MicroRNA-190b Targets RFWD3 in Estrogen Receptor–Positive Breast Cancer

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Elisabet Alexandra Frick[1], Karen Kristjansdottir[1], Snaedís Ragnarsdottir[1], Arnar Ingi Vilhjalmsson[1], Maria Rose Bustos[1], Linda Vidarsdottir[1], Thorkell Gudjonsson[2] and Stefan Sigurdsson[1]

[1]Department of Biochemistry and Molecular Biology, Cancer Research Laboratory, Biomedical Center, Faculty of Medicine, School of Health Sciences, University of Iceland, Reykjavik, Iceland.
[2]Biotech Research & Innovation Centre, University of Copenhagen, Copenhagen, Denmark.

ABSTRACT

BACKGROUND: In the year 2020, breast cancer was the most common form of cancer worldwide. Roughly 70% of breast cancers are estrogen receptor–positive (ER+). MicroRNA-190b (miR-190b) has previously been reported to be upregulated in ER+ breast cancers. Previously, we have demonstrated that miR-190b is hypomethylated in ER+ breast cancers, potentially leading to its upregulation.

OBJECTIVES: To further study the role of miR-190b in ER+ breast cancer and to identify its clinically relevant targets in breast cancer.

DESIGN: Patient cohort and cell line–based RNA-sequencing analysis.

METHODS: The Cancer Genome Atlas was used to obtain gene expression data and clinical information on patients with breast cancer. To identify messenger RNA (mRNA) targets for miR-190b, the ER+ breast cancer cell line T-47D was used to immunoprecipitate biotin-labeled miR-190b followed by RNA sequencing. Western blot was used to confirm miR-190b target. Patient survival based on miR-190b and selected target was studied using the Cancer Genome Atlas.

RESULTS: In this study, we confirm that miR-190b is overexpressed in breast cancer via differential expression analysis and show that high expression of miR-190b results in more favorable outcomes in Luminal A patients, hazard ratio (HR) = 0.29, 95% confidence interval [CI] = 0.12-0.71, $P = .0063$. MicroRNA-190b target analysis identified RING finger and WD repeat domain 3 (RFWD3) as one of miR-190b regulatory targets in ER+ breast cancer. Survival analysis of RFWD3 showed that elevated levels result in poorer overall survival in patients with Luminal A breast cancer (HR = 2.22, 95% CI = 1.33-3.71, $P = .002$). Gene ontology analysis of our sequencing results indicates 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 ER+ 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 patients with Luminal A breast cancer but not in patients with Luminal B breast cancer. These findings provide novel insights into miR-190b role in breast cancer and that its clinical relevance is subtype specific.

PLAIN LANGUAGE SUMMARY

MicroRNA-190b targets RFWD3 in ER-positive Breast Cancer

Breast cancer is the most common diagnosed type of cancer worldwide. Most of them, or 70%, overexpressed the estrogen receptor (ER) which can be targeted with drugs. MicroRNA-190b (miR-190b) is known to be overexpressed in these types of breast cancers, and we have shown that loss of DNA methylation within the genomic region of miR-190b occurs in these ER+ cancers as well, which potentially is the cause for its overexpression. We, therefore, aimed at understanding miR-190b further. To do so, we used a technique called immunoprecipitation to capture miR-190b targets and performed RNA sequencing to identify potential targets. Of the targets, we identified RFWD3 and performed a western blot to confirm whether it was a true target. Finally, we performed survival analysis using data from the Cancer Genome Atlas to see whether RFWD3 was important for patient prognosis. In summary, we identified RFWD3 to be a target of miR-190b in ER+ breast cancers and that its expression is lower when miR-190b is elevated. We also saw that lower levels of RFWD3 are linked to better outcomes in a subgroup of ER+ breast cancers called Luminal A. These findings help in understanding miR-190b and its role in breast cancer and show that its clinical relevance is subgroup specific.

KEYWORDS: Breast neoplasms, microRNAs, estrogen receptor alpha, immunoprecipitation, prognosis

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CORRESPONDING AUTHOR: Stefan Sigurdsson, Department of Biochemistry and Molecular Biology, Cancer Research Laboratory, Biomedical Center, Faculty of Medicine, School of Health Sciences, University of Iceland, Sturlugata 8, 101 Reykjavik, Iceland. Email: stefsi@hi.is

Introduction

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 fifth leading cause of global cancer–related mortality and accounted for 1 in 6 cancer deaths in women.[1] Breast cancer is commonly divided into 5 clinical subtypes based on genomic profiling and/or histopathologic parameters.[2] Patient prognosis, therapeutic response, and strategy vary with each subtype, which are thought to be a consequence of different oncogenic drivers and evolutionary paths.[3]

Most breast cancers, or approximately 70%, are estrogen receptor–positive (ER+) and are divided into the subtypes Luminal A (LumA) and Luminal B (LumB). The LumA subtype expresses the ER and progesterone receptor (PR), does not express the human epidermal growth factor 2 (HER2), and has low levels of the proliferation marker Ki-67. The LumB subtype expresses both ER and PR, does in some instances express HER2, and has high levels of Ki-67.[4] These cancers are most commonly treated by inhibiting the ER 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 with LumA patients.[6] Further characterization between the 2 luminal subtypes, as for all subtypes, is thus vital for understanding the oncogenic drive of these cancers and to improve patient outcomes.[4]

MicroRNAs (miRNAs) are small noncoding RNAs that play a role in gene silencing by post-transcriptionally binding to the 3′ untranslated region (UTR) of messenger RNA (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.[8-11]

It has been previously noted that microRNA-190b (miR-190b) is overexpressed in ER+ tumors.[12] In a differential expression analysis using The Cancer Genome Atlas (TCGA), miR-190b was among few miRNAs significantly associated with poor survival.[13] Overexpression of miR-190b promotes proliferation and migration.[13] 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 hypomethylated LumA breast tumors.[14] Although miR-190b impact on some cancers has been researched, such as impairing insulin signaling and gluconeogenesis through targeting Insulin-like growth factor 1 (IGF-1) in hepatocellular carcinoma[15] and mediating radiosensitivity in gastric cancer by targeting Bcl-2,[16] miR-190b 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 2 groups. We analyzed RNA-seq data from TCGA and showed miR-190b expression results that are consistent with what we had already seen for DNA methylation[14] 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 RING finger and WD repeat domain 3 (RFWD3), which plays a role in genome maintenance[17] to be a clinically relevant target of miR-190b.

Methods

The cohort

The Cancer Genome Atlas (https://www.cancer.gov/tcga) gene expression and clinical information from patients with breast cancer were 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.[18] File merging, setup, and statistical analysis were carried out using R statistical program[19] and RStudio.

Statistical and data analysis

To compare miR-190b expression between tissue samples, we used the nonparametric Kruskal-Wallis test followed by the post hoc Dunn 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 to 2013. Median follow-up was 2.21 years, and maximum follow-up was 19.35 years. For visual representation of survival, the Kaplan-Meier method was used to generate survival curves. The cutoff for definition of high vs low miR-190b was set at 4 based on the highest 95% expression value in normal tissue (range = 0.006-6.87; median = 2.51) and the lowest 10% expression value in ER+ breast tumors (range = 0.006-9.47; median = 6.14). The cutoff value for RFWD3 was at the first and third quartiles (10.7 and 11.7). Session info for R can be found in Supplemental Data 9. To determine the correlation between miR-190b and the expression of targets from the pulldown, Spearman nonparametric correlation analysis or multivariate linear regression correcting for confounding factors was performed. Differential expression analysis was carried out using the DESeq2.[20] 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 Supplemental 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 according to Supplemental data thus show log2 fold expression change induced by the miR-190b mimic compared with negative control. Gene ontology (GO) analysis was carried out using clusterProfiler.[21] Gene ontology 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) as it is considered a suitable representative of the LumA subtype[22] as well as diploid on chromosome 1 here miR-190b is situated. The cell line was cultured in RPMI according to ATCC guidelines. Twenty-four hours before transfection, cells were seeded on a 10-cm culture dish to reach maximum 80% confluency at the time of harvest. After the seeding period, the cells were transfected with 5 nM 3′ biotin-labeled miR-190b mimic (Qiagen, cat No. 39178) or scrambled control (Qiagen, cat No. 339125) using lipofectamine RNAiMax (Thermo Fisher, cat No. 13778150). Twenty-four hours after transfection, fresh media was substituted. Cell lysates were then harvested 48 hours after transfection with 1 mL of RIPA lysis buffer. The samples were incubated for 10 minutes and centrifuged at 12.000 g for 10 minutes 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 No. 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. The 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 10 µL RNA-seq-free water.

RNA sequencing

The RNA sequencing of the RNA from bio-miR-190b immunoprecipitation was carried out by DeCode Genetics. The samples were aligned using kallisto.[23] Data analysis was carried out as described above.

Western blot

Twenty-four hours before transfection, T-47D cells were seeded on a 10-cm culture dish to reach maximum 80% confluency at the time of harvest. After the seeding period, the cells were transfected with miR-190b mimic (50 nM, Qiagen, cat No. 219600), inhibitor (200 nM, Qiagen, cat No. 219300), or scrambled control (Qiagen, cat No. 1027271) using lipofectamine RNAiMax (Thermo Fisher, cat No. 13778150). Twenty-four hours after transfection, fresh media was substituted. Forty-eight hours after transcription, proteins were extracted using 2× Laemmmli 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 1× PBS buffer with 0.1% (v/v) Tween-20 and probed with primary antibodies (RFWD3, 1:500 Abcam, ab138030 and SMC1 (Structural Maintenance of Chromosome protein 1), 1:1000, Abcam, ab9262) overnight at 4°C. Subsequently, the membrane was washed with 1× PBS + 0.1% Tween-20 and secondary antibody (Santa Cruz, Mouse antirabbit IgG-HRP, sc-2357) at 1:10.000 dilution for 1 hour at room temperature. The membrane was developed with luminol reagent (Santa Cruz, sc-2048) and visualized using ChemiDoc XRS+ system (Bio-Rad) (see Supplemental Figure 1 for unprocessed figures of the western blots).

Results

MicroRNA-190b is overexpressed in ER+ tumors

Using RNA-seq data from the TCGA database, we confirm what we had previously observed in the Icelandic cohort.[14] MicroRNA-190b was significantly overexpressed in primary tumors overall (n = 1090) compared with 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, $P = 2.2^{16}$ followed by Dunn multiple comparison, median values: normal tissue = 2.51, metastatic tumors = 5.07, and primary tumors = 5.70) (Figure 1A). MicroRNA-190b was significantly higher expressed in ER+ tumors (n = 810) compared with both ER– tumors (n = 237) and normal tissue (n = 104), while there was no significant difference between ER– tumors and normal tissue (Kruskal-Wallis, $P = 2.2^{16}$ followed by Dunn multiple comparison, median values: normal tissue = 2.51, ER– = 2.33, and ER+ = 6.14) (Figure 1B). Finally, miR-190b was overexpressed in both LumA and LumB compared with other subtypes which is in harmony with our previously published methylation data (Kruskal-Wallis, $P = 2.2^{16}$ followed by Dunn 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; and LumB = 6.31, n = 203) (Figure 1C).

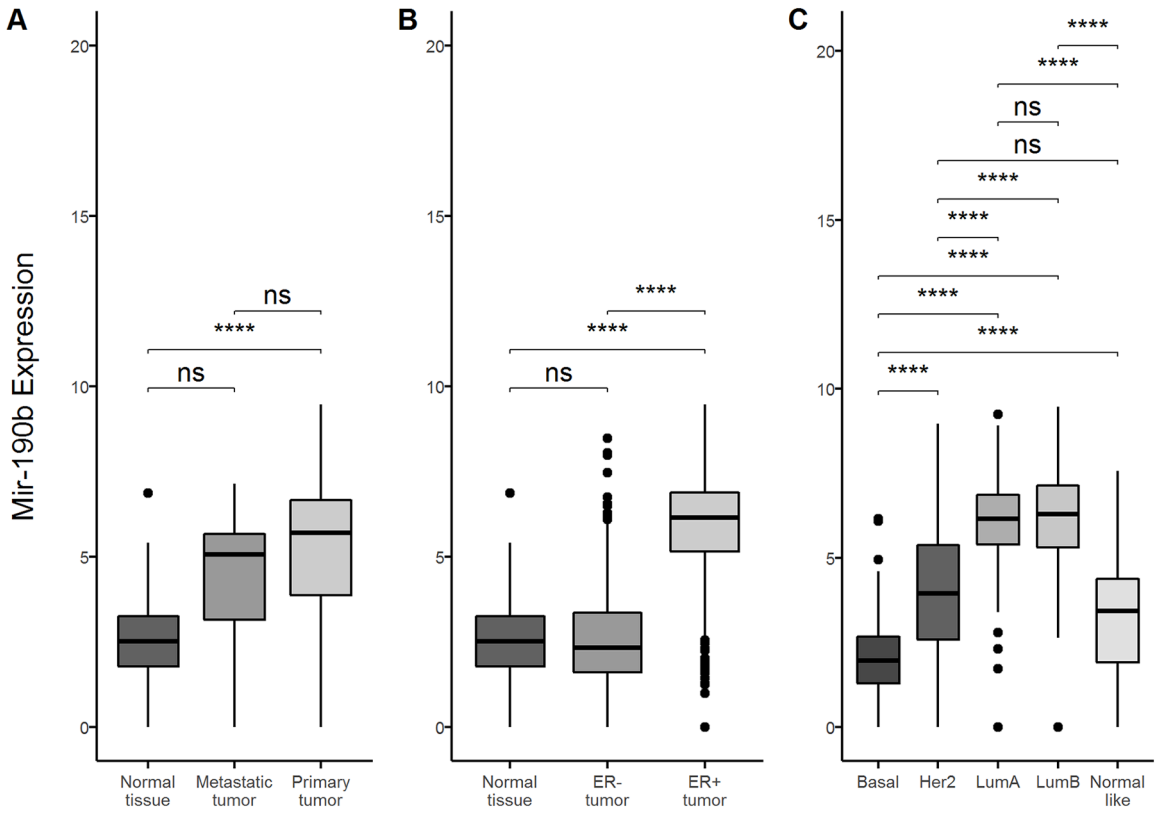

Figure 1. miR-190b expression in breast cancer from TCGA. (A) miR-190b expression is higher in primary tumors (n = 1090) compared with normal breast tissue (n = 104). There was no significant difference between primary tumor samples and metastatic tumor samples (n = 7) nor metastatic and normal samples (Kruskal-Wallis, $P = 2.2^{16}$ followed by Dunn multiple comparison). (B) miR-190b expression is higher in ER+ tumors (n = 810) compared with ER– tumors (n = 237) and normal tissue (n = 104) while there was no significant difference between ER– tumors and normal tissue (Kruskal-Wallis, $P = 2.2^{16}$ followed by Dunn multiple comparison). (C) miR-190b is significantly overexpressed in the ER+ subtypes, LumA (n = 564) and LumB (n = 203), compared with 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, $P = 2.2^{16}$ followed by Dunn multiple comparison).

MicroRNA-190b is significantly overexpressed in differential expression analysis

Differential miRNA-expression analysis of primary tumors compared with solid normal tissue from TCGA resulted in 353 (22%) upregulated and 227 (14%) downregulated miRNAs (False discovery rate (FDR) <0.05) out of 1626 nonzero total read counts (Supplemental Data 1). In total, 152 (9.3%) of the miRNAs had a log2-fold change ±1. MicroRNA-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 with 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. MicroRNA-190b 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. 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 patients with breast cancer based on miR-190b expression levels.[14] 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 where high expression indicates more favorable outcome (hazard ratio [HR] = 0.55, 95% confidence interval [CI] = 0.32-0.95, $P = .033$). On 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 and that of Dai et al,[13] significantly better survival in patients with high expression of miR-190b in LumA tumors (HR = 0.29, 95% CI = 0.12-0.71, $P = .0063$). No difference was seen in patients with LumB nor ER– tumors in general.

Messenger RNA 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 58 222 nonzero total read counts, 5294 read counts (9.1%) were upregulated and 11 440 (20%)

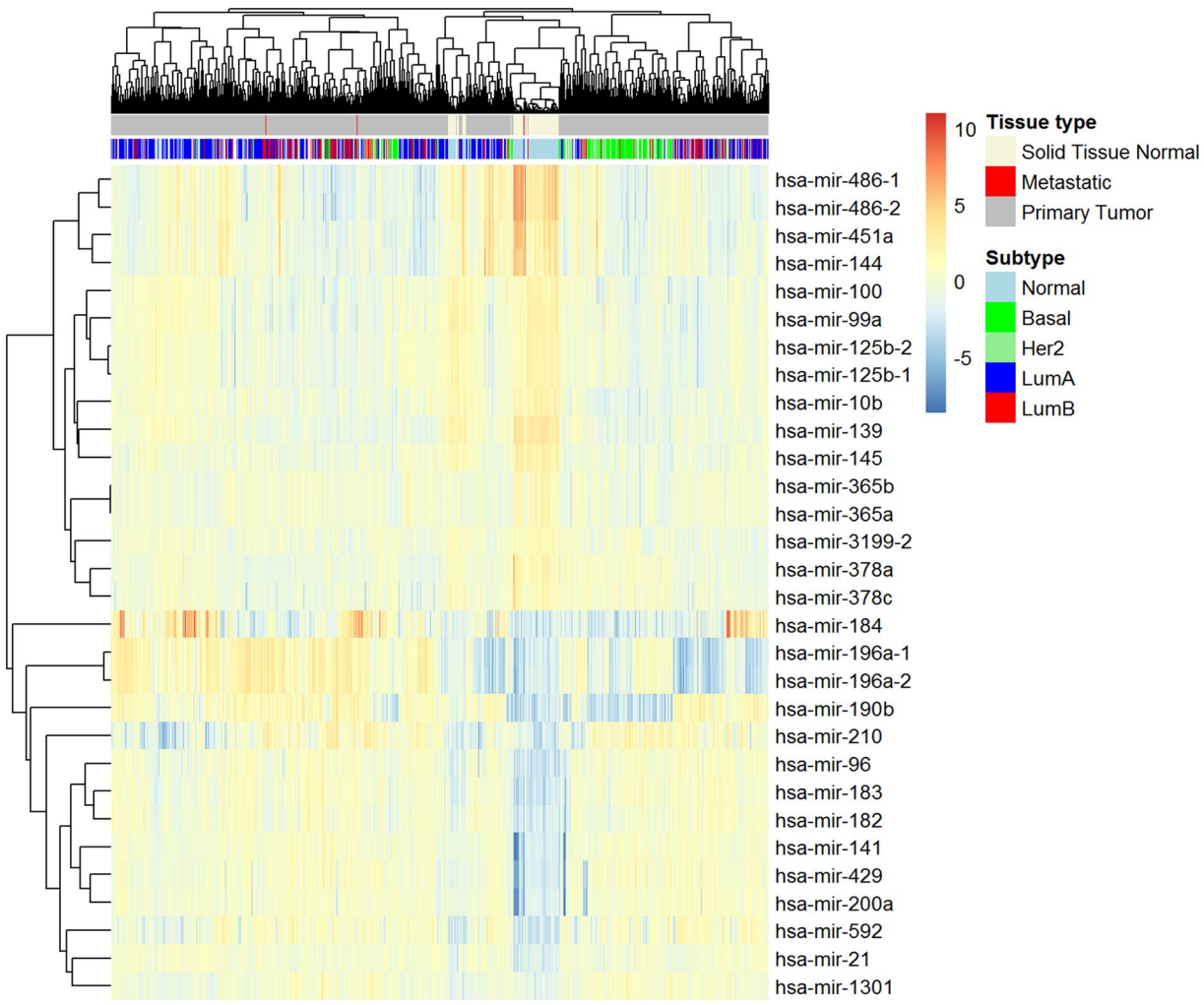

Figure 2. Heatmap of the top 30 differentially expressed miRNA in the TCGA cohort. Differential expression analysis was performed to compare primary breast cancer to normal tissue. The normalized expression values of each gene were used in the heatmap for all samples available in the cohort. miR-190b is distinctly lower in basal tumors and normal tissue.

were downregulated (FDR <0.05) (Supplemental Data 2). Gene ontology enrichment analysis resulted in 100 significant pathways (Supplemental Data 3) (Supplemental Figure 2).

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 (Supplemental Data 4). Gene ontology term enrichment resulted in 233 significant pathways (Supplemental Figure 3).

MicroRNA-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 35 437 nonzero read counts (Supplemental 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 (Figure 4) (Supplemental Data 6).

Clinical impact of miR-190b–associated targets

Of the 133 positively enriched targets from the bio-miR-190b pulldown, 77 were significantly correlated with miR-190b expression in the TCGA data and had a slope greater than ±0.1 ($P < .05$, Spearman 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 ER status (HR = 0.55-2.36, $P < .05$) (Supplemental Figure 4). When looking exclusively at ER+ cases, 40 targets were significant (HR = 0.27-3.16, $P < .05$). In LumA patients, 19 targets were significant (HR = 0.27-2.89, $P < .05$) while in LumB 16 were significant (HR = 0.25-5.20, $P < .05$). Eight of the significant targets overlapped between LumA and

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)
Sex (%)	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)
	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)

(Continued)

Table 1. (Continued)

		OVERALL
	NA	1 (0.1)
Menopause status (%)	Indeterminate (neither premenopausal nor postmenopausal)	33 (3.0)
	Peri (6-12 mo 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 mo since LMP and no prior bilateral ovariectomy and not on estrogen replacement)	226 (20.6)
	NA	92 (8.4)
PR 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)
	60-69	19 (1.7)
	70-79	43 (3.9)
	80-89	30 (2.7)
	90-99	106 (9.7)
	NA	662 (60.3)
ER 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)	

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor 2; LumA, Luminal A; LumB, Luminal B; IQR, interquartile range; MiR-190b, microRNA-190b; NA, not applicable; PR, progesterone receptor.

LumB. Only 3 targets were significant in ER– tumors (HR = 0.48-1.71, $P < .05$) (Supplemental Data 7).

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

RFWD3 was confirmed as a miR-190b target

Of the 70 significant terms from GO enrichment analysis from the pulldown, RFWD3 belongs to 7 of them, all being DNA replication and/or repair terms. We thus picked RFWD3 for further analysis. As expected, we observed a reduction in RFWD3 protein levels on miR-190b overexpression via miRNA mimic by western blot (Figure 5) (see Supplemental Figure 1 for uncropped western blots).

Using omics data in DepMap from the Broad institute,[24] we also see a negative trend between miR-190b expression and RFWD3 protein levels in breast cancer cell lines (Supplemental Figure 5A), which is in line with the western blot findings. We, furthermore, see negative correlation between RFWD3 and miR-190b RNA levels in TCGA (multivariate linear regression corrected for ER status, $P = .0002$, $R^2 = .18$, n = 1028) (Supplemental Figure 5B). When looking into RFWD3 RNA expression by subtype, we see that it is significantly underexpressed in LumA and normal-like compared with LumB and HER2 (Supplemental Figure 6).

Overall survival analysis based on RFWD3 expression shows less favorable outcomes in patients with breast cancer with elevated RFWD3 RNA levels in TCGA (HR = 1.61, 95% CI = 1.22-2.12, $P = .0007$). When looking into specific subgroups, we see that survival in ER+ patients was similar

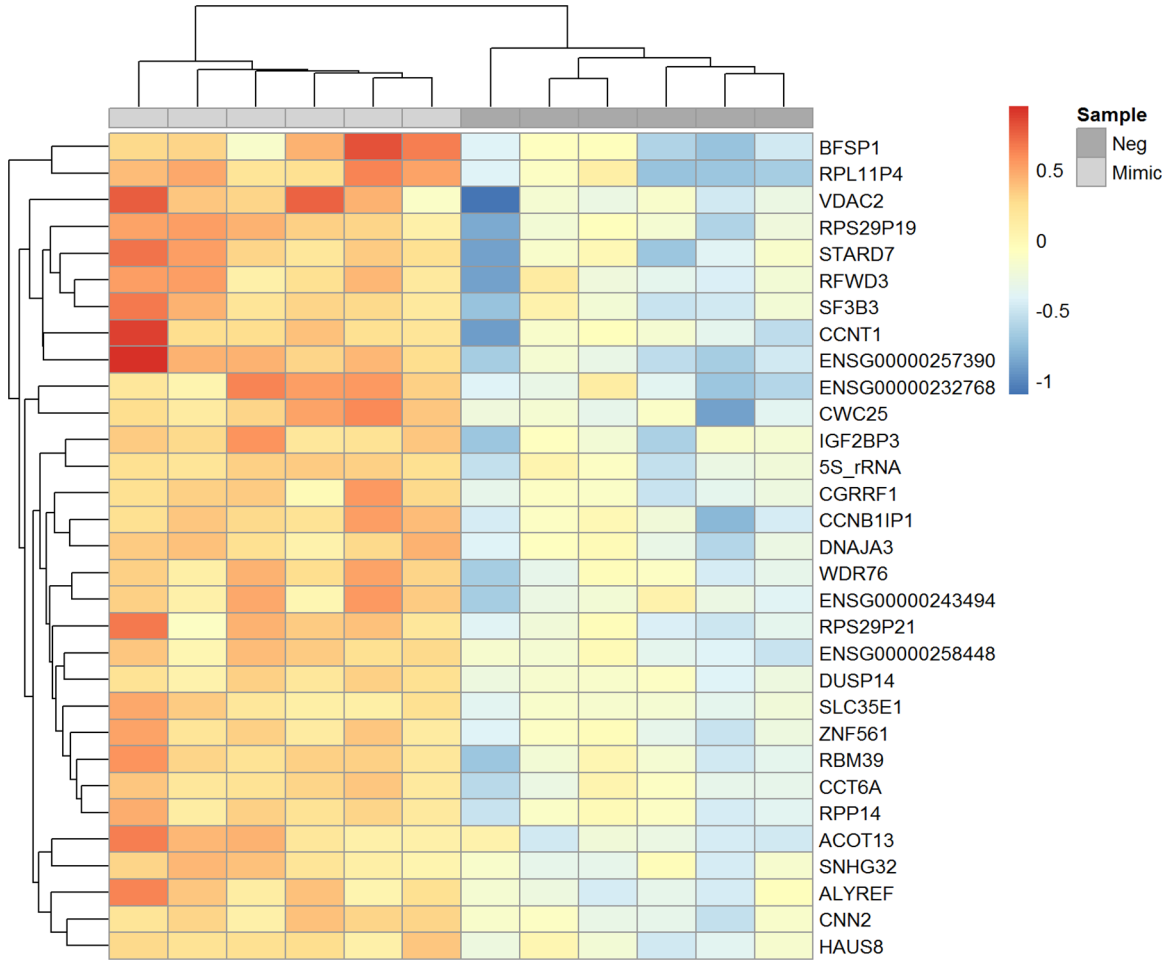

Figure 3. Heatmap of the top 30 positively enriched genes in bio-miR-190b pulldown-seq. Biotin-labeled miR-190b mimic, an RNA fragment that mimics endogenous miR-190b, was transfected into T-47d cells. miR-190b targets were immunoprecipitated in a 2-step process, first with anti-Ago2 and secondly with streptavidin-coated magnetic beads. Finally, the immunoprecipitated RNA was sequenced and differential expression analysis used for target discovery.

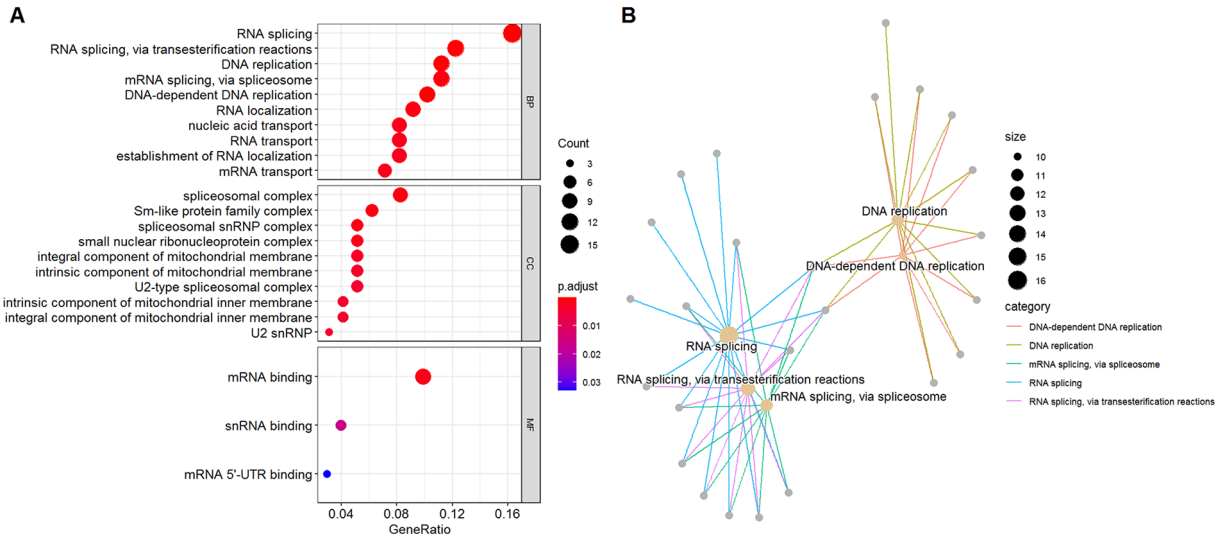

Figure 4. Gene ontology enrichment analysis from bio-miR-190b pulldown-seq. (A) Top 23 GO results from differential expression analysis based on bio-miR-190b pulldown-seq divided by GO category. (B) Gene-concept network of the top 5 results from GO enrichment analysis.

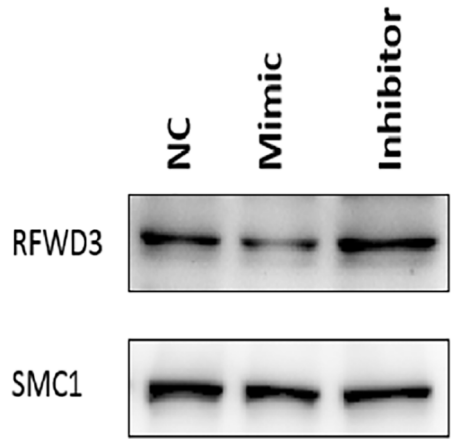

Figure 5. RFWD3 is a target of miR-190b. Western blot of RFWD3. After using miR-190b mimic, we see downregulation of RFWD3. After using miR-190b inhibitor, we see upregulation of RFWD3. Molecular weight was estimated using a protein ladder containing prestained-sized markers, which were used as a reference. SMC1 was used as a loading control. The figure has been cropped (see Supplemental Figure 5 for original blots).

(HR = 1.78, 95% CI = 1.24-2.56, *P* = .002) (Figure 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* = .002) (Figure 6B). Survival analysis in LumB and ER– patients was not significant, similar to what we saw in miR-190b (Figure 6C).

Discussion

We have previously demonstrated that frequently in ER+ breast cancers, miR-190b is hypomethylated which is correlated with high expression. We showed that patients with LumA breast cancer have poorer outcomes when this does not occur. Here, we reconfirm our findings regarding miR-190b expression and patient survival with data from TCGA. Findings from Dai et.al, furthermore, support our findings. In their publication from 2019, they found a similar trend in patient survival when looking at miR-190b expression in patients with breast cancer overall. They, furthermore, observed, on an experimental level, that miR-190b overexpression increased cell proliferation and reduced apoptosis which contradicts Cizeron-Clairac et al findings from 2015.[12,13]

Our findings suggest that miR-190b is more clinically relevant on a subtype level. We, furthermore, show that miR-190b is within the top 30 most significantly differentially expressed miRNAs in the cohort which is in consensus to what can be found in the literature.

Here, we report RFWD3 as a novel miR-190b target gene. We see a clear trend of miR-190b downregulating RFWD3, and we see negative correlation in the expression data from TCGA. RING finger and WD repeat domain 3 is enriched in the bio-miR-190b pulldown-seq indicating that miR-190b binds to it; RFWD3 protein levels are diminished in western blot on miR-190b mimic transfection, and we see that patient survival analysis by RFWD3 expression reflects survival seen

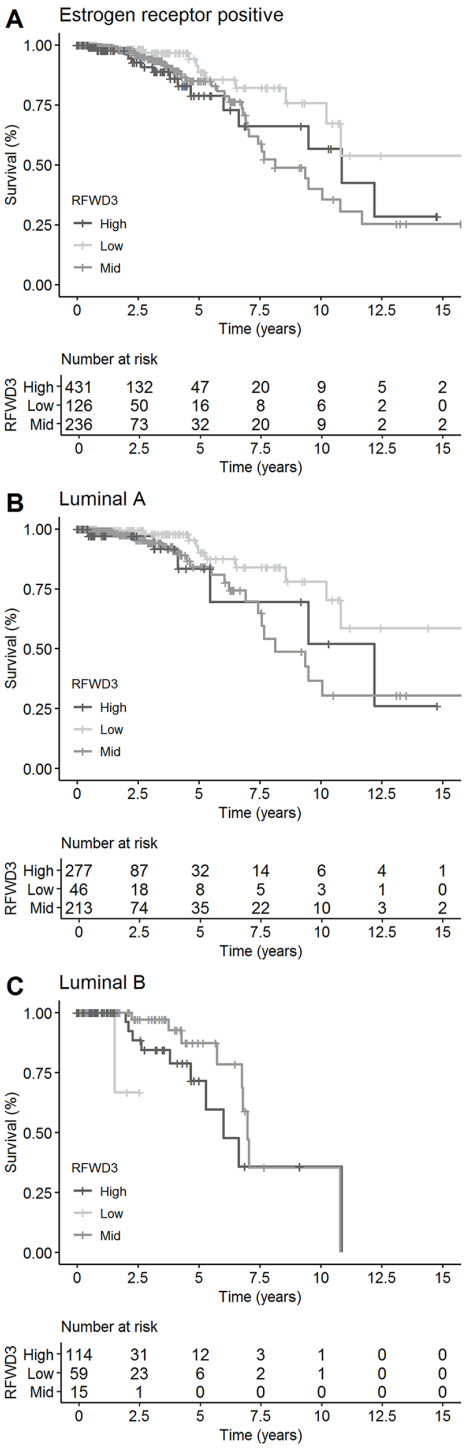

Figure 6. Overall survival associated with RFWD3 expression subdivided by ER+ status, LumA, and LumB. The Cox proportional hazards models represent RFWD3 expression as a continuous variable. For visual representation, RFWD3 expression split into 3 groups: low represents expression below the first quartile (0%-25%), mid between the first and third quartiles (25%-75%), and high above the third quartile (75%-100%). (A) Higher levels of RFWD3 (continuous) are associated with less favorable outcomes in ER+ patients with breast cancer (Cox proportional hazards: HR = 1.78, 95% CI = 1.24-2.56, *P* = .002). (B) The same was seen in patients with LumA breast cancer where higher levels of RFWD3 (continuous) were associated with poorer outcome (Cox proportional hazards: HR = 2.22, 95% CI = 1.33-3.71, *P* = .002). (C) There was no significant difference in overall survival related to RFWD3 (continuous) in patients with LumB breast cancer.

for miR-190b expression. RING finger and WD repeat domain 3 is an E3 ubiquitin ligase, necessary for DNA interstrand cross link repair as well as replication protein A (RPA)-mediated DNA damage signaling and repair through ubiquitination of RAD51 and the RPA complex at stalled replication forks. This results in their removal from DNA damage sites and promotion of homologous recombination.[17,25,26] RING finger and WD repeat domain 3 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. RING finger and WD repeat domain 3 has been suggested to do this by binding to and inhibiting MDM2 and catalyzing the formation of short ubiquitin chains on p53, not leading to degradation of p53.[27] A recent study shows that RFWD3 acts as modulator of stalled fork stability though SMARCAL1 in BRCA2-deficient cells via the hyperubiquitination of RPA.[28]

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 with ER+/$BRCA2^{wt}$ tumors.[14] These findings indicate that miR-190b may have a role in BRCA2 mutated cancers although further studies are needed to fully confirm this.

Through the different GO 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 regarding survival, 3 had a predicted 3′ UTR-binding site containing the seed region of miR-190b. Nineteen had a predicted seedless 3′ UTR-binding sites; 7 had a seed region–binding sites within CDSs (coding regions); 18 had seedless sites within CDSs; there were no seed-region sites found within 5′ UTRs; and there were 19 with a seedless binding site within 5′ UTRs (Supplemental Data 8) (STarMirDB).[29]

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 precise binding site within RFWD3 and the mechanism behind its regulation is needed.

RING finger and WD repeat domain 3 is significantly differentially expressed in the mRNA-sequencing analysis in the TCGA although the log fold change may seem minor (Supplemental 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. MicroRNA-190b regulation of RFWD3 is most likely not dependent on ER status while for other targets it most probably is. The strength of this study is that it is mostly based on the comprehensive TCGA database and the additional RNA-seq data provided by us is publicly available, making the study reproducible and transparent. A major limitation of the study is that the precise miR-190b-RFWD3 binding site was not characterized although we observed through the RNA-seq pulldown and western blotting binding between miR-190b and RFWD3. Furthermore, we could not verify the interplay between BRCA2 mutations, miR-190b, and RFWD3 within publicly available databases, and therefore, it needs further investigation within a laboratory setting.

Conclusions

We have confirmed that RFWD3 is a clinically relevant target of miR-190b in ER+ breast cancer which is associated with better survival in patients with LumA breast cancer but not in LumB patients. Other targets are yet to be confirmed from our data set. Gene ontology 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 2 luminal subtypes, LumA and LumB.

Declarations

Ethics approval and consent to participate

This does not apply as the data used in this article are from TCGA which is publicly available.

Consent for publication

This does not apply as the data used in this article are from TCGA which is publicly available.

Author Contributions

EAF contributed to conceptualization, data curation, formal analysis, methodology, writing—original draft, and writing—review and editing. KK contributed to investigation, methodology, and writing—review and editing. SR contributed to methodology, and writing—review and editing. AIV contributed to methodology, and writing—review and editing. MRB contributed to investigation, methodology, software, and writing—review and editing. LV contributed to conceptualization, investigation, validation, and writing—review and editing. TG contributed to conceptualization, methodology, project administration, validation, and writing—review and editing. SS contributed to conceptualization, funding acquisition, project administration, resources, supervision, validation, and writing—review and editing.

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Availability of data and materials
The data sets generated and analyzed during this study are available in the Gene Expression Omnibus (GEO) repository (GSE198723). Data from the TCGA were downloaded from https://www.cancer.gov/tcga.

Supplemental material
Supplemental material for this article is available online.

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