overall patient survival in a Cox proportional hazards model, regardless to ER status (HR = 0.55–2.36, P < 0.05) (Supplementary Fig. 3). When looking exclusively at ER + cases 40 targets were significant (HR = 0.27–3.16, P < 0.05). In LumA patients 19 targets were significant (HR = 0.27–2.89, P < 0.05) while in LumB 16 were significant (HR = 0.25–5.20, P < 0.05). 8 of the significant targets overlapped between LumA and LumB. Only 3 targets were significant in ER- tumors (HR = 0.48–1.71, $P < 0.05$) (Supplementary data 7).

We found no associations with miR-190b (GRCh38, chr1:154192665–154194743) in the NHGRI-EBI catalog of human genome-wide association studies (GWAS), indicating that miR-190b is well conserved.

RFWD3 was confirmed as a miR-190b target

Of the 70 significant terms from gene ontology enrichment analysis from the pulldown, RFWD3 belongs to 7 of them, all being DNA replication and/or repair terms. We thus picked this gene for further analysis and confirmed its knockdown upon miR-190b overexpression via miRNA mimic by western blot (Fig. 5) (see supplementary Fig. 5 for uncropped western blots).

Using omics data in DepMap from the Broad institute we also see a negative trend between miR-190b expression and RFWD3 protein levels in breast cancer cell lines (Supplementary Fig. 4A). We furthermore see negative correlation between RFWD3 and miR-190b RNA levels in TCGA (Multivariate linear regression corrected for ER status, P = 0.0002, R2 = 0.18, n = 1028) (Supplementary Fig. 4B). [15] .

Overall survival analysis based on RFWD3 expression shows less favorable outcomes in breast cancer patients with elevated RFWD3 RNA levels in TCGA (HR = 1.61, 95% CI 1.22–2.12, P = 0.0007). When looking into specific subgroups we see that survival in ER + patients was similar (HR = 1.78, 95% CI 1.24–2.56, P = 0.002) (Fig. 6A). The same trend was seen in LumA patients where elevated RFWD3 levels result in poorer outcomes (HR = 2.22, 95% CI 1.33–3.71, P = 0.002) (Fig. 6B). Survival analysis in LumB and ER- patients was not significant, similar to what we saw in miR-190b (Fig. 6C).

Discussion

We have previously demonstrated that frequently in ER + breast cancers, miR-190b is hypo-methylated which is correlated with high expression. We showed that LumA breast cancer patients have poorer outcomes when this does not occur. Here we reconfirm our findings regarding miR-190b expression with

data from TCGA and show that miR-190b is within the top 30 most significantly differentially expressed miRNAs in the cohort.

We see a clear trend of miR-190b downregulating RFWD3. We see negative correlation in the expression data from TCGA, RFWD3 is enriched in the bio-miR-190b pulldown-seq indicating that miR-190b binds to it, RFWD3 protein levels are diminished in western blot upon miR-190b mimic transfection, and we see that patient survival analysis by RFWD3 expression reflects survival seen for miR-190b expression. RFWD3 is an E3 ubiquitin ligase, necessary for DNA inter-strand cross link repair as well as RPA-mediated DNA damage signaling and repair through ubiquitination of RAD51 and the RPA (Replication Protein A) complex at stalled replication forks. This results in their removal from DNA damage sites and promotion of homologous recombination [16, 18, 19]. RFWD3 is also known to regulate the G1/S DNA damage checkpoint by mediating p53/TP53 stability via ubiquitination during the late response to DNA damage [20]. A recent study shows that RFWD3 acts as modulator of stalled fork stability though SMARCAL1 in BRCA2-deficiant cells via the hyper-ubiquitination of RPA [21]. Interestingly, as mentioned in our previous publication, there is a correlation between BRCA2 loss of heterozygosity (LOH) and miR-190b hypomethylation in breast tumors of individuals with the Icelandic $BRCA2^{999del5}$ founder mutation. We also found that miR-190b hypomethylation is less frequent in ER+/$BRCA2^{999del5}$ tumors compared to ER+/$BRCA2^{wt}$ tumors [13]. These findings indicate that miR-190b may have a role in BRCA2 mutated cancers though further studies are needed to fully confirm this.

Through the different gene ontology analyses we performed, we see many cancer related pathways emerge such as DNA repair, cell-cell adhesion, ion-transport, differentiation and more, all indicating that more is to be discovered regarding miR-190b and that it has a potential developmental role in breast cancer.

Expression of miRNAs is dynamic between tissues, morbidities, in which mRNAs they target and how. A great focus, both in scientific literature and in online miRNA target databases/algorithms, has been set on 3‘UTR seed-bound miRNA interactions for target discovery. Of the 30 enriched targets that were clinically relevant with regards to survival, 3 had a predicted 3‘UTR binding site containing the seed region of miR-190b. 19 had a predicted seed-less 3‘UTR binding sites, 7 had a seed-region binding sites within CDS‘s (coding regions), 18 had seed-less sites within CDS‘s, there were no seed-region sites found within 5‘UTRs and there were 19 with a seed-less binding site within 5‘UTRs (Supplementary data 8) (STarMirDB [22]). For RFWD3 we saw 15 potential seedless binding sites within its 3’UTR and 19 seedless binding sites within the CDS. Further research on miR-190b‘s precise binding site within RFWD3 and the mechanism behind its regulation is needed.

RFWD3 is significantly differentially expressed in the mRNA sequencing analysis in the TCGA though the log fold change may seem minor (supplementary data 3). It is worth noting that the log fold change represents log fold change in RFWD3 per unit of miR-190b expression change. The effect is thus greater than it might seem at first. Why RFWD3 is not significant in differential expression analysis of the

ER/miR-190b interaction is open to interpretation. MiR-190b regulation of RFWD3 is most likely not dependent on ER status while for other targets it most probably is.

In this study we have defined RFWD3 as a clinically relevant target of miR-190b in ER + breast cancer. Other targets are yet to be confirmed from our dataset. GO terms from our analysis indicate miR-190b to potentially contribute to moderating breast cancer development or tumorigenesis, making miR-190b worthy of further research which could prove useful for patient treatment and/ or characterization between the two Luminal subtypes, LumA and LumB.

Conclusions

In this study we confirmed that miR-190b is overexpressed in ER + breast cancer. We showed that miR-190b targets RFWD3 in the ER + breast cancer cell line T-47d leading to lower RFWD3 protein levels. Differential expression analysis based on miR-190b expression in the samples from the TCGA furthermore confirmed that RFWD3 is significantly under expressed when miR-190b expression is elevated in tumors. Lastly we saw that low levels of RFWD3 is associated with better survival in Luminal A breast cancer patients but not in Luminal B patients.

Materials And Methods

The cohort

TCGA (https://www.cancer.gov/tcga) gene expression and clinical information from breast cancer patients was downloaded, May 2018, using GDS-data transfer tool client. All data files requested were listed on a manifest file retrieved from TCGA (https://portal.gdc.cancer.gov/). PAM50 subtyping was extracted using „TCGAbiolinks::TCGAquery_subtype("brca")“ in R [23]. File merging, setup and statistical analysis was carried out using R statistical program[24] and RStudio.

Statistical and data analysis

To compare miR-190b expression between tissue samples we used the non-parametric Kruskal-Wallis test followed by the post-hoc Dunn‘s multiple comparison test. Survival analysis was carried out with Cox proportional hazards regression over time, using expression values as continuous variables and correcting for age of diagnosis. Overall survival was defined as time from diagnosis to end of follow-up or death. The time range of initial diagnosis was 1988–2013. Median follow up was 2.21 years and maximum follow up was 19.35 years. For visual representation of survival, Kaplan-Meier method was used to generate survival curves. The cut-off for definition of high vs low miR-190b was set at 4 based on the highest 95% expression value in normal tissue (ranging from 0.006–6.87, median = 2.51) and the lowest 10% expression value in ER + breast tumors (ranging from 0.006–9.47, median = 6.14). The cut-off value for RFWD3 was at the first and third quartile (10.7 and 11.7). Session info for R can be found in the supplementary data 9. To determine the correlation between miR-190b and the expression of targets from the pulldown, Spearman’s non-parametric correlation analysis or multivariate linear regression correcting

for confounding factors was performed. Differential expression analysis was carried out using the DESeq2 [25]. In the differential expression analysis for miRNAs the design formula was based on tumor and normal samples, using normal samples as reference. In the differential expression analysis for mRNA, miR-190b expression was used as a continuous variable. Thus, the reported log2 fold change in the according supplementary data is per unit of miR-190b expression change. In the bio-miR-190b pulldown, negative control (scrambled) was set as reference in the design formula. The results in according to supplementary data thus show log2 fold expression change induced by the miR-190b mimic compared to negative control. Gene ontology analysis was carried out using clusterProfiler [26]. GO analysis for the mRNA from TCGA was carried out using all the significantly differentially expressed genes. For the pulldown analysis, the significantly positively enriched genes were used in the GO analysis exclusively.

RNA-immunoprecipitation

The Cell line T-47D, used in this study, was obtained from the American Type Culture Collection (ATCC). The cell line was cultured in RPMI according to ATCC guidelines. 24h before transfection, cells were seeded on a 10cm culture dish to reach maximum 80% confluency at the time of harvest. After the seeding period, the cells were transfected with 5nM 3‘ biotin-labeled miR-190b mimic (Qiagen, cat:39178) or scrambled control (Qiagen, cat:339125) using lipofectamine RNAiMax (Thermo-Fisher, cat: 13778150). 24h after transfection, fresh media was substituted. Cell lysates were then harvested 48h after transfection with 1ml of RIPA lysis buffer. The samples were incubated for 10mins and centrifuged at 12.000g for 10 min at 4°C. The supernatant was transferred into a new tube. A double immunoprecipitation was performed, first using Ago2 protein to precipitate miRNAs bound in RISC, the following precipitation was done to isolate mRNA bound to the biotin-labeled miR-190b mimic. Dynabeads G (Thermo-Fisher, cat: 10004D) with rat Ago2 antibody (Thermo-Fisher, SAB4200085-200ul) was used for precipitation of Ago2 following wash and elution steps recommended by the manufacturer. The eluted samples were then directly loaded for the second precipitation. Streptavidin beads were used for precipitation, wash and elution steps were carried out according to manufacture protocol. RNA from the eluted samples was finally isolated using phenol-chloroform-isoamyl alcohol mixture following the wash steps according to manufacture protocol. The RNA was suspended in 10ul RNAse free water.

RNA-sequencing

RNA-sequencing of the RNA from bio-miR-190b immunoprecipitation was carried out by DeCode Genetics. The samples were aligned using kallisto [27]. Data analysis was carried out as described above.

Western blot

24h before transfection, T-47d cells were seeded on a 10cm culture dish to reach maximum 80% confluency at the time of harvest. After the seeding period, the cells were transfected with miR-190b mimic (50nM, Qiagen, cat:219600), inhibitor (200nM, Qiagen, cat:219300) or scrambled control (Qiagen, cat:1027271) using lipofectamine RNAiMax (Thermo-Fisher, cat: 13778150). 24h after transfection, fresh media was substituted. 48h post transcription, proteins were extracted using 2xLaemmmli sample buffer

(Santa Cruz, sc-286963) and treated with benzonase nuclease (Sigma, E1014). Samples were electrophorized using 8% acrylamide gel followed by transfer to nitrocellulose membrane (Santa Cruz, sc-286963). Membrane was blocked in 5% (w/v) skim milk in 1xPBS buffer with 0,1% (v/v) Tween-20 and probed with primary antibodies (RFWD3, 1:500 Abcam, ab138030 and SMC1, 1:1000, Abcam, ab9262) overnight at 4°C. Subsequently the membrane was washed with 1xPBS + 0,1% Tween-20 and secondary antibody (Santa Cruz, Mouse anti-rabbit IgG-HRP, sc-2357) at 1:10.000 dilution for 1h at room temperature. The membrane was developed with Luminol Reagent (Santa Cruz, sc-2048) and visualized using ChemiDoc XRS + system (Bio-Rad). See supplementary Fig. 5 for unprocessed figures of the western blots.

Abbreviations

CI – Confidence interval

ER – Estrogen receptor

Lum A – Luminal A

Lum B – Luminal B

miRNA – microRNA

mRNA – messengerRNA

HR – Hazard ratio

TCGA – The cancer genome Atlas

UTR – Untranslated region

GSEA – Gene set enrichment analysis

GO – Gene ontology

Declarations

Ethics approval and consent to participate

This does not apply as the data used in this manuscript is from The Cancer Genome Atlas which is publicly available.

Consent for publication

This does not apply as the data used in this manuscript is from The Cancer Genome Atlas which is publicly available.

Availability of data and materials

The datasets generated and analysed during the current study are available in the Gene Expression Omnibus (GEO) repository, (GSE198723). Data from the TCGA was downloaded from (https://www.cancer.gov/tcga).

Competing interests

The authors declare that they have no competing interests

Funding

This work was funded by the Icelandic Cancer Research Fund: 2018-05, The Icelandic Research Fund: ID #184969 and The Eimskip University fund.

Author´s contributions

EAF contributed to the study design, bioinformatic and statistical analysis, and writing of the manuscript. SS oversaw the study design, coordination and writing of the manuscript. TG and LV oversaw project coordination and were involved in analysis and interpretation of data as well as revising the manuscript. AIV and MRB conducted all cell work described. MRB furthermore oversaw uploading the RNA sequencing data to the Gene Expression Omnibus. KK implemented the RNA immunoprecipitation experiments and western blots. SR performed the western blots. All authors read and approved of the manuscript.

Acknowledgements

Not applicable

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Figures

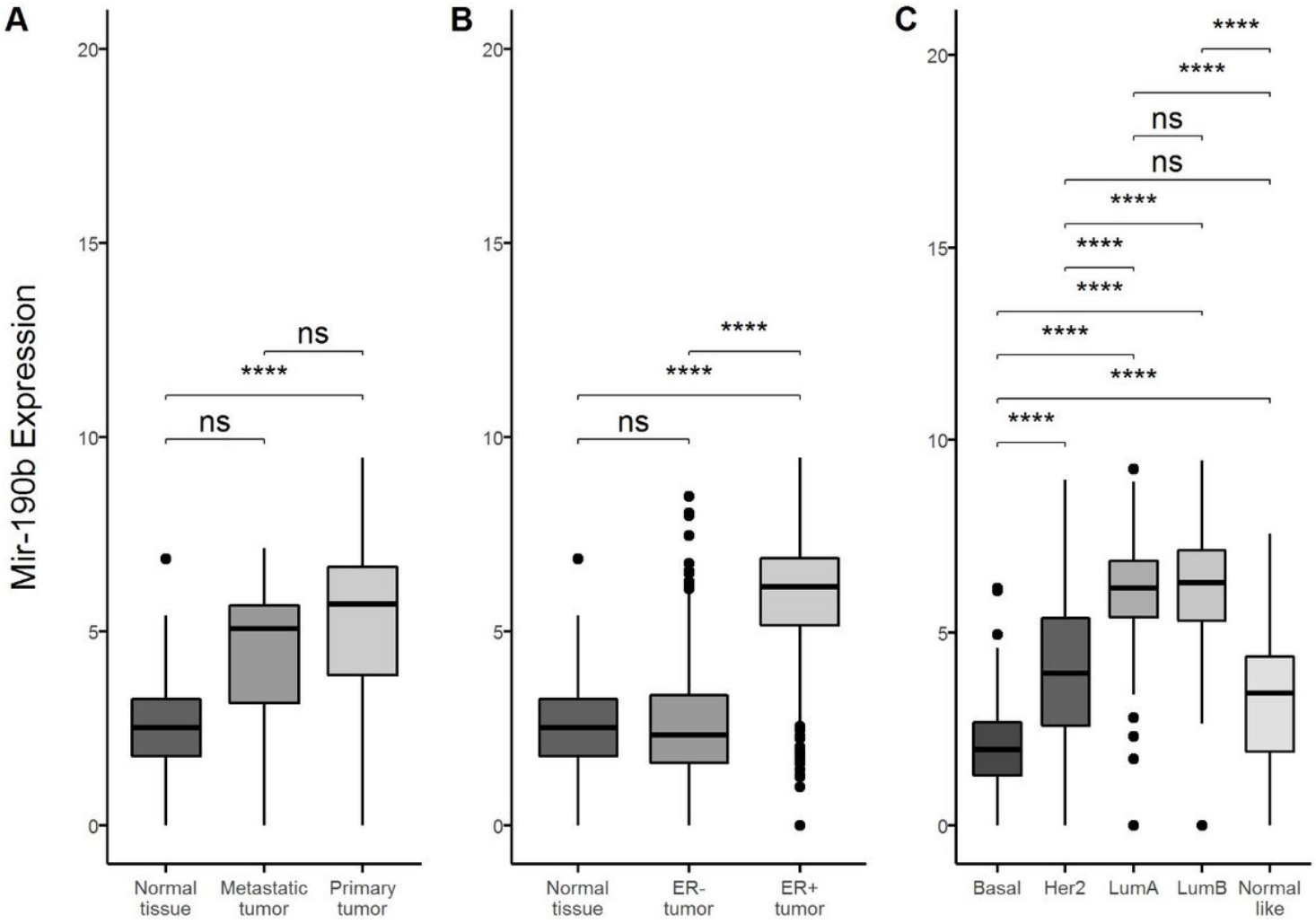

Figure 1

MiR-190b expression in breast cancer from TCGA. A) MiR-190b expression is higher in primary tumors (n=1090) compared to normal breast tissue (n=104). There was no significant difference between primary tumor samples and metastatic tumor samples (n=7) nor metastatic and normal samples (Kruskal-Wallis, P value=2.2^{-16} followed by Dunn's multiple comparison. B) MiR-190b expression is higher in ER+ tumors (n=810) compared to ER- tumors (n=237) and normal tissue (n=104) while there was no significant difference between ER- tumors and normal tissue (Kruskal-Wallis, P value=2.2^{-16} followed by Dunn's multiple comparison). C) MiR-190b is significantly overexpressed in the ER+ subtypes, LumA (n=564) and LumB (n=203), compared to the others, Normal like (n=40), Her2 (n=81), and Basal (n=188). There was no significant difference between LumA and LumB as well as between Her2 and normal like (Kruskal-Wallis P value=2.2^{-16} followed by Dunn's multiple comparison).

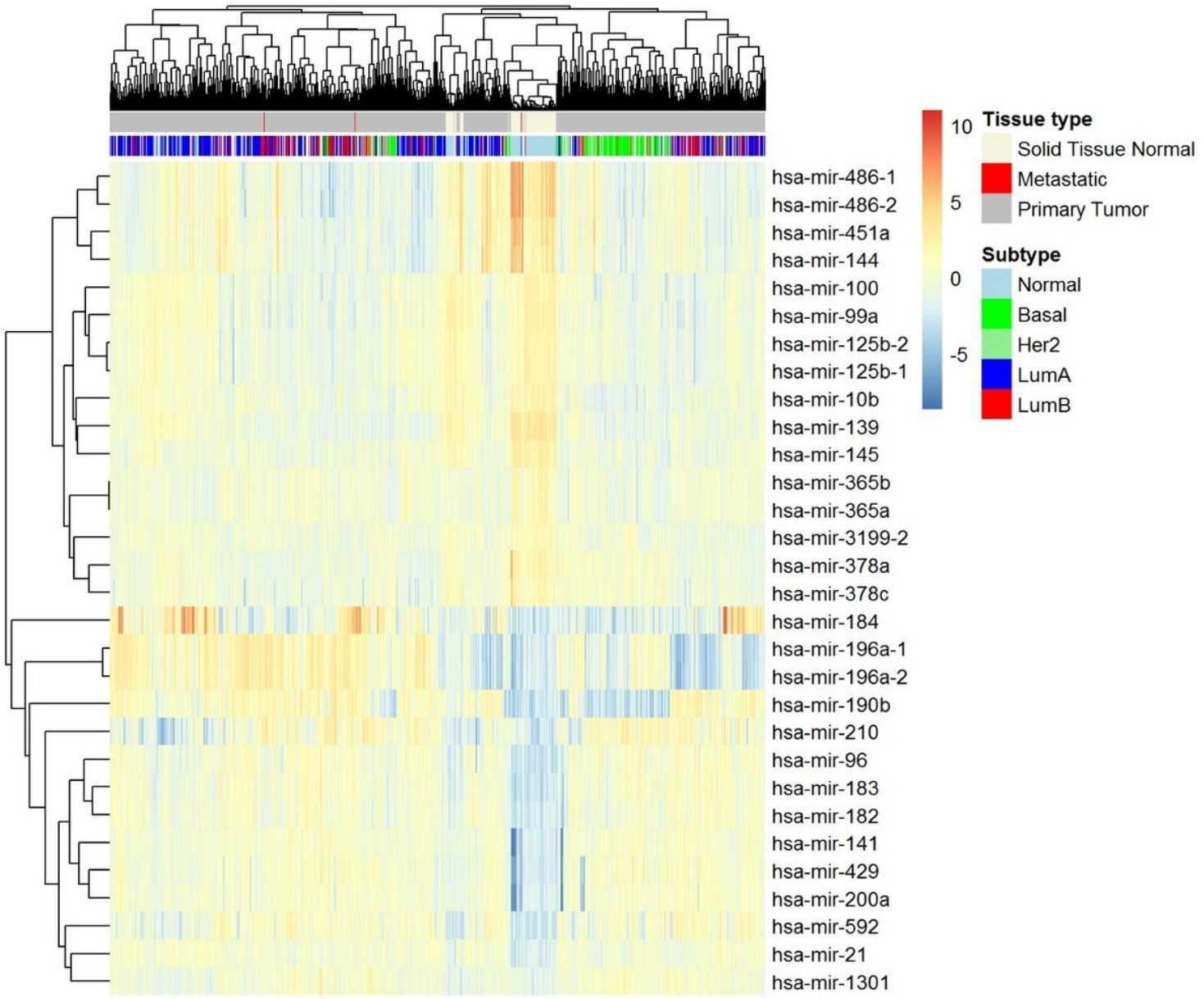

Figure 2

Heatmap of the top 30 diferentially expressed miRNA in the TCGA cohort. Differential expression analysis was performed to compare primary breast cancer to normal tissue. The normalized expression values of each gene were used in the heatmap for all samples available in the cohort. MiR-190b is distinctly lower in Basal tumors and normal tissue.

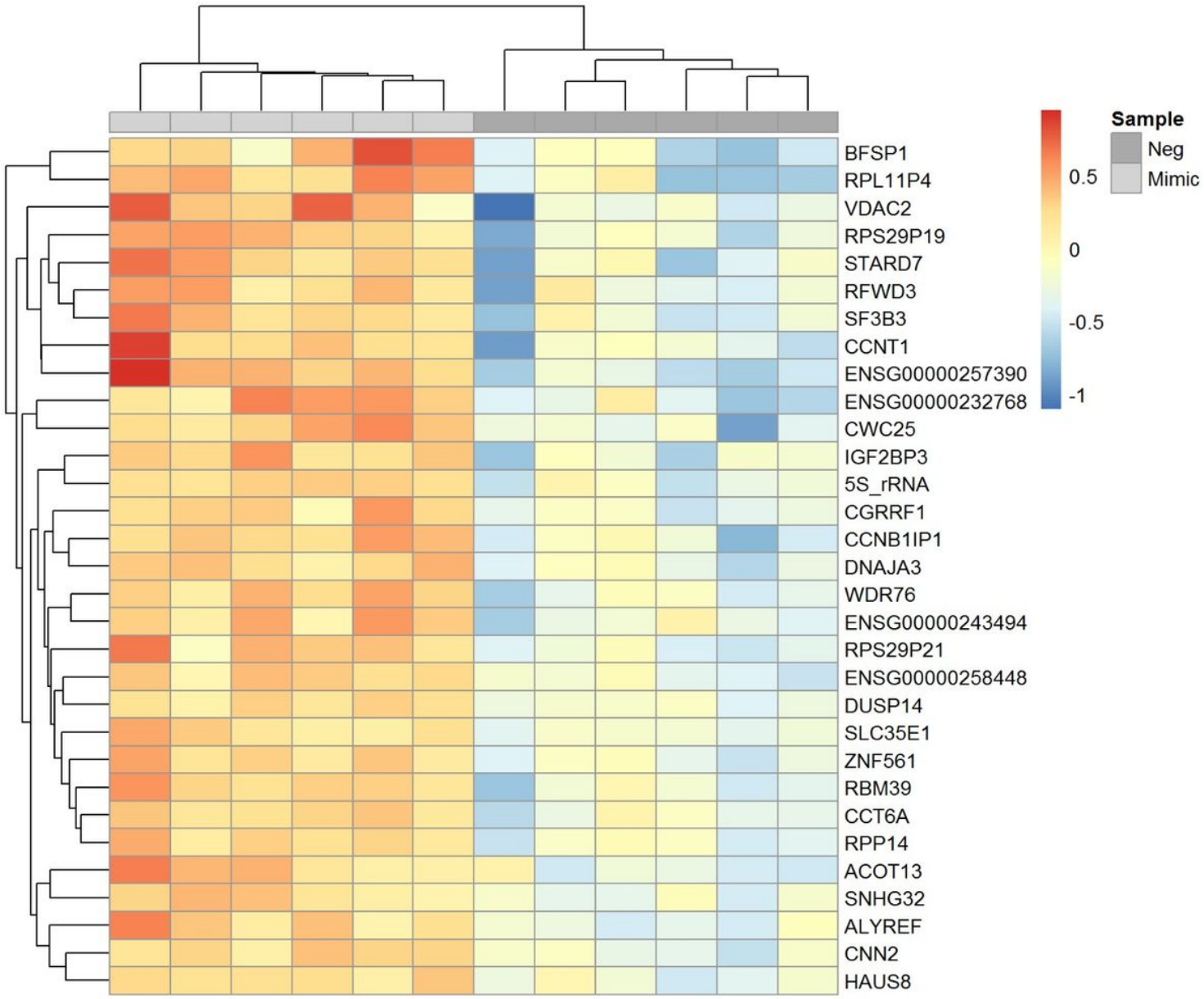

Figure 3

Heatmap of the top 30 positively enriched genes in bio-miR-190b pulldown-seq. Biotin labelled miR-190b mimic, an RNA fragment which mimics endogenous miR-190b, was transfected into T-47d cells. miR-190b targets were immunoprecipitatied in a two step process, first with anti-AGO2 and secondly with streptavidin-coated magnetic beads. Finally the immunoprecipitated RNA was sequenced and differential expression analysis used for target discovery.

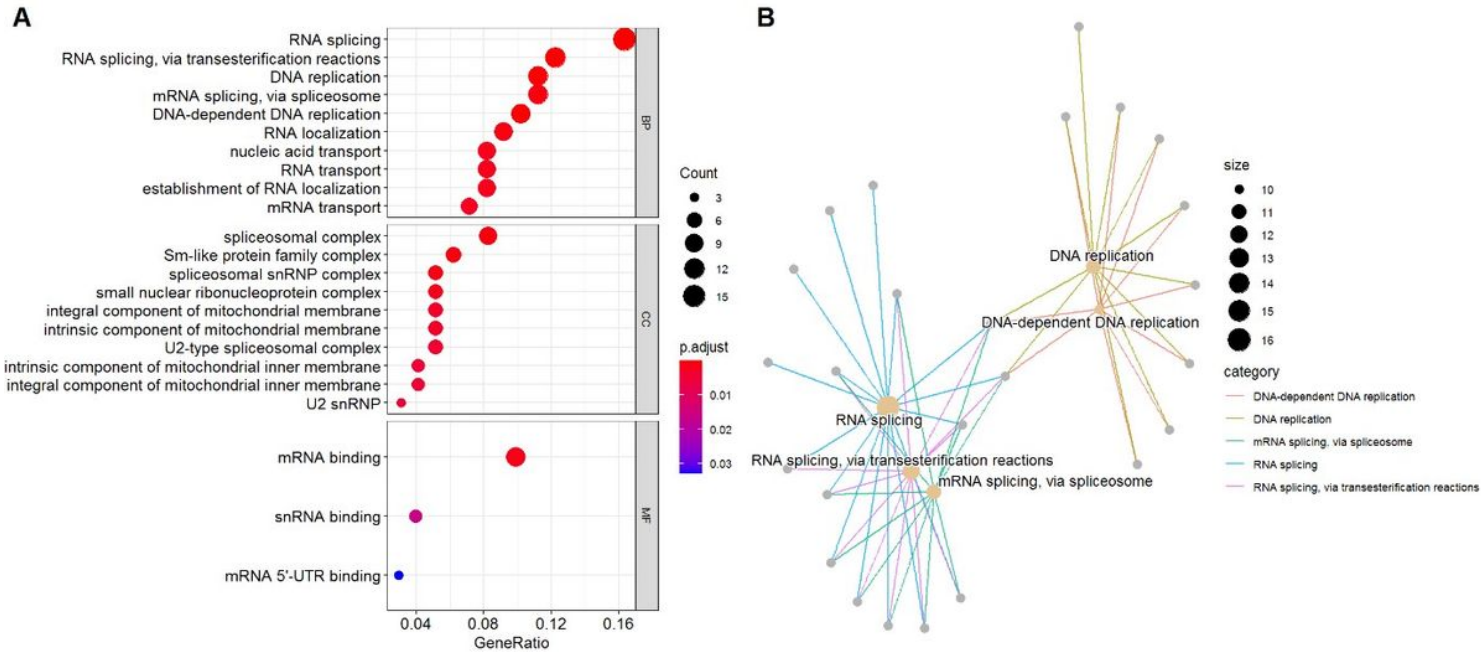

Figure 4

Gene ontology enrichment analysis from bio-miR-190b pulldown-seq A) Top 23 gene ontology results from differential expression analysis based on bio-miR-190b pulldown-seq divided by GO category. B) Gene-Concept network of the top 5 results from gene ontology enrichment analysis.

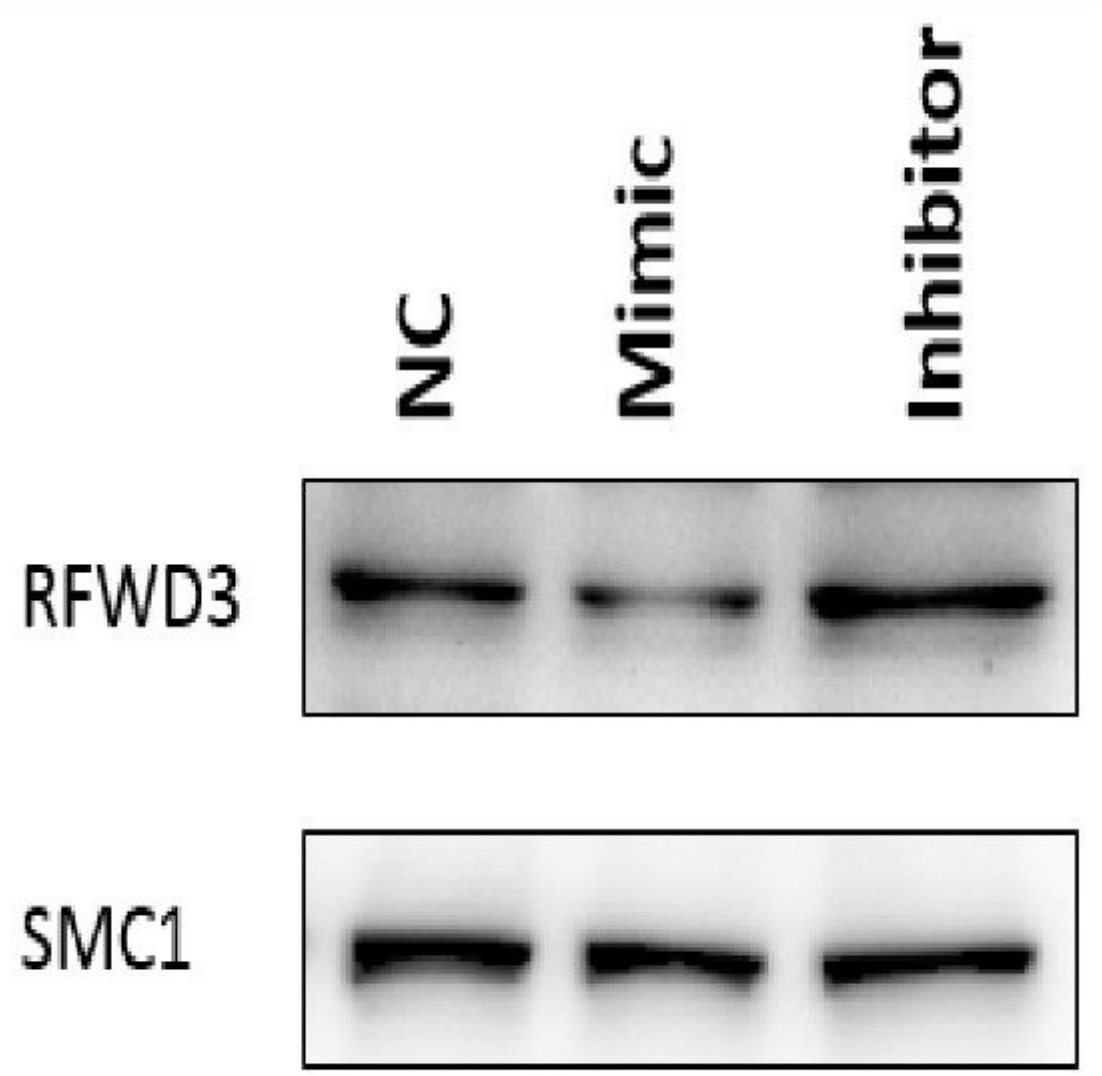

Figure 5

RFWD3 is a target of miR-190b. Westernblot of RFWD3. After using miR-190b mimic we see downregulation of RFWD3. After using miR-190b inhibitor we see upregulation of RFWD3. The figure has been cropped, see supplementary figure 5 for original blots.

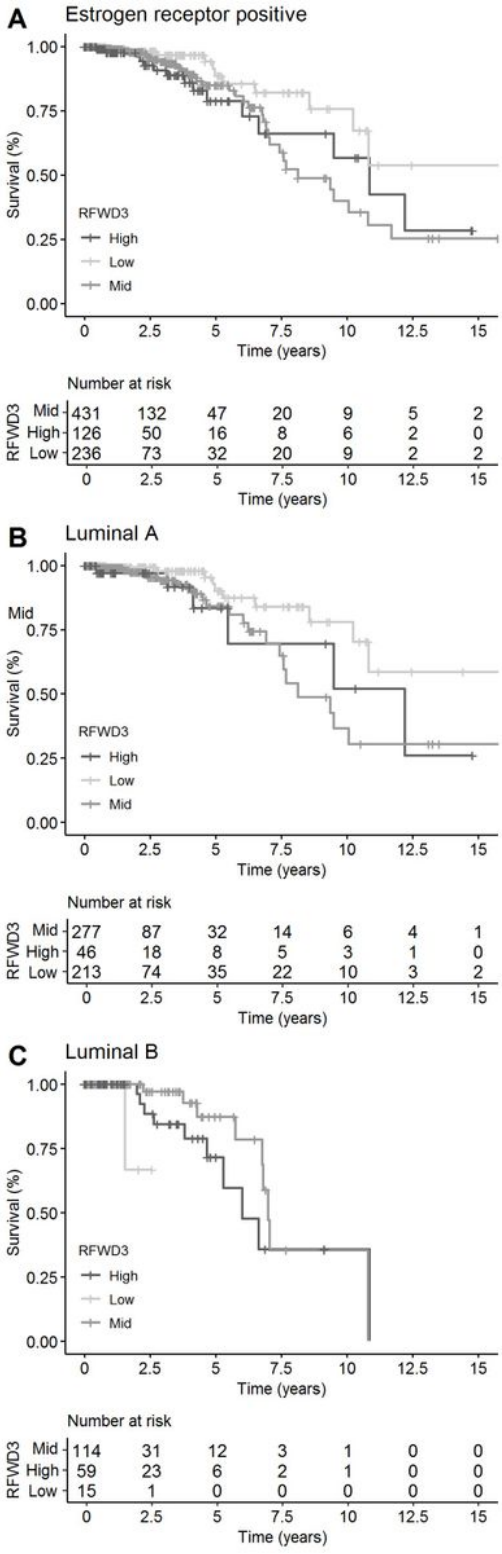

Figure 6

Overall survival associated with RFWD3 expression subdivided by ER+ status, LumA and LumB. The Cox proportional hazards models represent RFWD3 expression as a continous variable. For visual representation, RFWD3 expression split into 3 groups, Low represents expression below the first quartile (0-25%), Mid between the first and third quartile (25-75%) and High above the third quartile (75-100%). A) Higher levels of RFWD3 are associated with less favourable outcomes in ER+ breast cancer patients (Cox

proportional hazards: HR = 1.78, 95% CI 1.24-2.56, P = 0.002). B) The same was seen in LumA breast cancer patients where higher levels of RFWD3 were associated with poorer outcome (Cox proportional hazards: HR = 2.22, 95% CI 1.33-3.71, P = 0.002). C) There was no significant difference in overall survival related to RFWD3 in LumB breast cancer patients.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata1.xlsx
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