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Original Article

Low expression of miR-195 is associated with cell proliferation, glycolysis and poor survival in estrogen receptor (ER)-positive but not in triple negative breast cancer

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Abstract: MiR-195 is a tumor suppressive microRNA in breast cancer. Its clinical relevance remains debatable as it has only been studied via in vitro experiments or small cohort studies. We analyzed a total of 2,038 patients in the TCGA and METABRIC cohorts to assess whether low miR-195 expressing tumors are associated with aggressive cancer characteristics and poor prognostic outcomes. The median cutoff of miR-195 expression was used to split the groups into miR-195 high and low groups. Low miR-19 expressing tumors demonstrated high cell proliferating features by enriching the gene sets associated with cell proliferation, MKI67 expression and pathological grade. One-third of the top target miR-195 genes were related to cell proliferation. Low miR-195 expressing tumors were associated with both pro-cancerous and anti-cancerous immune cells. Low miR-195 expressing tumors were associated with enhanced glycolysis and poor survival in ER-positive tumors, but not other subtypes of breast cancer. In conclusion, low expression of miR-195 in ER-positive breast cancer was associated with enhanced cancer cell proliferation, glycolysis, and worse overall survival.

Keywords: MicroRNA, miR-195, cell proliferation, glycolysis, breast cancer, TCGA, METABRIC, GSEA

Introduction

Breast cancer is the most common cancer in women and accounts for the second most common cancer-related cause of death in the world [1, 2]. Among the subtypes of breast cancer, estrogen receptor (ER)-positive tumors are the most frequent subtype [3]. ER-positive tumors also have the most favorable outcome among all the subtypes. However, it has a late onset recurrence rate ranging from 11% to 40% at 20-years follow up [4, 5]. Therefore, patients remain concerned about disease recurrence and death for an extended period of time after treatment. A novel prognostic biomarker that can predict the likelihood of recurrence of ER-positive tumors is needed to select the patients who may require further interventions.

MicroRNAs (miRNAs) have recently attracted attention in their role as diagnostic and prognostic biomarkers. MicroRNAs (miRNAs) are well-known post-transcriptional regulators of

gene expression [6]. A number of in vitro studies have shown that miR-195 targets oncogenes and suppresses several types of cancer, including breast, lung, prostate, colorectal, and gastric cancer [7]. In breast cancer, miR-195 was reported to function as a tumor suppressive miRNA that targets cell cycle related genes [7, 8]. One study reported that lower miR-195 in plasma was associated with advanced grade and more lymph node metastasis, suggesting its potential role as a diagnostic biomarker [9]. Luo et al reported that miR-195 inhibited breast cancer cell proliferation by directly targeting cyclin E1 [8]. Singh et al demonstrated that miR-195 suppressed lipogenesis-related genes, which led to the inhibition of cell proliferation, migration, and invasion of breast cancer cells [10]. Though many studies have elucidated the anti-cancer effects of miR-195, they were conducted using *in-vitro* cell culture systems or small patient cohorts. There have been no studies to date that have demonstrated the clinical relevance of miR-195 expression in breast cancer using multiple large patient cohorts.

Recently, our group has been utilizing an *in-silico* translational research approach to clarify the clinical relevance of several miRNAs, such as tumor suppressive miRNAs in breast cancer patients [11-16]. For instance, we demonstrated that the overexpression of miR-30a and miR-200c, which are both tumor suppressive, is associated with favorable outcomes in breast cancer patients [14]. We also reported that miR-143 is associated with a favorable tumor immune microenvironment and improved survival in ER-positive breast cancer patients [11].

In addition to cancer cell proliferation, deregulated metabolism is a cornerstone of oncogenesis [17] and is named as one of the hallmarks of cancer [18]. Cancer cells are known to generate their energy by using aerobic glycolysis through a process known as the "Warburg effect" [19]. To date, there has been no publication that has linked miR-195 expression with cancer metabolism using large patient cohorts.

In the current study, we aimed to investigate the role of miR-195 in breast cancer progression, cancer metabolism and patient survival.

Materials and methods

Data acquisition of breast cancer cohorts

Base line characteristics and the expression data of messenger RNA (mRNA) of 2,038 patients were obtained from The Cancer Genome Atlas (TCGA) Pan-Cancer study (TCGA PanCancer Atlas) and The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) as previously described [20-30]. Also, microRNA-195 (miR-195) expression and survival data were obtained from those two large cohorts. Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.ih.gov/geo/) was utilized to obtain the miR-195 expression data from Lee CH et al (GSE45666; n = 116) [31]. Given that all the cohorts mentioned above are publicly accessible and the patients' information are anonymized, Institutional Review Board (IRB) approval was waived for the current study.

Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) is the software provided by Broad Institute (http://software.broadinstitute.org/gsea/index.jsp). As we have previously published, the Hallmark groups of MSigDB collections were utilized for the current study [24, 28, 29]. A false discovery rate (FDR) of 0.25 was set as statistical significance, as recommended by the GSEA developer (Broad Institute).

The composition of immune cells in the tumor microenvironment

Computational algorithm xCell was used to identify and estimate the abundance of the immune cells; CD4+ T cell, CD8+ T cell, Th1 cells, Th2 cells, Tregs, NK cells, M1 macrophage, and M2 macrophage using expression profile of 158, 116, 36, 24, 39, 101, 188, and 159 unique cell marker genes, respectively, based on the work of Aran D et al [32] as we have reported previously [29, 30, 33-38]. Cytolytic activity (CYT) was calculated utilizing expression data of granzyme A (GZMA) and Perforin 1 (PRF1) as reported previously [12, 39-42].

Prediction target genes of miR-195

We utilized the online database miRDB (http://mirdb.org) to obtain the top 30 predicted target genes of miR-195 [43].

Table 1. Distribution of base line characteristics by miR-195 expression

Characteristic	whole cohort (n = 755)		*p* value
	miR-195 High n = 378	miR-195 Low n = 377	
Age			
< 65 y	270	259	0.474
≥ 65 y	108	117	
Unknown	0	1	
Stage			
I/II/II/V	74/203/95/3	63/227/76/6	0.153
Unknown	3	5	
pT			
T1/T2/T3/T4	115/194/57/11	97/229/39/12	0.0488
Tx	1	0	
pN			
N0/N1/N2/N3	174/132/40/28	183/126/37/24	0.858
Nx	4	7	
M			
M0/M1	305/3	289/6	0.331
Mx	70	82	
ER Status			
Positive	296	248	< 0.001
Negative	62	103	
Unknown	20	26	
PgR Status			
Positive	269	216	< 0.001
Negative	87	137	
Unknown	22	24	
HER2 Status			
Positive	43	81	< 0.001
Negative	285	251	
Unknown	50	45	

Statistical analysis

R software (http:///www.r-project.org/) (version 4.0.2) was used for purposes of statistical analyses. The significant difference was calculated utilizing Fisher's exact test or one-way ANOVA method. Also, the analysis of one-way ANOVA was utilized for the box plots of the current study. The black line inside boxplots (Tukey type) demonstrates median value and the span of rectangle demonstrates inter-quartile ranges. A two-sided $P < 0.05$ was considered statistically significant. Kaplan Meier curves were plotted for survival analysis.

Results

miR-195 expression is associated with ER and PR expression

Patients were divided into groups of high and low miR-195 expression by the median cutoff. Given that miR195 expression was previously reported as a suppressive miRNA in several cancers [7], we expected that high miR-195 expression would be associated with aggressive clinicopathological factors. Among the analyzed categories, there were statistically significant differences in estrogen receptor (ER), progesterone receptor (PR), and HER2 status (**Table 1**). The low miR-195 expressing group had a greater number of patients with a negative ER and PR status, as well as a greater number of patients with positive HER2 status. Interestingly, both of these receptor subtypes are known to be biologically aggressive. There were significantly more patients with a T2 stage tumor, as classified by the American Joint Committee on Cancer (AJCC), in the low miR-195 expression group as compared to the high miR-195 expression group. The association between low miR-195 expression tumors, aggressive breast cancer subtypes and larger tumors are in agreement with the notion that miR-195 is a tumor suppressive miRNA.

The association between miR-195 expression, Nottingham pathological grade

Understanding that miR-195 is a tumor suppressive miRNA, we hypothesized that low miR-195 expressing tumors are associated with cell proliferative features. We first confirmed that miR-195 expression was significantly lower in tumors compared to normal breast tissue in TCGA as well as the GSE45666 cohort (**Figure 1**; both $P < 0.001$). A higher Nottingham pathological grade was associated with lower expression of miR-195 in both the whole group as well as ER-positive patients of TCGA (**Figure**

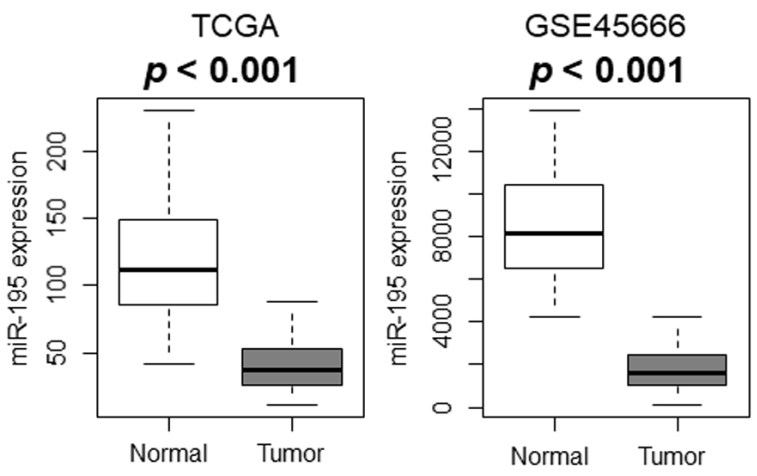


Figure 1. Tumors expressed lower levels of miR-195 compared with normal tissue samples. Left: TCGA cohort (Normal: n = 87, Tumor: n = 87). Right: GSE445666 cohort (Normal: n = 15, Tumor: n = 101 samples). To determine *p*-value, one-way ANOVA method was utilized. The black line inside boxplots (Tukey-type) demonstrate the median value. The span of rectangle demonstrates inter-quartile level values.

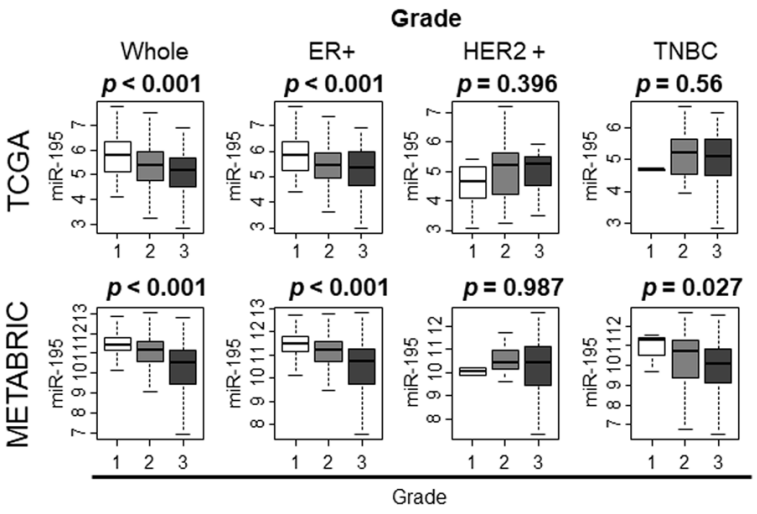


Figure 2. Association of miR-195 expression levels and Nottingham pathological grade. Nottingham pathological grade and miR-195 expression levels in Whole cohort, ER-positive (ER+), HER2-positive, and triple negative (TN) subtypes in TCGA and METABRIC cohorts. Open box (Grade 1), shaded box (Grade 2), Closed box (Grade 3). Open box (low miR-195) and shaded box (high miR-195). To determine *p*-value, one-way ANOVA method was utilized. The black line inside boxplots (Tukey-type) demonstrate the median value. The span of rectangle demonstrates inter-quartile level values. ER+, ER-positive; HER2+, HER2-positive; TN, triple negative.

2; P < 0.001 and P < 0.001, respectively). These results were validated in the METABRIC cohort (**Figure 2**; P < 0.001 and P < 0.001, respectively). This trend was also noted in the triple negative breast cancer (TN) subtype of METABRIC cohort.

Breast cancer with low miR-195 expression enriched cell proliferation-related gene sets and miR-195 expression associated with MKI67 expression

To investigate the underlying mechanisms of low miR-195 expressing breast cancer's association with enhanced cell proliferation, we performed gene sets enrichment analysis (GSEA). In concordance with its association to grade, low miR-195 expressing groups across the whole group of patients, as well as ER-positive and TNBC patients of TCGA demonstrated enrichment of cell proliferation related gene sets (**Figure 3**, Supplementary Figure 1). For instance, E2F Targets and G2M Checkpoint, Myc Targets V1 and Myc Targets V2 were among gene sets which are enriched. This result was validated in the METABRIC cohort (**Figure 3**, Supplementary Figure 1). Interestingly, PI3K-Akt-mTOR signal pathway was enriched in the low miR-195 expressing tumors in ER-positive patients alone and not with TNBC patients (**Figure 3**).

We also analyzed the association between miR-195 and MKI67 gene expression, which encodes Ki67. The group with low miR-195 expression was associated with a higher expression level of MKI67 across the whole group. The same trend was observed among the ER-positive and TNBC in TCGA (**Figure 3**; P < 0.001, P = 0.011, P = 0.034, respectively). Furthermore, this result was validated in the METABRIC cohort (**Figure 3**; P < 0.001, P < 0.001, P = 0.003, respectively) indicating that the tumors with lower expression of miR-195 were associated with enhanced cancer cell proliferation.

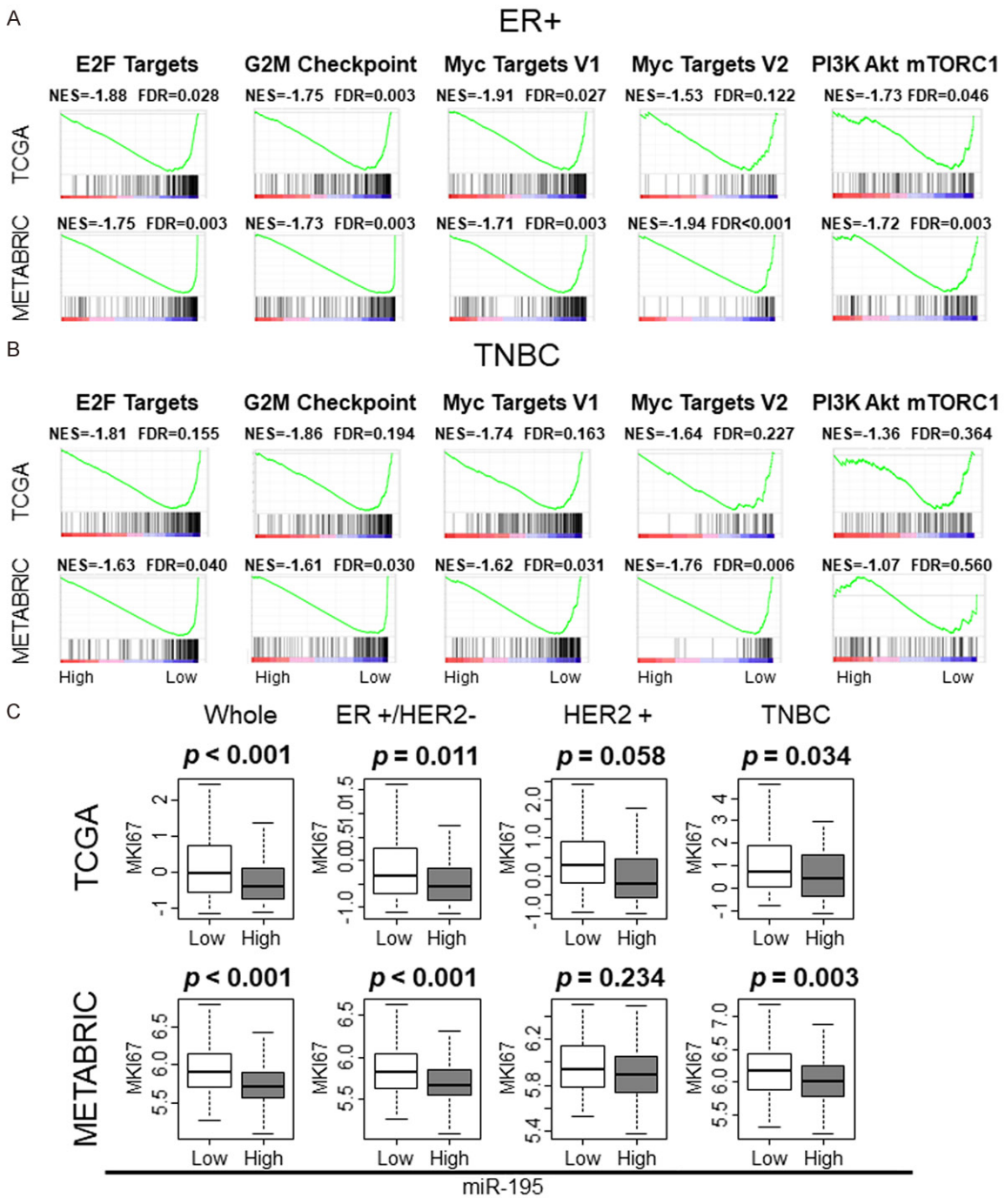
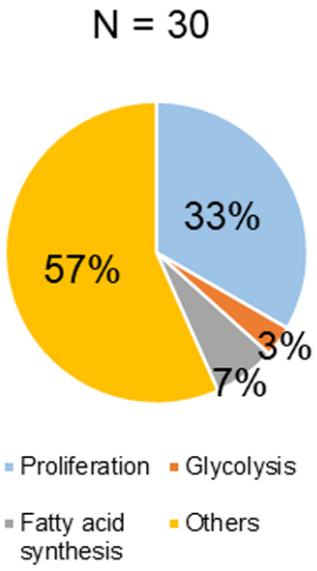


Figure 3. Gene set enrichment analysis (GSEA) of miR-195 low expressing breast cancer in TCGA and METABRIC cohorts and association of miR-195 expression levels and MKI67 expression. A. ER-positive subtype in TCGA and METABRIC. B. TN subtype in TCGA and METABRIC. The group was divided into two groups (miR-195-High and -Low) by utilizing median cutoff. C. MKI67 expression was shown in Whole cohort, ER+, HER2+ and TN breast cancers in TCGA and METABRIC cohorts. The statistical significance was decided using FDR < 0.25. ER+, ER-positive; TN, triple negative breast cancer; NES, normalized enrichment score; FDR, false discovery rate.

Cell proliferation related genes are among the target genes of miR-195

Given that low miR-195 expressing tumors enriched cell proliferation related gene sets, we hypothesized that cell proliferation-related genes are among the predicted target genes of miR-195. We utilized the publicly accessible database miRDB (http://mirdb.org) to obtain the top 30 predicted genes of miR-195 [43].

A



B

Gene Symbol	Gene Description	Function
PAPPA	pappalysin 1	Proliferation
FASN	fatty acid synthase	Fatty acid synthesis
UNC80	unc-80 homolog, NALCN channel complex subunit	Proliferation
FGF2	fibroblast growth factor 2	Proliferation
TNRC6B	trinucleotide repeat containing 6B	Others
PTPN4	protein tyrosine phosphatase, non-receptor type 4	Proliferation
PHF19	PHD finger protein 19	Others
DESI1	desumoylating isopeptidase 1	Others
UBE2Q1	ubiquitin conjugating enzyme E2 Q1	Proliferation
LSM11	LSM11, U7 small nuclear RNA associated	Others
NECTIN1	nectin cell adhesion molecule 1	Others
GAREM1	GRB2 associated regulator of MAPK1 subtype 1	Proliferation
ANKUB1	ankyrin repeat and ubiquitin domain containing 1	Others
FBXO21	F-box protein 21	Others
CCNE1	cyclin E1	Proliferation
ATG14	autophagy related 14	Others
LUZP1	leucine zipper protein 1	Others
SLC13A3	solute carrier family 13 member 3	Fatty acid synthesis
ARIH1	ariadne RBR E3 ubiquitin protein ligase 1	Others
MGAT4A	alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase A	Glycolisis
EPHB2	EPH receptor B2	Others
BTRC	beta-transducin repeat containing E3 ubiquitin protein ligase	Others
SPRYD3	SPRY domain containing 3	Others
ARL2	ADP ribosylation factor like GTPase 2	Others
CASK	calcium/calmodulin dependent serine protein kinase	Others
NUP50	nucleoporin 50	Proliferation
DCLK1	doublecortin like kinase 1	Proliferation
CYB561A3	cytochrome b561 family member A3	Others
ZBTB46	zinc finger and BTB domain containing 46	Others
FGF7	fibroblast growth factor 7	Proliferation

Figure 4. Distribution and list of top 30 predicted target genes of miR-195. A. Distribution of predicted genes of miR-195. B. List of top 30 predicted target genes.

Concordant with our expectation, 33% of those predicted genes were related to cell proliferation (**Figure 4A**). Among the top 30 predicted genes, only cyclin E1 and nucleoporin 50 were included in the gene sets analyzed in **Figures 3** and **4B**. Ten percent of predicted genes were associated with cancer metabolism, such as glycolysis and fatty acid synthesis. The remain-

ing 17 genes were miscellaneous genes that were difficult to categorize. All the target genes are listed in **Figure 4B**. This result implies that miR-195 impedes the proliferation of cancer cells by inhibiting the multiple cell proliferation genes, most of which are not included in the analyzed gene set.

The association between miR-195 and the tumor immune microenvironment

It has recently become clear that tumor infiltrating immune cells play a significant role in cancer progression. Given that low miR-195 expression tumors are highly proliferative and aggressive, it was of interest to investigate infiltration of associated immune cells as related to miR-195 expression. As an example, miR-195 has been linked with the activation of CD8+ cells and inhibition of regulatory T cells (Treg) in prostate cancer [44]. Our group recently reported that highly proliferative G2M Checkpoint activated breast cancer has an increased infiltration of unfavorable immune cells [45]. Given that low miR-195 expressing tumors enriched the G2M Checkpoint gene set, we expected that they may be associated with an unfavorable and pro-cancerous tumor immune environment.

We found that the infiltration of pro-cancerous immune cells, T helper type 2 (Th2) cells, Tregs and M2 macrophages (M2), were all higher in the low miR-195 expression groups amongst the ER-positive subtype patients but not in TNBC patients. In contrast, Th2 cells were significantly higher in low miR-195 expressing tumors in TNBC (**Figure 5B**). To our surprise, low miR-195 expressing tumors were also associated with a higher infiltration of anti-cancerous immune cells such as T helper type 1 (Th1) cells and M1 macrophages (M1) in ER-positive patients, whereas only M1 was elevated in TNBC patients (**Figure 5A**). The cytolytic activity score, which evaluates the overall immune cell killing activity, was no different across the expression levels of miR-195 in either of the breast cancer subtype (**Figure 5C**).

Low expression miR-195 tumors were associated with glycolysis in ER-positive but not in TNBC

Cancer cells have more active glycolysis as compared to normal cells, which is more commonly recognized as Warburg effect. The glucose transporter that delivers glucose into the cell and glycolysis-related products, such as hexokinases, lactate dehydrogenase, and glucose-6-phohphate, are upregulated in cancer cells [46, 47]. As miR-195 has been shown to target glycolysis-related genes in vitro [7], it was of interest to investigate whether glycolysis was associated with the expression of miR-195 in breast cancer patients. We found that the glycolysis gene set was significantly enriched by low miR-195 expression tumors in the ER-positive subtype consistently in both TCGA and METABRIC cohorts (**Figure 6A**). This was not the case in patients with TNBC (**Figure 6C**). We analyzed the expression levels of Hexokinase 2 (HK2), Lactate dehydrogenase A (LDHA), glucose-6-phosphate dehydrogenase (G6PD), and Glucose transporter 1 (GLUT1) as representative genes for glycolysis. All these genes except for GLUT1 were significantly expressed in low miR-195 expression ER-positive tumors in both the METABRIC and TCGA cohorts (**Figure 6B**). Only LDHA was consistently elevated in low miR-195 expression TNBC patients in both cohorts (**Figure 6D**). These results indicate that glycolysis is strongly associated with low miR-195 expressing ER-positive patient, but not with the TNBC subtype.

Low miR-195 expression was associated with poor survival amongst ER-positive breast cancer patients.

We assessed the association between miR-195 expression and clinical outcomes utilizing Kaplan-Meier analysis. It has been previously shown that the expression of glycolysis-related genes was associated with a poor prognosis in breast cancer [48]. Additionally, as the upregulation of glycolysis was observed in low miR-195 expressing ER-positive/HER2-negative tumors, we expected that these tumors would demonstrate worse survival compared to high miR-195 expressing tumors. The low miR-195 expressing group demonstrated inferior overall survival (OS) across the whole cohort. This result was concordant with ER-positive subtype of patients, but inferior survival was not observed across other subtypes in the TCGA cohort (**Figure 7**; $P = 0.016$ and $P = 0.005$, respectively). These findings were also noted in the METABRIC cohort (**Figure 7**; $P = 0.016$ and $P = 0.005$, respectively).

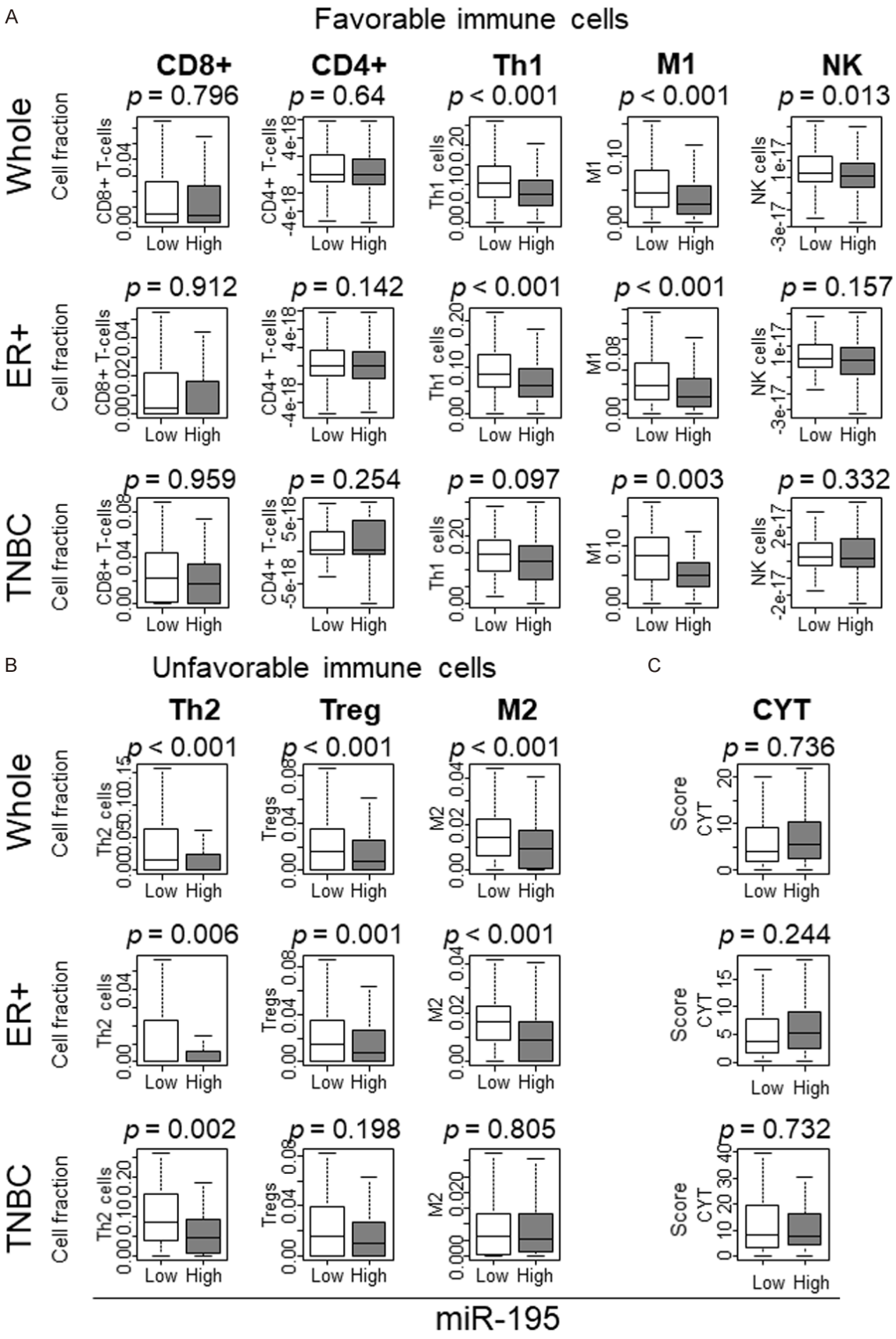

A
Favorable immune cells
CD8+
CD4+
Th1
M1
NK
Whole
ER+
TNBC
Cell fraction
p = 0.796
p = 0.64
p < 0.001
p < 0.001
p = 0.013
p = 0.912
p = 0.142
p < 0.001
p < 0.001
p = 0.157
p = 0.959
p = 0.254
p = 0.097
p = 0.003
p = 0.332
CD8+ T-cells
CD4+ T-cells
Th1 cells
M1
NK cells
Low High
B
Unfavorable immune cells
Th2
Treg
M2
Whole
ER+
TNBC
Cell fraction
p < 0.001
p < 0.001
p < 0.001
p = 0.006
p = 0.001
p < 0.001
p = 0.002
p = 0.198
p = 0.805
Th2 cells
Tregs
M2
Low High
C
CYT
p = 0.736
p = 0.244
p = 0.732
Score
CYT
Low High
miR-195

Figure 5. The relationship between the levels of miR-195 and infiltrating myeloid cells and lymphoid cells within the bulk tumor in TCGA and METABRIC cohort. A. Analysis of favorable immune cells. B. Analysis of unfavorable immune cells. C. Analysis of CYT score. CD8+, CD8 positive T cell; CD4+, CD4 positive T cell; Th1, type 1 helper T cell; Th2, type 2 helper T cell; M1, Macrophage M1; M1, Macrophage M1; NK, natural killer cells; Treg, regulatory T cell; CYT, cytolytic activity score. ER+, ER-positive and HER2-negative; TN, triple negative breast cancer.

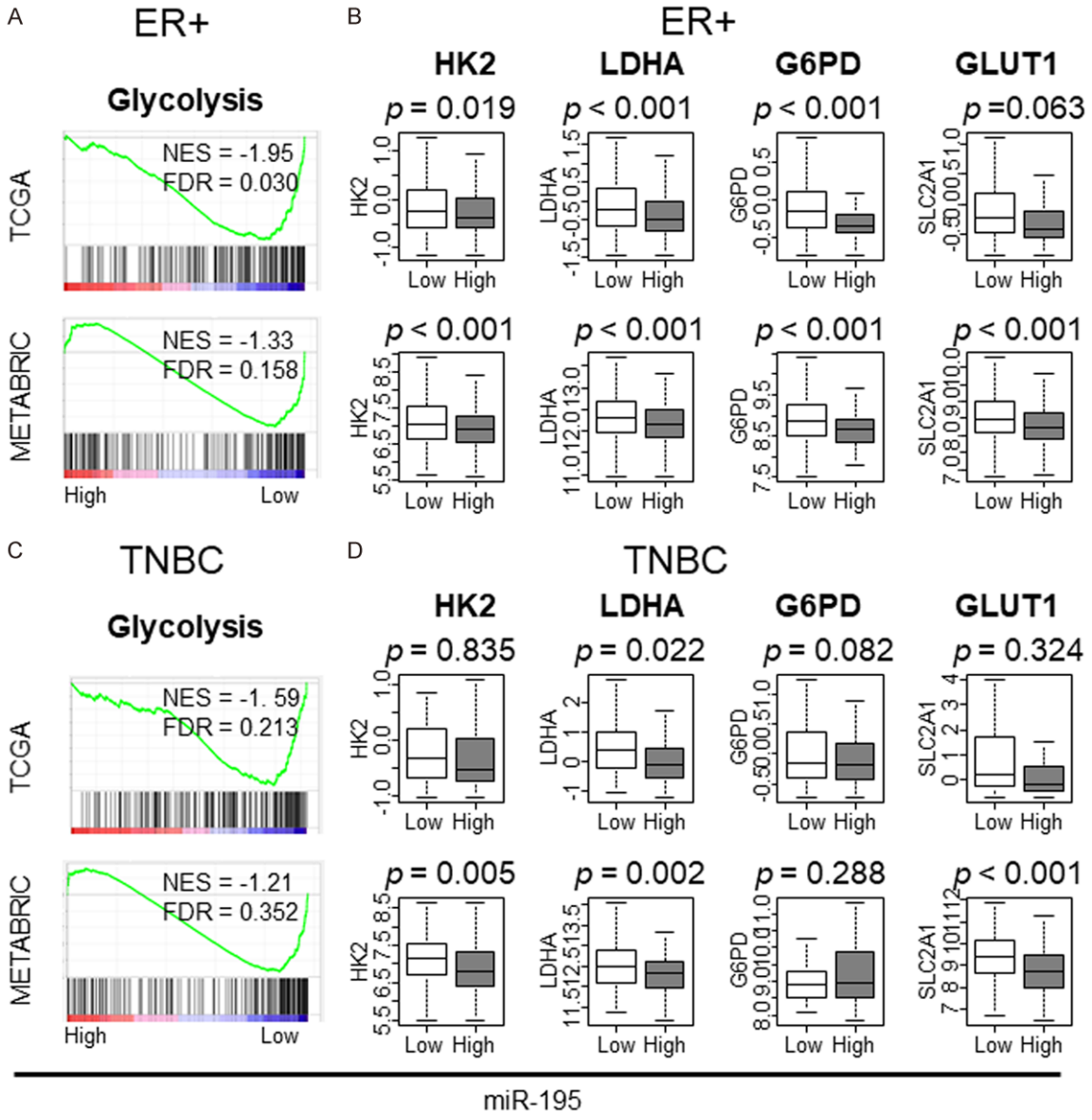


Figure 6. The relationship between the levels of miR-195 and the glucose metabolism related gene set as well as related genes. A. Gene set enrichment analysis of miR-195 low expressing ER+ breast cancer. B. Analysis of glycolysis related genes of ER+ breast cancers. Closed box (high miR-195), Open box (low miR-195). C. Gene set enrichment analysis of miR-195 low expressing TN breast cancer. D. Analysis of glycolysis related genes of TN breast cancers. Closed box (high miR-195), Open box (low miR-195). HK2, Hexokinase 2; LDHA, Lactate dehydrogenase A; G6PD, glucose-6-phosphate dehydrogenase; GLUT1, Glucose transporter 1.

Discussion

In this study, we demonstrated that the low expression of miR-195 in ER-positive breast cancer is associated with enhanced cancer cell proliferation, glycolysis and worse OS. MiR-195 expression was downregulated in breast cancer compared with normal breast tissue, which

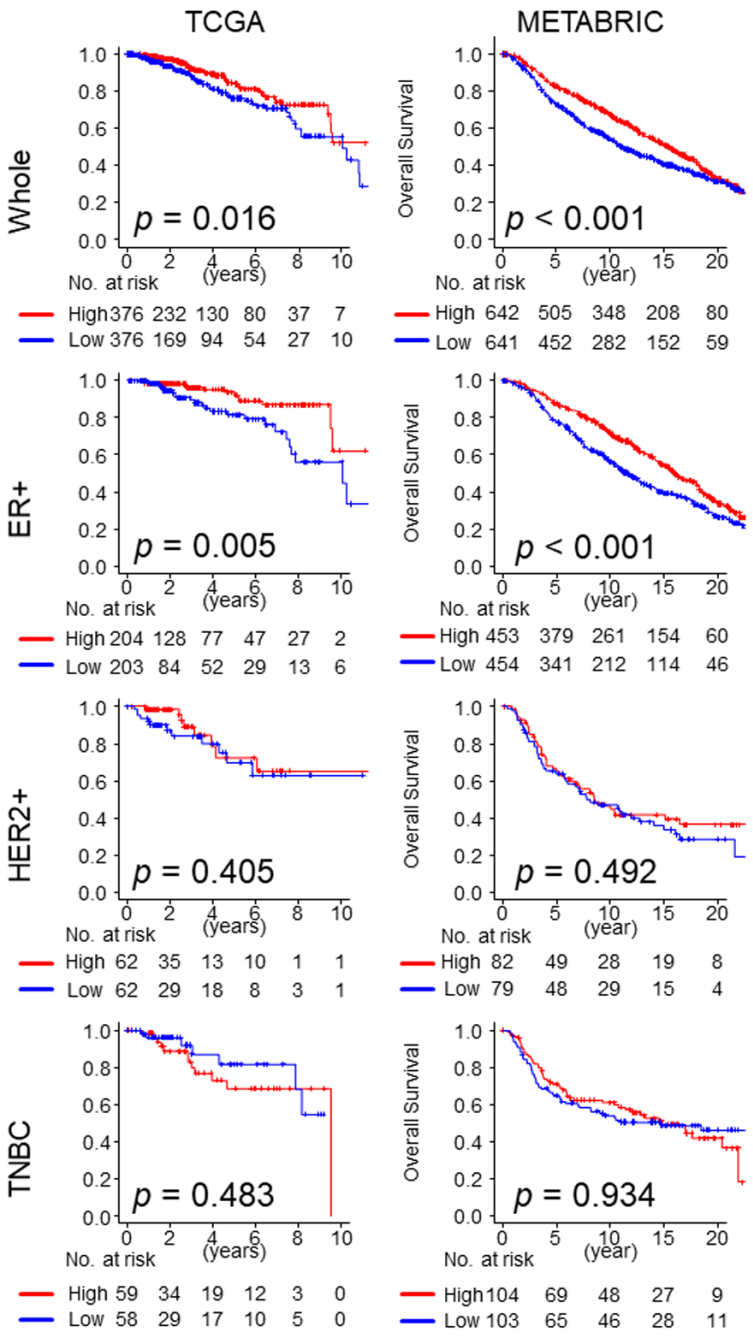


Figure 7. Kaplan Meier survival analysis of miR-195 low expressing (blue line) and high expressing breast cancer (red line) in Whole cohort, ER+, HER2+, and TNBC of TCGA and METABRIC cohorts.

is consistent with the role of miR-195 as a tumor suppressor miRNA. ER and PR negativity and HER2 positivity were associated with low miR-195 expressing breast cancer. Low MiR-195 expressing tumors demonstrated high cell proliferation features by enriching the gene sets associated with cell proliferation. They also associated with higher MKI67 expression as well as higher pathological grade. Approximately 33% of the top target genes of miR-195 are related with cell proliferation, but only two of them were included in cell proliferation gene sets. The low miR-195 expressing group demonstrated an association with higher infiltration of both anticancerous as well as pro-cancerous immune cells as compared to the high miR-195 expressing group. Low miR-195 expressing tumors were associated with enhanced glycolysis and poor survival in ER-positive patients. However, this was not the case in the other subtypes of breast cancer patients.

We found that low miR-195 expressing tumors enriched the gene sets which demonstrate the proliferation of cancer cells. For instance, E2F Targets, G2M Checkpoint, and Myc Targets were enriched in both ER-positive and TNBC. We demonstrated that the 33% of top 30 predicted genes of miR-195 was associated with cell proliferation. This result implied that miR-195 inhibits cell proliferation by targeting multiple cell proliferation-related genes. This finding was only possible because we utilized an *in-silico* translational approach and analyzed multiple genes as scores. The enrichment of E2F Targets gene set in miR-195 expressing tumors is understandable given that previous studies have reported that miR-195 inhibited cell cycle transition of G1 to S phase by directly targeting cyclin E1 [8], where E2F plays a critical role. E2F interacts with other factors such as cyclin D, cyclin E, and cyclin-dependent kinases, to suppress the G1 to S phase transition of the cell cycle. In fact, miR-195 was shown to suppress cell proliferation by targeting cell cycle

regulators such as Raf-1 and cyclin D1 [49]. We have translated these mechanistic studies into the clinical setting by assessing MKI67 expression, the most widely used cell proliferation marker, as well as the Nottingham pathological grade. Both measures are routinely used to assess cell proliferation by histological analysis in the clinical setting. Strikingly, these clinical assays (MKI67 and Nottingham pathological grade) were perfectly concordant with low miR-195 expression in ER-positive patients by transcriptomic analysis utilizing GSEA.

We recently reported that a high score of G2M Checkpoint and E2F Targets was associated with a higher infiltration of immune cells [25, 45]. Given these results, we expected that low miR-195 expression tumors, which enriched G2M Checkpoint and E2F Targets gene sets, to have a greater immune cell infiltration compared to high miR-195 expression tumors. As expected, low miR-195 expressing tumors revealed a higher infiltration of both pro-cancerous (Th2, Treg, and M2) and anti-cancerous (Th1 and M1) immune cells. Interestingly, this finding was more prominent in the ER-positive subtype of breast cancer as opposed to the TN subtype, the latter of which is known to have a high infiltration of immune cells in the tumor microenvironment [50]. However, there was no difference of the estimated overall immune cell killing activity as measured by CYT in the bulk tumor, which suggests that the tumor immune microenvironment is not the primary mechanism behind the aggressiveness of low miR-195 expression breast cancer. Given the relationship between miR-195 expression and the tumor immune microenvironment, it was of interest to investigate the source cells of miR-195 expression, whether it be from cancer cells, immune cells or stromal cells in human breast cancer. However, we were unable to find a single-cell sequence data set with microRNA expression levels in the public domain. We also tried to generate a single-cell microRNA sequence cohort ourselves without success.

Numerous miRNAs have been reported to negatively regulate glycolysis by inhibiting key enzymes such as GLUT1, HK2, and LDHA [51-53]. For instance, Fong et al reported that miR-122 targets GLUT1 to affect glucose uptake and eventually inhibit breast cancer metastasis [51]. Also, Li et al reported that miR-30a inhibits glycolysis in breast cancer cells by targeting LDHA [52]. To the best of our knowledge, this is the first study to report the association of miR-195 to glycolysis. The current study demonstrates that low miR-195 expressing tumors enriched glycolysis gene sets. In correlation, the expression of a key enzyme which functions at the first step of glycolysis, HK2, was higher in low miR-195 expressing tumors. This result is very understandable given that low miR-195 expressing tumors enriched the gene set relating to PI3K/AKT/mTOR pathway. The stimulation of this pathway has been previously reported to increase glycolysis [54]. A meta-analysis of 86 studies reported that the increased protein expression of key enzymes in glycolytic pathway, such as HK2, was an indicator of poor survival in breast cancer [48]. For instance, Dong et al reported that the positive protein expression of G6PD was associated with worse overall survival in triple negative breast cancer patients [55]. This implied that the upregulation of glycolysis pathway is a prognostic factor of worse survival.

Increased cell proliferation by activated MYC signaling was shown to induce DNA damage as a mechanism for oncogene-induced genetic instability [56]. Given previous reports that high mutation loads generate neoantigens that attract immune cells into the tumor microenvironment [57], it is reasonable to see an infiltration of immune cells in low miR-195 expression tumors that have enhanced cell proliferation. With the Warburg effect, an aggressive cancer with high cell proliferation is expected to have high glycolytic activity. However, this does not explain why glycolysis was not different across vaying miR-195 expression in TNBC, or why the types of infiltrating immune cells were different across breast cancer subtypes; M1, Th2, Treg and M2 were infiltrated in low miR-195 expressing ER-positive and the whole cohort, but only M1 and Th2 were infiltrated in TNBC. We see this as infiltration of less immune cell types in TNBC. Glycolysis promotes the activation of M1 macrophages, thus it is reasonable to see low miR-195 expressing ER-positive tumors with activated glycolysis to have high M1 macrophage infiltration [58, 59]. On the other hand, given that TNBC is known to be highly proliferative and immunogenic as compared to ER-positive tumors, the clinical impact may not be as relevant as it is in ER-positive tumors. We

cannot help but speculate that miR-195 expression is more clinically relevant in the ER-positive subtype with less cell proliferation and immunogenicity as a baseline to activate glycolysis and stimulate more immune cell infiltration.

Even though we have demonstrated a new finding in this study, it does possess certain limitations. This study is retrospective and utilized two large publicly accessible cohorts, TCGA and METABRIC. Despite containing large numbers of the patients, these cohorts lack some key clinical information such as the type of treatment provided. Further, it will be of interest to clarify the mechanism of overexpression/downregulation of miR-195 in breast cancer cell lines, as well as the mechanism of cell proliferation, apoptosis, and glycolysis by *in vitro* experiments. However, we do not have the capability to conduct these pre-clinical experiments at this point. Thus, further experimental analyses are needed to validate our finding of observational associations.

Conclusions

In conclusion, low miR-195 expressing tumors are associated with cell proliferation, glycolysis, and poor prognostic outcomes in ER-positive breast cancer.

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Disclosure of conflict of interest

None.

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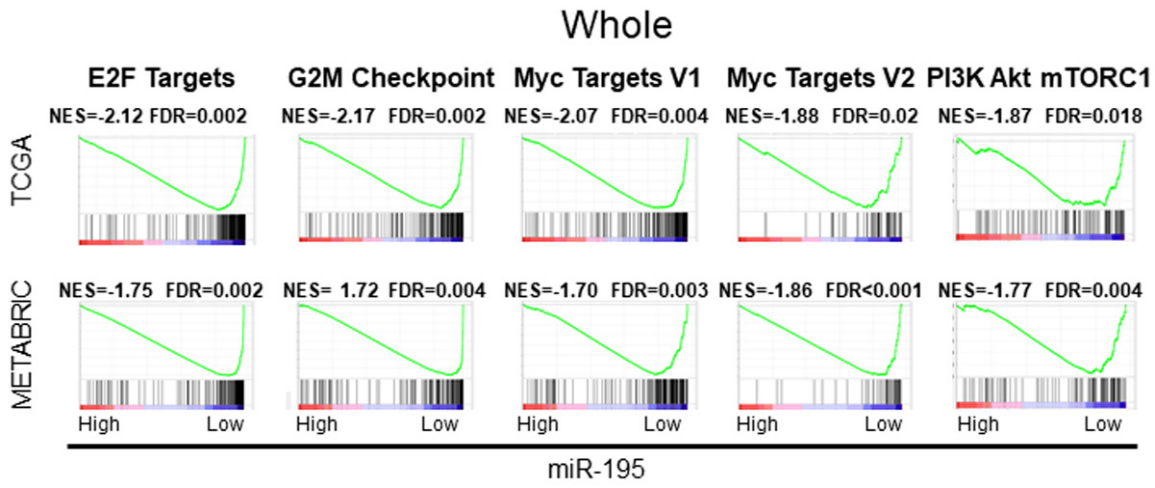

Supplementary Figure 1. Gene set enrichment analysis of miR-195 low expressing breast cancer of Whole cohort. Upper row represent of TCGA cohort and lower row represent of METABRIC. FDR < 0.25 is considered to be statistically significant. NES, normalized enrichment score; FDR, false discovery rate.