

**ANALYSIS OF ERBIN GENE EXPRESSION REGULATION BY
MICRORNAS IN BREAST CANCER**

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By
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August 2025

ANALYSIS OF ERBIN GENE EXPRESSION REGULATION BY MICRORNAS
IN BREAST CANCER

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August 2025

We certify that we have read this thesis and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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Breast cancer is a heterogeneous disease characterized by distinct molecular subtypes, defined by specific gene markers and mutations associated with different prognoses and treatment responses. The ERBIN (ERBB2IP) gene encodes a basolateral scaffold/adaptor protein that maintains epithelial cell polarity and adhesion, while also regulating key signaling pathways, including MAPK and TGF- β signaling. Its expression changes across diseases, including HER2-positive breast cancer. However, the mechanisms underlying aberrant ERBIN expression levels remain poorly investigated. This study aimed to identify specific miRNAs regulating ERBIN expression in distinct breast cancer subtypes and to understand further regulatory interactions within the miRNA-ERBIN axis. ERBIN expression patterns in publicly available breast cancer patient datasets were analyzed across molecular subtypes, TP53 mutation status, and hormone receptor status to address this aim. Expression correlation analysis between ERBIN and miRNA expression, and the results from miRNA target prediction tools, identified potential miRNAs that may regulate ERBIN expression in breast cancer. These candidates were further analyzed for their expression in aggressive subgroups to evaluate their inverse association with ERBIN, considering clinical characteristics. In addition, miRNA-seq analysis following ERBIN knockdown in MDA-MB-231 cells revealed potential regulatory feedback loops between ERBIN and selected miRNAs. Overall, the results provide comprehensive insight into the post-transcriptional regulation of ERBIN in breast cancer and suggest that the miRNA-ERBIN relationships provide a potential biomarker or therapeutic target.

Keywords: ERBIN, ERBB2IP, Breast Cancer, microRNA, miRNA-seq, MDA-MB-231 breast cancer cell line



ÖZET

MEME KANSERİNDE ERBİN GEN İFADESİNİN MİKRORNA'LER TARAFINDAN DÜZENLENMESİ

Özge Yüksel

Moleküler Biyoloji ve Genetik, Yüksek Lisans

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Meme kanseri, farklı hastalık sonucu tahminleri ve tedavi yanıtlarıyla ilişkili olan farklı moleküler alt tipleri, belirli gen belirteçleri ve mutasyonlarla karakterize heterojen bir hastalıktır. ERBIN (ERBB2IP) geni, epitel hücre polaritesini ve adezyonunu koruyan, bazolateral konumlu bir iskelet/adaptör proteinini kodlar ve ayrıca MAPK ile TGF- β dahil olmak üzere birçok temel sinyal yollarını düzenler. ERBIN gen ifade seviyesi, HER2-pozitif meme kanseri dahil olmak üzere, hastalıklarda duruma bağlı olarak değişiklik göstermektedir. Ancak, anormal ERBIN ifade düzeylerine yol açan mekanizmalar yeterince araştırılmamıştır. Bu çalışma, farklı meme kanseri alt tiplerinde ERBIN geninin ifadesini düzenleyen belirli miRNA'ları belirlemeyi ve miRNA-ERBIN eksenindeki ek düzenleyici etkileşimleri araştırmayı amaçlamaktadır. Bu amaçla, kamuya açık meme kanseri hasta veri setlerindeki ERBIN gen ifadesi; moleküler alt tip, TP53 mutasyon durumu ve hormon reseptörü durumuna göre analiz edilmiştir. ERBIN ile miRNA ekspresyonu arasındaki korelasyon analizi, çeşitli miRNA hedef tahmin araçlarının sonuçlarıyla birleştirilerek ERBIN gen ifadesini potansiyel olarak düzenleyen miRNA adayları belirlenmiştir. Bu adayların, klinik özelliklere göre ERBIN ile ters ilişkilerini anlamak amacıyla, agresif alt gruplarda ifade durumları değerlendirilmiştir. Ayrıca, MDA-MB-231 hücrelerinde ERBIN gen ekspresyonunun baskılanması sonrasında yapılan miRNA-seq çalışması, ERBIN ile seçilmiş miRNA'lar arasındaki potansiyel düzenleyici geri besleme döngülerini ortaya koymuştur. Genel olarak, bu çalışmanın sonuçları agresif meme kanserinde ERBIN'in post-transkripsiyonel düzenlenmesine dair kapsamlı bir bakış sunmakta ve miRNA-ERBIN ilişkilerinin potansiyel biyobelirteç veya terapötik hedefler olabileceğini göstermektedir.

Anahtar Kelimeler: ERBIN, ERBB2IP, Meme Kanseri, mikroRNA, miRNA dizileme, MDA-MB-231 meme kanseri hücre hattı





To my family...

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ABBREVIATIONS

3'UTR	3' untranslated region of mRNA
5'UTR	5' untranslated region of mRNA
AGO	Argonaute protein family
AChR	Acetylcholine receptor
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
APS	Ammonium persulfate
BCL2	B-cell lymphoma 2
BCL11A	B-cell lymphoma/leukemia 11A
CDS	Coding sequence
CEBPA	CCAAT/enhancer-binding protein α
CEBPB	CCAAT/enhancer-binding protein β
CRC	Colorectal cancer
DGCR8	DiGeorge syndrome critical region 8
DCP2	Decapping protein 2
dPBS	Dulbecco's phosphate-buffered saline
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DMFS	Distant metastasis-free survival
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
ER α	Estrogen receptor α
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERBB2IP/ERBIN	Erbin / ErbB2-interacting protein
ERK	Extracellular signal-regulated kinase
ESR1	Estrogen receptor one gene
FBS	Fetal bovine serum

FDR	False discovery rate
FHL2	Four-and-a-half LIM domains protein 2
FO XK2	Forkhead box protein K2
FO XM1	Forkhead box protein M1
GEO	Gene Expression Omnibus
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
HO XB13	Homeobox protein HO XB13
HO XD10	Homeobox protein HO XD10
HR	Hazard ratio
IHC	Immunohistochemistry
JNK	c-Jun N-terminal kinase
KLF5	Krüppel-like factor 5
KTN1-AS1	KTN1 antisense RNA 1
LAP	Leucine-rich repeat and PDZ
LATS2	Large tumor suppressor kinase 2
LRR	Leucine-rich repeat
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MDA-MB-231	Triple-negative human breast-cancer cell line
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MITF	Microphthalmia-associated transcription factor
MREs	miRNA response elements
NGS	Next-generation sequencing
OS	Overall survival
PAM50	Prediction Analysis of Microarray 50
PDZ	PSD-95/Discs Large/ZO-1 domain
PI3K	Phosphoinositide 3-kinase

PR	Progesterone receptor
PGR	Progesterone receptor gene
qRT-PCR	Quantitative reverse-transcription PCR
RB	Retinoblastoma protein
RBP	RNA-binding protein
RECK	Reversion-inducing cysteine-rich protein with Kazal motifs
RFS	Relapse-free survival
RISC	RNA-induced silencing complex
RNA22	miRNA target prediction algorithm
RPK	Reads per kilobase
SFKs	Src family kinases
TCGA	The Cancer Genome Atlas
TCGA-BRCA	TCGA Breast Invasive Carcinoma
TF	Transcription factor
TGF- β	Transforming growth factor beta
TNBC	Triple-negative breast cancer
TP53	Tumor protein p53 gene
TPM	Transcripts per million
TRBP	Transactivation response element RNA-binding protein
UCSC	University of California, Santa Cruz
UTR	Untranslated region
WT	Wild type
XRN	5'–3' exoribonuclease
lncRNA	Long non-coding RNA

CHAPTER 1: INTRODUCTION

1.1. Cancer

Cancer is a group of diseases marked by uncontrolled cell proliferation, invasion, and potential metastasis. The Hallmarks of Cancer framework defines six core capabilities: (i) sustaining proliferative signaling, (ii) evading growth suppressors, (iii) resisting cell death, (iv) enabling replicative immortality, (v) inducing angiogenesis, and (vi) activating invasion and metastasis (Hanahan & Weinberg, 2011). The model was later expanded to include avoiding immune destruction, tumor-promoting inflammation, genome instability and mutation, and deregulated cellular metabolism (Hanahan, 2022).

Normal cells maintain a balanced regulation of growth signals to control cell division and growth, while cancer cells dysregulate these signals and activate pathways such as PI3K/AKT and MAPK (Duronio & Xiong, 2013), which promote uncontrolled proliferation and resistance to cell death, eventually affecting survival mechanisms and energy metabolism. Cancer cells escape growth-suppressive signals by inactivating genes such as retinoblastoma-associated (RB) and TP53, which control cell cycle progression and apoptosis (Hanahan & Weinberg, 2011). In this context, the TGF- β signaling pathway promotes RB and p53 functions, interfering with proliferation and enhancing apoptosis in early stages. At the same time, it drives tumor progression and metastasis due to the rewiring of TGF- β signaling in later stages (Seoane & Gomis, 2017).

Cancer cells resist apoptosis by regulating anti-apoptotic and pro-apoptotic protein levels, for example, B-cell lymphoma 2 (BCL2), caspases, and Bax and Bak proteins, as part of apoptosis regulation (Dandoti, 2021). Tumor cells invade surrounding tissues and metastasize to distant sites through morphological changes with epithelial-mesenchymal transition (EMT) and detach from neighboring cells and the extracellular matrix (ECM) (Hanahan & Weinberg, 2011). This EMT process occurs through the activation of specific transcription factors (e.g., Snail, Slug, Twist, Zeb1, and Zeb2) and loss of proteins, including E-cadherin, and an increase in mesenchymal markers such as N-cadherin (Lamouille *et al.*, 2014).

1.2. Breast Cancer

Breast cancer is the most common cancer type affecting women across the world. Its incidence continues to increase because of higher reproductive and hormonal risk factors such as later age at first birth, fewer number of children, menopausal hormone therapy, and lifestyle risk factors, including alcohol intake and physical inactivity (Sung *et al.*, 2021). Breast cancer is highly heterogeneous regarding the variety of proteomic, genomic, epigenetic, and transcriptomic features, leading to alterations in apoptosis, proliferation, invasion, and treatment response (L. Guo *et al.*, 2023; Saleem *et al.*, 2020). This heterogeneity leads to different results from patient to patient in gene expression levels when it has context-dependent characteristics.

Based on immunohistochemistry (IHC), that used protein-level staining, breast cancer is classified into four clinical groups: Luminal A-like (ER-positive, PR-positive, HER2-negative, low Ki-67), Luminal B-like (ER-positive with either high Ki-67 or HER2 positivity), HER2-enriched (HER2-positive, ER/PR-negative), and triple-negative breast cancer (TNBC; ER-negative, PR-negative, HER2-negative) (Tang & Tse, 2016). According to Prediction Analysis of Microarray 50 (PAM50), which is based on gene expression profiles derived from microarrays, RNASeq, or qRT-PCR (Dank *et al.*, 2023), patient tumor samples are classified differently from the IHC method, by excluding TNBC type but including Basal-like type, and defines HER2-positive breast cancer as HER-enriched, and determines the molecular subtypes by considering biological properties and prognoses of tumor samples. According to PAM50, HER2-enriched and Basal-like types are aggressive, while the tumor samples with the best prognosis are Luminal A (Kensler *et al.*, 2018). Breast cancer patients can also be classified by single gene markers, ER and HER2 status, and are considered in treatment decisions. For instance, ER-positive tumors can respond to endocrine therapy, ER-negative tumors do not, and HER2-positive tumors can respond to targeted anti-HER2 therapy (Rakha *et al.*, 2022).

In breast cancer, several canonical signaling pathways are frequently altered, such as WNT/ β -catenin, TGF- β /SMAD, NOTCH, Hedgehog, IL-6/STAT3, and receptor tyrosine kinase pathways such as HER2(ErbB2), EGFR, and VEGF signaling (Figure 1). The alterations in these pathways promote proliferation, EMT, metastasis, and resistance to treatment.

Basal-like tumors overlap with triple-negative cancer, but they are not the same classification and differ from each other due to different markers and features (Badve *et al.*, 2010). Among the major biological processes and signaling pathways (Figure 1), basal-like tumors, which are characterized as an aggressive form of breast tumor, involve angiogenesis, dysregulated cell cycle, proliferation, survival processes, and invasion-related mechanisms. Genes associated with these pathways are generally overexpressed, TP53 mutations are more frequent, and PTEN expression levels are reduced (Bertucci *et al.*, 2011). Most of the basal-like breast cancers have high histological grades, increased proliferation and invasion, frequent apoptosis of cells, and poor prognosis in patients with shorter metastasis-free survival (MFS) and overall survival (OS) (Bertucci *et al.*, 2011). As another example of aggressive breast cancer, in the TNBC type, the PI3K/Akt/mTOR, as a member of key signaling pathways (Figure 1), is reported to promote chemoresistance (Hassan & Aubel, 2025).

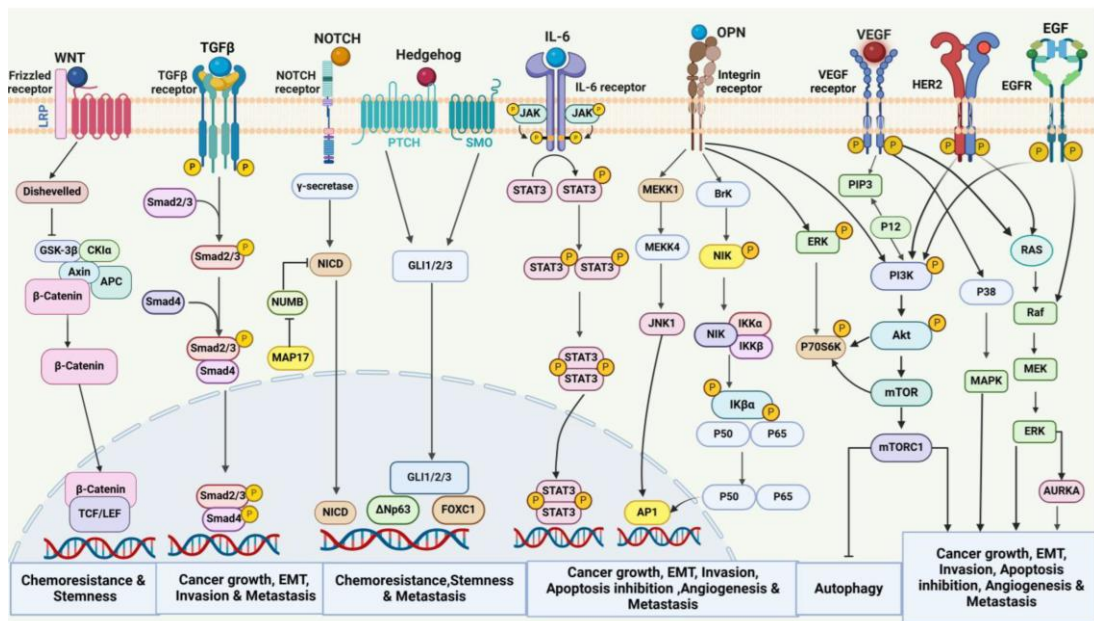


Figure 1. Biological processes and associated signaling pathways play key roles in breast cancer.

The primary biological processes resulting from breast cancer include chemoresistance, stemness, cancer growth, EMT, invasion, metastasis, apoptosis inhibition, angiogenesis, and autophagy. The signaling pathways that regulate these processes include Wnt, TGF- β , Notch, Hedgehog, JAK/STAT, NF- κ B, PI3K/AKT/mTOR, and MAPK/ERK. The figure is adapted from Panda *et al.* (2025), licensed with CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

1.3. MicroRNAs and Post-Transcriptional Regulation

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs with around 19-25 nucleotides long and regulate gene expression. These small RNAs have direct control over the silence of genes at the post-transcriptional level together with RNA-binding proteins (RBPs), and have transcriptional, through transcription factors, and epigenetic regulatory roles, with epigenetic modifiers (J. Liu *et al.*, 2022). The dysregulation of miRNA-mRNA interactions plays a role in the initiation and progression of many diseases, but the most relevant one is reported as cancer (Ankasha *et al.*, 2018). In breast cancer, miRNAs have been extensively studied and indicated to act on distinct expression patterns, especially in subtypes of breast tumors (Muñoz *et al.*, 2023).

1.3.1. MicroRNA Biogenesis

The biogenesis of miRNAs starts with transcribing the primary miRNA (pri-miRNA) in the nucleus. It goes through multiple steps, eventually forming the mature miRNA in the cytoplasm and functioning as a regulatory molecule (Figure 2). The pri-miRNA is transcribed by RNA polymerase II/III from DNA as a hairpin structure (Georgakilas *et al.*, 2014). This structure is then cleaved by the ribonuclease (RNase) III enzyme Drosha and its cofactor RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8), forming the microprocessor complex together. This complex cleaves the pri-miRNA into a smaller hairpin-shaped pre-miRNA (Gregory *et al.*, 2004). In this process, the microprocessor complex recognizes the pri-miRNA from its m⁶A methylated GGAC motifs and other short sequence motifs with DGCR8 and cleaves a 2-nucleotide 3' overhang on pre-miRNA with Drosha, which is required to be recognized by exportin-5 (Alarcón *et al.*, 2015; Choudhury & Li, 2012).

The pre-miRNA is exported to the cytoplasm from the nucleus with the exportin-5 protein only in the presence of Ran-GTP (Yi *et al.*, 2003). In the cytoplasm, another RNase III enzyme, Dicer, cleaves the terminal loop of pre-miRNA with the help of its cofactor, TRBP (Vergani-Junior *et al.*, 2021), resulting in the mature miRNA in a double-stranded RNA (dsRNA) form. In the resulting dsRNA, the strand derived from the 5' end of the pre-miRNA, the “5p” strand, and the strand originating from the 3' end, “3p” strand (O'Brien *et al.*, 2018).

The mature miRNA in its double-stranded form is loaded into the Argonaute (AGO) subfamily proteins (Ago1-4 in human), which are the core proteins of the RISC and require ATP to load small-RNA duplexes (Yoda *et al.*, 2009). The AGO proteins then unwind the miRNA duplex, and only one strand (guide strand) remains, while the other (passenger strand) is degraded. The selection of which strand, 5p or 3p arm, will be processed is based on the thermodynamic characteristics of the miRNA 5'end, and the strand with the lower 5'end stability is retained in the AGO (Khvorova *et al.*, 2003). AGO2 cleaves the passenger strand only if it has a complete or near-complete matching base pair. On the other hand, AGO2 does not slice them, and they are unwound from the guide strand and degraded by the general RNA degradation machinery, such as exonucleases (O'Brien *et al.*, 2018). This miRNA biogenesis is accepted as the canonical pathway, while there are also non-canonical mechanisms independent of Drosha/DGCR8 or Dicer.

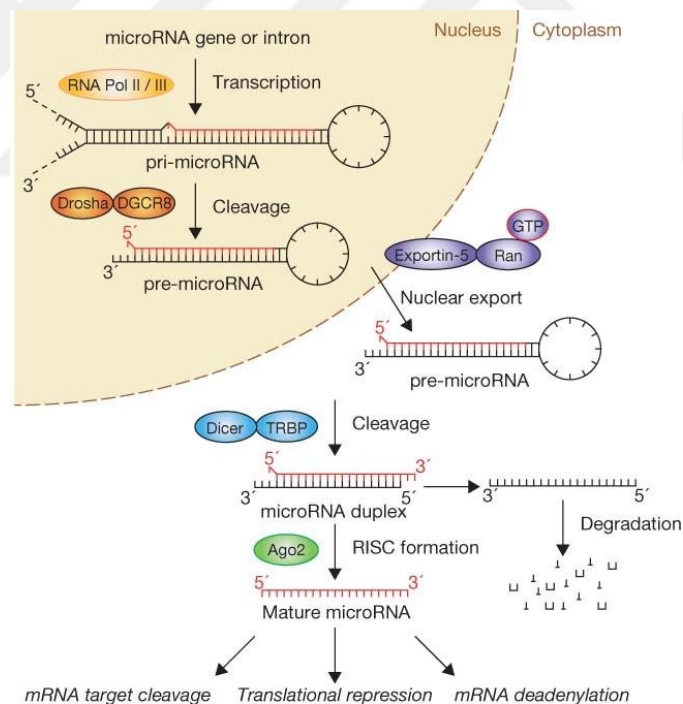


Figure 2. Canonical miRNA biogenesis process.

RNA polymerase II/III transcribes pri-miRNAs, followed by cleavage with Drosha-DGCR8 complex in the nucleus, forming pre-miRNA, and continues with nuclear export to the cytoplasm. Dicer cleaves the molecule to form a miRNA duplex, and AGO2 forms the mature miRNA as a protein component of the RISC. The other miRNA strand is degraded in the duplex form, and the mature miRNA is processed further. The figure is taken with permission from Winter *et al.* (2009), *Nature Cell Biology*, Springer Nature.

1.3.2. MicroRNA Mechanism of Action

MiRNAs regulate gene expression primarily post-transcriptionally by translational repression and target mRNA degradation. The recognition of mRNA occurs mainly with the 6-8 nucleotide seed region at the 5' end of the miRNA, and miRNA binds to the short complementary sequences known as miRNA response elements (MREs) (O'Brien *et al.*, 2018). The miRNAs canonically bind to the 3' UTR of their target mRNAs, while it is known that miRNAs can also bind to mRNA regions through their 5' UTR or coding sequence (CDS), and silence the target gene (O'Brien *et al.*, 2018). Additionally, miRNAs can bind to the promoter region of mRNA and induce transcription and regulate gene expression at the transcriptional level (Xu *et al.*, 2014; Lytle *et al.*, 2007; Hausser *et al.*, 2013).

In the silencing process, the guide strand is loaded into the RISC complex through AGO proteins, and the passenger strand is inactivated by degradation, forming the miRNA-induced silencing complex (miRISC). This complex binds to the MRE in target mRNAs through base-pairing, which is complementary (not entirely complementary in animal cells) to the seed region of the miRNA sequence or sometimes to more of the seed region sequence, so that miRISC can bind (O'Brien *et al.*, 2018).

Target degradation occurs with complete or near complete base-pairing in plants, leading to the direct exonucleolytic cleavage of the target and direct degradation, a process that is mainly seen in plants and is similar to the action mechanism of siRNAs (Yekta *et al.*, 2004). This cleavage can interfere with the binding of AGO proteins with the 3' end of the miRNA and can lead to the miRNA strand degradation (Ameres *et al.*, 2010). Target degradation happens with partial complementarity in animals, mainly with deadenylation and decapping mechanisms. The miRISC recruits the GW182 family of proteins and acts as a scaffolding protein for the proteins in the mRNA degradation mechanism. The PAN2-PAN3 (initial) and CCR4-NOT, the poly(A)-deadenylase complexes, perform deadenylation. Decapping protein 2 (DCP2) and related proteins are also recruited to decapping the mRNA, and eventually, exoribonuclease 1 (XRN) degrades the mRNA in the 5'–3' direction. In translational repression, miRISC prevents the association of the eIF4F, a component of the translational initiation complex, to the 5' cap of mRNA and prevents ribosome assembly (O'Brien *et al.*, 2018).

1.3.3. Role of microRNAs in Cancer

MiRNAs can function as oncomirs, their increased expression promotes tumorigenesis, or as anti-oncomirs, their decreased expression induces cancer cell growth, and aberrant expression of these miRNAs leads to carcinogenesis (Ankasha *et al.*, 2018). The diverse roles of miRNAs are studied in cancer and shown to modulate key cancer mechanisms, including cellular proliferation, differentiation, and apoptosis (Forterre *et al.*, 2020).

The miR-15 and miR-16 were first reported to be associated with cancer in chronic lymphocytic leukemia tumors (Calin *et al.*, 2002). Further studies revealed that miRNAs have relevance to cancer mechanisms as follows. The let-7 and miR-21 miRNA families influence cancer cells to respond to proliferative signaling, through removing RAS inhibition or suppressing PTEN, respectively, and leading to excessive activation of PI3K/AKT signaling (Choudhury & Li, 2012). On the other hand, the miR-17-92 cluster and miR-195 lead to insensitivity to signals, such as TGF- β -dependent signals, that usually stop the growth of cells by stopping cell division or triggering cell cycle arrest (Forterre *et al.*, 2020). The miRNAs in the miR-17-92 cluster are overexpressed in many cancer types, and target inhibitors of the cell cycle, including p21 and p57, while miR-195 miRNAs are downregulated in cancer and target cyclins, leading to cell proliferation (Hui *et al.*, 2013; Mestdagh *et al.*, 2010; Sekiya *et al.*, 2010).

The primary miRNAs that play a role in cancer by regulating apoptosis escape are miR-34a, miR-185, and miR-15/miR-16 by targeting pro-apoptotic and anti-apoptotic genes, such as BCL2 (Forterre *et al.*, 2020). In invasion and metastasis, processes such as disruption of cell-cell adhesion, degradation of extracellular matrix, and EMT are also targeted by miRNAs, for example, miR-10b through HOXD10 and RHOC, miR-200 family miRNAs on EMT by targeting ZEB1 and ZEB2, miR-21 on invasion through PTEN and RECK (Guan *et al.*, 2018; Y. Wang *et al.*, 2016; L. Xu *et al.*, 2014). In conclusion, miRNAs are critical in multiple cancer hallmark processes by their ability to function as oncogenes or tumor suppressors.

1.3.4. MicroRNAs in Breast Cancer

MiRNAs have been extensively studied in breast cancer due to their aberrant expression in the tissue and blood of patients with breast cancer. MiRNA expression profiles differ in normal, precancer, and cancer (Jelski *et al.*, 2025). In breast cancer, miRNAs show a particular pattern of expression according to different subtypes because of each patient's tumor heterogeneity and characteristics (Blenkiron *et al.*, 2007).

Oncomirs, identified in breast cancer cells, have been found to induce breast cancer development by targeting the function of tumor suppressor genes and regulatory pathways (Muñoz *et al.*, 2023). Validated examples of oncomirs include miR-9, which directly targets the mRNA of the E-cadherin protein and induces metastasis and angiogenesis (Ma *et al.*, 2010) and is upregulated in extracellular vesicles of breast cancer cell lines (Berti *et al.*, 2023). The miR-10b promotes proliferation, migration, and invasion by targeting TBX5, PTEN, and HOXD10 (Kim *et al.*, 2016).

The miR-21 regulates BCL2, leading to tumor growth (Si *et al.*, 2006). The miR-155 targets RhoA and plays a role in TGF- β -induced EMT, cell migration, and invasion in breast cancer (Kong *et al.*, 2008). Its expression is found to be higher in the peripheral blood of breast cancer patients compared to standard controls (Al-Sharabass *et al.*, 2025). The miR-135b promotes cell proliferation and interrupts cell cycle progression through the LATS2, CDK2, and p-YAP targets (Hua *et al.*, 2016). Notably, miR-135b is found to be upregulated by circ_0000977 (lncRNA) in TNBC, downregulates GATA3, APC, LZTSL1, and SMAD5, and may control proliferation, migration through EMT, and invasion (Bertoli *et al.*, 2021; Darbeheshti *et al.*, 2023).

The miR-17-92 cluster, acting both as an oncogene and a tumor suppressor in cancer, miRNAs in this cluster are found to be expressed at higher levels in TNBC, and associated with poor patient outcomes, while being reduced in estrogen receptor-positive breast cancer with better outcomes (M. M. Hossain *et al.*, 2020). In this study, miR-17-92 is also shown to inhibit cell growth and invasion of TNBC cells, while having a role in chemoresistance in the HER2-positive breast cancer cell line.

The miR-301a is another oncomir that plays a role in EMT and autophagy, which is found to enhance breast tumor invasion and is upregulated in TNBC tissues (H. Liu & Wang, 2021; Shad *et al.*, 2023).

1.4. ERBIN

1.4.1. Structure and function of Erbin

Erbin is identified as a protein that interacts with ErbB2 (HER2) tyrosine kinase in the basolateral membrane of epithelial cells (Borg *et al.*, 2000; Huang *et al.*, 2001; Jaulin-Bastard *et al.*, 2001). Erbin protein has a PDZ domain in its C terminus and 16 Leucine-Rich-Repeats (LRR) in its N-terminal (Borg *et al.*, 2000); therefore, Erbin is the LAP (leucine-rich repeat and PDZ) protein superfamily (Santoni *et al.*, 2002). The fundamental role of PDZ domain proteins is to scaffold protein complexes at specific membrane sites, including receptors and channels, especially in epithelial cells (Borg *et al.*, 2000). Particularly, the recruitment of PDZ domain proteins provides a functional specificity to RTKs because these proteins have functional diversity, can only bind to specific receptors, scaffold different regulators, and localize to subcellular compartments so that they anchor and gather receptors and their modulators (Nourry *et al.*, 2003).

As a PDZ domain protein, Erbin can ensure that ErbB2 signaling happens in the right place and the correct order. Erbin localizes and aids signaling via its PDZ domain, and it binds the unphosphorylated C-terminus of ErbB2, which allows ErbB2 to be restricted to the basolateral membrane of epithelial cells before ligand-induced activation or dimerization (Borg *et al.*, 2000). The LRR region of Erbin mediates its localization to the basolateral domain instead of the cytosolic region or apical membrane of epithelial cells (Legouis *et al.*, 2003). With LRR, palmitoylation at cysteines 14 and 16 aids Erbin plasma membrane targeting (Izawa *et al.*, 2008).

In desmosomes and adherens junctions, the PDZ domain of Erbin directly interacts with the carboxyl-terminus of p0071 (plakophilin-4), an armadillo repeat protein involved in cell–cell adhesion. This interaction occurs with higher affinity than Erbin and ErbB2 (Izawa *et al.*, 2002; Jaulin-Bastard *et al.*, 2002). In addition, Izawa *et al.* (2002) showed that overexpression of Rho family GTPases (Cdc42, Rac1, or RhoA), key proteins that regulate cadherin-mediated cell-cell adhesion, resulted in Erbin accumulation at cell-cell contact sites in epithelial cells, suggesting Rho family proteins may regulate the localization of Erbin within the cell.

Erbin also interacts with the cytoplasmic domain of the $\beta 4$ -integrin subunit of $\alpha 6\beta 4$ -Integrin and bullous pemphigoid antigen 1 (eBPAG1), which are components of hemidesmosomes, and proteins that link the cell cytoskeleton to the extracellular matrix (Borradori & Sonnenberg, 1996) and connect Erbin to the cytoskeleton via intermediate filaments (Favre *et al.*, 2001). Guo *et al.* (2006) demonstrated that in ErbB2-mediated mammary tumorigenesis, the $\beta 4$ -integrin subunit, instead of being a part of hemidesmosomes as in normal mammary ducts, functions as a signaling molecule. Activation of ErbB2 leads to phosphorylation of the cytoplasmic domain of $\beta 4$ -integrin via Src family kinases (SFKs), resulting in amplification of ErbB2 signaling. As a result, c-Jun and STAT3, downstream effectors of the ErbB2 pathway, are activated, contributing to JNK-mediated cell proliferation, enhanced survival despite the loss of adhesion and polarity, and STAT3-driven invasion.

In Schwann cells, Erbin is found to form a complex with the merlin-binding partner, NHERF1 (EBP50), and Merlin proteins, and its depletion enhances activation of the MAPK pathway through this complex (Rangwala *et al.*, 2005). NHERF1 binds with merlin (moesin-ezrin-radixin-like protein), a protein that localizes at the cell membrane, interacts directly with the actin cytoskeleton, and indirectly with other cytoskeleton proteins (Abeyesundara *et al.*, 2014; McClatchey & Giovannini, 2005). Rangwala *et al.* (2005) demonstrated that silencing Erbin in primary cultures of Schwann cells disrupts the cell-cell contacts and alters the morphology of cells, and found the dissociation of β -catenin from E-cadherin, but their expression is not affected. In the same study, for the Erbin-silenced cells, an increase in the levels of Ki-67 and phosphorylated ERK is recorded, which supports that Erbin prevents proliferation and preserves cell-cell contact in Schwann cells.

ERBIN has been demonstrated to function as a tumor suppressor or an oncogene, depending on the cancer type and cellular context. In colorectal cancer (CRC), studies suggest the tumor suppressor role of ERBIN, as its expression is downregulated. Its low expression leads to disruption of epithelial polarity, enhancing MAPK and Akt signaling and inducing EMT (Figure 3), which eventually results in elevated cell migration and invasion (P. Dai *et al.*, 2005; Y. Z. Huang *et al.*, 2003; Stevens *et al.*, 2018). In cervical cancer, decreased expression level of ERBIN induced cell proliferation and tumor growth by promoting progression from G1 to S phase in the cell cycle by altering p27 and p21 expression (Hu *et al.*, 2013; H. Huang *et al.*, 2015).

Additionally, in a colorectal cancer lung metastasis model, low ERBIN expression was found to induce mitochondrial oxidative phosphorylation in B cells and resulted in metastasis of colorectal cancer to the lung (Qiu *et al.*, 2024). On the other hand, high ERBIN expression destabilizes the ER α protein and inhibits its signaling, inducing tumorigenesis and is associated with tamoxifen resistance in hepatocellular carcinoma (Wu *et al.*, 2017).

In breast cancer, ERBIN is reported to function both as an oncogene and a tumor suppressor. In HER2-positive breast cancer, the Erbin protein stabilizes ErbB2 at the membrane, and low expression of the ERBIN gene decreases ErbB2 signaling, suggesting ERBIN plays a role as an oncogene by increasing the ErbB2 signaling-dependent cellular proliferation (Jeong *et al.*, 2025; Tao *et al.*, 2014). On the other hand, D. Liu *et al.* (2013) showed that ERBIN expression is decreased in breast cancer tissues, resulting in increased heregulin-induced AKT activation, and inducing cell migration, invasive features of cell lines, and trastuzumab resistance, suggesting its tumor-suppressor role.

1.4.2. Erbin in Signaling Pathways

In the MAPK pathway, Erbin, through the LRR domain, is found to inhibit Ras-Raf interaction only when Ras is activated by growth factors, resulting in decreased phosphorylation and activation of ERK (Huang *et al.*, 2003). Erbin interacts with Sur-8 (Shoc2), a scaffold protein that facilitates Ras-mediated activation of Raf. By competing with Raf or Ras for Sur-8 binding, Erbin prevents the formation of Sur-8-Ras-Raf-PP1 complex, thereby inhibiting the ERK activation and altering the expression of downstream targets such as acetylcholine receptor (AChR) subunit (Dai *et al.*, 2005; Young *et al.*, 2013).

As a representation of signaling pathways affected by Erbin is demonstrated in Figure 3, including its competitor molecule Shoc2, another LRR scaffold protein that competes for binding with the Ras/Raf complex (Jang *et al.*, 2020).

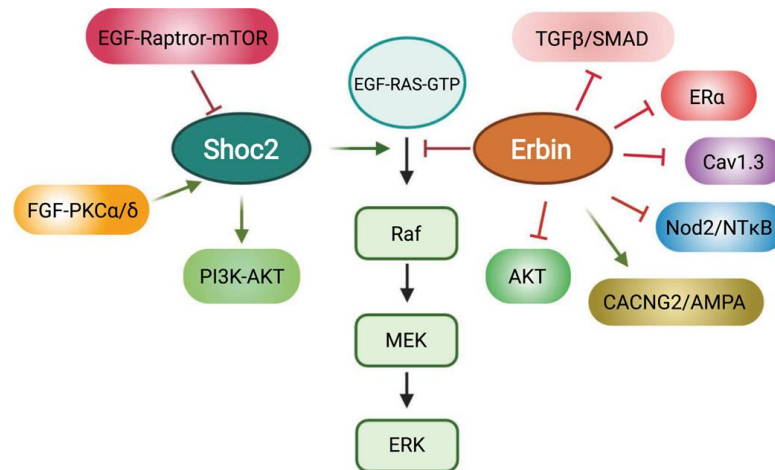


Figure 3. Signaling pathways regulated by Erbin and Shoc2.

Erbin is shown to negatively regulate TGFβ/SMAD, ERα, Cav1.3, and Nod2/NFκB, AKT pathways, and positively regulate CACNG2/AMPA signaling. ERBIN also negatively regulates EGFR-RAS-GTP in coordination with Shoc2. Copyright permission is taken from Jang *et al.* (2020), *FEBS Journal*, John Wiley & Sons.

F. Dai *et al.* (2007) and Warner *et al.* (2003) demonstrated that ERBIN inhibits TGF-β signaling by interacting with the phosphorylated SMAD2/3 complex through the MH2 domain of SMAD3 and blocking the nuclear translocation of this complex. TGF-β signaling relies on translocating the SMAD2/3-SMAD4 complex from the cytosol to the nucleus. When the TGF-β type I receptor is phosphorylated, the SMAD2/3 forms a complex with SMAD4, and this complex translocates into the nucleus to regulate the transcription of TGF-β signaling target genes (Massagué, 2012). In addition, Sflomos *et al.* (2011) showed that ERBIN binds with SARA protein and stated that the ERBIN binding with SARA may lead to the occupation of its SMAD-binding surface or ERBIN may directly bind to SMAD2/3. Therefore, the overexpression of ERBIN may prevent SMAD2/3 and SARA binding and negatively affect the phosphorylation of SMAD2/3 or bind with SMAD3 and sequester it in the cytosol, and block SMAD4 binding (Massagué, 2012; Sflomos *et al.*, 2011). In a recent paper, Erbin's role is explicitly shown to reduce EMT by coordinating TGF-β (with TGF-β type I receptor kinase) and EGFR signaling in breast and lung cancer cell lines (C. Li *et al.*, 2025). They also suggested that the function of Erbin in the TGF-β pathway through SMAD proteins also affects the TGF-β-related MAPK pathway through ERK phosphorylation.

1.4.3. Regulation of ERBIN Expression

D. Liu et al. (2012) showed that c-Myb regulates ERBIN expression by binding its core promoter region and has a positively correlated expression relation. They found that this interaction increases during the G2/M phase. ERBIN regulation is also studied in cardiac hypertrophy, and it is reported that microphthalmia-associated transcription factor (MITF), together with Four and a half LIM domains protein 2 (FHL2), targets ERBIN by binding its promoter and decreasing its expression (Rachmin *et al.*, 2015). Moreover, the study focusing on ERBIN and Nod2 interaction in monocyte cells found that LPS, TNF- α , or MDP stimulation increased ERBIN mRNA levels (McDonald *et al.*, 2005).

1.4.4. MicroRNA-Mediated Regulation of ERBIN

Across diseases, several microRNAs are reported to target ERBIN directly. In lupus nephritis, miR-130b-3p binds the ERBIN 3' UTR and suppresses its expression, thereby promoting EMT in renal tubular cells. In the same model, TGF- β 1 is validated to up-regulate ERBIN at the mRNA and protein levels (W. Wang et al., 2015). In human hepatocellular carcinoma (HCC), ERBIN mRNA is found to be directly targeted by miR-23c from its 3'-UTR, and it is involved in miR-23c inhibiting cellular proliferation and enhancing apoptosis (L. Zhang *et al.*, 2018). Additionally, the long non-coding RNA KTN1 antisense RNA 1 (KTN1-AS1) is found to regulate ERBIN through miR-23c by sponging and negatively regulating it, preventing cellular tumor growth in HCC (L. Zhang, Wang, Wang, Chen, *et al.*, 2018). In acute myeloid leukemia (AML), miR-183-5p activates MAPK and PI3K/Akt signaling by negatively regulating ERBIN expression and eventually increasing cell proliferation in leukemia cells (Zheng et al., 2019). These studies demonstrate that microRNAs regulate ERBIN in a context-dependent manner that shapes EMT and oncogenic signaling.

Overall, studies showed that the Erbin protein acts as an anchor for the ERBB2 receptor at the basolateral membrane of epithelial cells and functions as a scaffold in various ERBB2-dependent and -independent signaling pathways. Depending on the type of cancer and biological context, the ERBIN gene is a tumor suppressor in colorectal cancer, HER2-positive breast cancer via inhibition of Akt signaling, and cervical cancers with Akt-Skp2 activation, and is an oncogene in HER2-positive breast cancer through HER2 stabilization and MAPK signaling, and in colorectal

cancer metastasis. This context-dependent role of ERBIN expression indicates its complex biological functions, especially in breast cancer, and shows the importance of understanding its expression regulation in cancer. Therefore, even though downstream mechanisms of ERBIN have been investigated, its upstream expression regulation, particularly by miRNAs in breast cancer, remains poorly understood, limiting our understanding of the aberrant expression patterns of ERBIN in cancer.

Accordingly, this study hypothesizes that ERBIN shows distinct expression patterns across molecular breast cancer subtypes, specifically downregulation in aggressive subtypes, and specific miRNAs contribute to this downregulation, and the loss of ERBIN expression displays regulatory feedback on specific miRNAs.

1.5. Aim of the Study

This study aimed to identify the specific miRNAs that potentially regulate the ERBIN expression in breast cancer subtypes and explore the regulatory feedback network between ERBIN and its potential miRNA regulators by altering ERBIN expression in breast cancer cell lines.

ERBIN acts as an oncogene in early HER2-positive tumors and a tumor suppressor in other cancer types. The first aim is to characterize ERBIN expression in molecular subtypes of breast cancer with bioinformatics analyses of the publicly available datasets and their association with patient survival. The second aim of this study is to identify miRNAs that bind to specific regions of ERBIN mRNA using well-known target prediction tools and have expression correlation with ERBIN in a breast cancer GEO dataset. Additionally, different breast cancer patient datasets are used to explore miRNA expressions in aggressive types of breast cancer and their potential relation with ERBIN expression downregulation. Finally, the study aimed to explore the regulatory feedback between ERBIN and its potential miRNA regulators by analyzing small RNA-seq data generated following ERBIN silencing in the MDA-MB-231 cell line.

This study is expected to provide insight into how miRNAs would modulate ERBIN expression in breast cancer to understand miRNA regulation in breast tumors better and help identify potential biomarkers and targets for the prognosis and treatment of patients, especially in aggressive breast cancer.

CHAPTER 2: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Computational Analysis

2.1.1.1. Publicly Available Datasets

Publicly available datasets are used to analyze ERBIN and miRNA expression patterns in breast cancer. These datasets are obtained from the Gene Expression Omnibus (GEO) database (GSE19783 (Enerly *et al.*, 2011), GSE1456 (Pawitan *et al.*, 2005), GSE45827 (Gruosso *et al.*, 2016), GSE40267 (De Rinaldis *et al.*, 2013), GSE37963 (Ye *et al.*, 2023), GSE25066 (Hatzis, 2011), GSE20685 (Kao *et al.*, 2011), GSE42568 (Clarke *et al.*, 2013)), E-MTAB-365 (Guedj *et al.*, 2011), The Cancer Genome Atlas (TCGA) (Koboldt *et al.*, 2012), and the METABRIC (Curtis *et al.*, 2012) study. Dataset summaries are provided in Table 1.

Table 1. Publicly Available Datasets used in gene and miRNA expression analyses and their summary.

Dataset	Platform	Summary
GSE19783	GPL6480 - Agilent-014850 Whole Human Genome Microarray GPL8227 - Agilent-019118 Human miRNA Microarray	The data includes human primary breast tumor samples with 489 miRNA and genome-wide mRNA profiles and clinical information.
GSE1456	GPL96 - Affymetrix Human Genome U133A Array	The data includes the mRNA expression profile of Swedish breast cancer patients receiving surgery and their clinical information.
GSE45827	GPL570 - Affymetrix Human Genome U133 Plus 2.0 Array	The data show mRNA expressions from primary breast cancer samples, normal tissues, and cell lines with subtype information.
GSE40267	GPL10850 - Agilent - 021827 Human miRNA Microarray (V3)	The data provides a non-coding RNA profile from 181 breast tumor samples from 173 patients with their clinical information.
GSE37963	GPL14126 - ORB Sanger 15 miRNA Microarray	The data presents a non-coding RNA profile from 52 breast cancer cases and from 4 normal breast tissues with clinical information.
GSE25066	GPL96 - Affymetrix Human Genome U133A Array	The data includes more than 500 breast cancer samples, including clinical information.
GSE20685	GPL570 - Affymetrix Human Genome U133 Plus 2.0 Array	The data has the gene expression profile of 327 breast cancer samples with molecular and clinical features.

Dataset	Platform	Summary
GSE42568	GPL570 - Affymetrix Human Genome U133 Plus 2.0 Array	The data is composed of 104 breast cancer samples and clinical information.
E-MTAB-365	A-AFFY-44 - Affymetrix GeneChip Human Genome U133 Plus 2.0	The data includes 537 breast cancer patients with molecular subtypes and clinical information.
METABRIC	Illumina HT-12 v3 platform - cDNA Microarray profiling	The data includes around 2000 breast tumors with long-term survival data and clinical information.
TCGA-BRCA	Illumina HiSeq (2000). RNA-Seq	The data give mRNA gene expression, copy number alteration, and clinical data information for over 1000 breast tumors.

2.1.1.2. Transcriptomic Dataset

To explore downstream regulatory effects of ERBIN expression downregulation in breast cancer, a small RNA-seq dataset was generated from the total RNA of ERBIN siRNA- and non-targeting RNA-transfected MDA-MB-231 cells. The sequencing was performed on the Illumina NovaSeq 6000 platform, and the dataset information is given in Table 2.

Table 2. The dataset built and used in downstream analyses of ERBIN silencing in MDA-MB-231 cells

Dataset Type	Platform	Read	Summary
Small RNA-seq	Illumina NovaSeq 6000	10 million reads per sample, 150 bp single-end read	miRNA expression profiles of MDA-MB-231 cells with three replicates of siERBIN and control treatment

2.1.1.3. Reference Genome and Annotation Resources

In small RNA-seq analysis, as a reference genome assembly, “Homo sapiens Hg38” is also used. The mature and precursor miRNA sequences were retrieved from the miRbase database (version 22) to quantify miRNA expression.

The reference genome assembly “*Homo sapiens* GRCh38.p13” used in alignment is downloaded from the Ensembl database in FASTA format. The gene annotation dataset “GENCODE Release 40” is downloaded from GENCODE in GTF format. In addition, the miRNA reference sequences (v22) are downloaded from the miRBase database in a FASTA file format.

2.1.1.4. Software and Tools

The statistical analyses of gene and miRNA expression, visualization, and data integration are performed using R version 4.3.2 and RStudio. The R packages used in these analyses are listed in Table 3. For the miRNA target prediction, the online tools in Table 4 are used, and each tool was accessed through its web platform, and the default filtering options of each tool are used. For the ERBIN gene and candidate miRNA Kaplan-Meier survival analyses, the KMplotter online tool (<https://kmplot.com/analysis/>) is used (Györffy, 2024a; Györffy, 2024b), which provides processed and analyzed results from large-scale breast cancer datasets such as METABRIC and TCGA. The TAM 2.0 (Tool for Annotations of human MicroRNAs, <http://www.lirned.com/tam2/>) is utilized to classify candidate miRNAs into families. This web-based tool can group human miRNAs based on similar seed sequences, biological functions, and disease associations (Li *et al.*, 2018; Lu *et al.*, 2010).

Table 3. R packages used in gene and miRNA expression analyses

Package	Version	Source
GEOquery	2.70.0	(Davis & Meltzer, 2007)
dplyr	1.1.4	(Wickham <i>et al.</i> , 2023)
ggplot2	3.5.2	(Wickham, 2016a)
ggsignif	0.6.4	(Ahlmann-Eltze & Patil, 2021)
ggpubr	0.6.0	(Kassambara, 2023)
dunn.test	1.3.6	(Dinno, 2024)
stringr	1.5.1	(Wickham, 2023)
pheatmap	1.0.13	(Kolde, 2025)
readxl	1.4.5	(Wickham & Bryan, 2025)
VennDiagram	1.7.3	(Chen, 2022)
gridExtra	2.3	(Auguie, 2017)
miRBaseConverter	1.26.0	(Xu <i>et al.</i> , 2018)
reshape2	1.0.11	(Wickham, 2007)
forcats	1.4.4	(Wickham, 2016b)
TCGAbiolinks	2.30.4	(Mounir <i>et al.</i> , 2019)
SummarizedExperiment	1.32.0	(Morgan <i>et al.</i> , 2023)
UCSCXenaTools	1.4.8	(Wang <i>et al.</i> , 2019)
UpSetR	1.4.0	(Conway, Lex, & Gehlenborg, 2017)
biomaRT	2.56.0	(Durinck <i>et al.</i> , 2005)
readr	2.1.5	(Wickham <i>et al.</i> , 2024)

Package	Version	Source
tibble	3.2.1	(Müller & Wickham, 2023)
dorothea	1.20.0	(Garcia-Alonso <i>et al.</i> , 2019)
OmnipathR	3.16.0	(Türei <i>et al.</i> , 2021)
multiMiR	1.31.0	(Ru <i>et al.</i> , 2014)
igraph	2.1.4	(Csárdi <i>et al.</i> , 2006)
ggraph	2.2.1	(Pedersen, 2017a)
tidygraph	1.3.1	(Pedersen, 2017b)
tidyverse	2.0.0	(Wickham, 2016c)
ggrepel	0.9.6	(Slowikowski, 2016)

Table 4. miRNA-mRNA prediction tools used to identify miRNAs targeting ERBIN mRNA.

Tool	Access/Version	Summary
TargetScan	Web tool/v8.0	The prediction of miRNA targets is based on the presence of conserved sites in the 3'UTR and ORFs that match the seed region of each miRNA. It ranks target sites using the biochemical model that estimates target efficiency. The sequence alignment uses UCSC (Agarwal, Bell, Nam, & Bartel, 2015).
miRWalk	Web tool/v3.0	It predicts binding sites across the complete gene sequence, including 3'UTR, CDS, and 5'UTR regions. It used a machine learning approach based on random forest models trained on validated interactions from miRTarBase. The default filtering is based on thermodynamic parameters (Dweep & Gretz, 2015).
miRDB	Web tool	The predictions are made through MiRTarget, a bioinformatics tool that predicts miRNA-target interactions from high-throughput sequencing experiments and is based on machine learning methods (Chen & Wang, 2020).

Tool	Access/Version	Summary
RNA22	Web tool	The predictions are based on sequence patterns instead of evolutionary conservation. It identifies putative miRNA binding sites using a two-step algorithm, which detects sequence motifs in mRNA that can be a miRNA target and finds the miRNAs that match these motifs. It allows for non-canonical seed matches, G: U wobbles, and thermodynamic stability (Miranda <i>et al.</i> , 2006).
MicroT-CDS	Web tool	DIANA Lab develops the tool, and the prediction algorithm is trained on miRNA Recognition Elements (MREs) located in the 3'UTR and CDS regions. It integrated seed region binding, evolutionary conservation, and site accessibility (Paraskevopoulou <i>et al.</i> , 2013).

2.1.2. Laboratory Materials

The general laboratory materials comprise various chemicals, reagents, solutions, kits, and laboratory equipment utilized during the experiments. The catalog numbers and the company names are provided in Table 5. The solutions and the kits used in this thesis are given in Tables 6 and 7, respectively.

2.1.2.1. Chemicals and Reagents

Table 5. List of laboratory materials, catalog numbers, and company names.

Material Name	Catalog Number	Company
2-mercaptoethanol	A1108	PanReac AppliChem (Germany)
Agarose	BHE500	Prona (Spain)
Ammonium persulfate (APS)	A3678	Sigma Aldrich (USA)
30% Acrylamide/Bis-acrylamide Solution, 29:1	1610156	Bio-Rad Laboratories (USA)
Bovine Serum Albumin Fraction V (BSA)	10735078001	Roche (USA)
Bromophenol blue	B5525	Sigma Aldrich (USA)
Crystal Violet	V5265	Sigma Aldrich (USA)

Material Name	Catalog Number	Company
DEPC-treated water	M6082	Genaxxon Bioscience (Germany)
Ethidium Bromide	17898	Thermo Scientific (USA)
EDTA	A3562	Applichem (Germany)
ECL Prime System	RPN2232	Life Sciences (USA)
Gene Ruler DNA Ladder (1 kb)	SM0311	Thermo Scientific (USA)
Gene Ruler DNA Ladder (50 bp)	SM373	Thermo Scientific (USA)
Glycine	G8898	Sigma Aldrich (USA)
KH ₂ PO ₄	4243	Sigma Aldrich (USA)
NaCl	31434	Sigma Aldrich (USA)
Nitrocellulose Transfer Membrane	1212632	GVS Filter Technology (Italy)
Proteinase K	P2308	Sigma Aldrich (USA)
Proteinase inhibitor cocktail	87786	Sigma Aldrich (USA)
PageRuler Prestained Protein Ladder (250kDa)	26619	Thermo Scientific (USA)
Rnase A	R6513	Sigma Aldrich (USA)
SDS	71725	Sigma Aldrich (USA)
Taq DNA Polymerase	EP0402	Sigma Aldrich (USA)
TEMED	1.10732.0100	Merck (Germany)
Tris	T1503	Sigma Aldrich (USA)
Tween-20	39796	Serva (Germany)
4X Laemmli Sample Buffer	1610747	Bi-Rad Laboratories (USA)

2.1.2.1.1. Solutions

Table 6. Solutions and buffer recipes used in laboratory procedures.

Solutions	Recipe
RIPA buffer (1 ml)	1 µl 1 M Tris-HCl (pH=8.0), 20 µl EDTA, 300 µl 5M NaCl, 100 µl NP-40, 40 µl 25X protease inhibitor.
10X Running Buffer (1 L)	30 g Tris-Base, 144 g Glycine, 10 g SDS in dH ₂ O
10X Transfer Buffer (1 L)	30.3 g Tris-Base, 144.1 g Glycine, 20 % methanol in dH ₂ O.
10X TBS (1 L)	30 g Tris-base, 80 g NaCl, 2 g KCl in dH ₂ O (pH = 7.4).

Solutions	Recipe
TBS-T (0.2%) (500ml)	1ml Tween-20 in 50 ml 10X TBS in ddH ₂ O.
Blocking solution (10 ml) (3%)	0.3 mL of milk powder in 10 mL of 1X TBS-T.
Ponceau S staining solution	1g Ponceau S; 50 ml acetic acid in ddH ₂ O.
50x TAE (1 L)	242 g Tris-Base, 57.1 mL acetic acid, 100 mL 0.5 M EDTA.
Resolving Buffer (1 L)	1.5 M Tris-Base, 1L ddH ₂ O, pH adjusted to 8.8 with HCl.
Stacking Buffer (1 L)	0.5 M Tris-Base, 1L ddH ₂ O, pH is adjusted to 6.8 with HCl.

2.1.2.1.2. Kits

Table 7. The Kits used in the experiments.

Kit	Catalog Number	Company Name
Tripure	EP013	ELK Biotech (China)
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific (USA)
PowerUp SYBR Green Master Mix	A25742	Applied Biosystems (USA)
BCA Protein Assay Kit	23225	Thermo Scientific (USA)
miRCURY LNA RT Kit	339340	Qiagen
miRCURY LNA SYBR Green PCR Kit	339345	Qiagen
MiRNA PCR Assays	YP02119464, YP00204603, YP00204618, YP00204363, YP02119310	Qiagen
EZ-PCR Mycoplasma Detection Kit	20-700-20	Biological Industries Israel, Beit Haemek Ltd. (Israel)

2.1.2.1.3. Primers

Primers of RTq-PCR included in Table 8 are synthesized and purchased from Oligomer (Ankara, Türkiye). The primers in Table 8 are dissolved in nuclease-free water, diluted to 100 µM in their final concentration, and stored at -20 °C.

Table 8. Primers and their information are included in qRT-PCR for mRNA expression.

Primer	Sequence (5'-3')	Tm
ERBIN F	“CTAATCAGATTGAAGAGCTTCC”	57 °C
ERBIN R	“AACTCCTGTATTCCATTCTTGC”	
GAPDH F	“GGCTGAGAACGGGAAGCTTGTCAT”	64 °C
GAPDH R	“CAGCCTTCTCCATGGTGGTGAAGA”	

2.1.2.1.4. Antibodies

The antibodies used in the Western blot experiment are included in Table 9. The working concentrations are prepared according to the manufacturer's specifications.

Table 9. The details of the antibodies used in the Western blot experiment.

Antibody Name	Catalog Number	Company
Rabbit anti-ERBIN Antibody *	NA	NA
Mouse anti-Actin Antibody	A5441	Sigma-Aldrich
Anti-rabbit IgG-HRP	A6154	Sigma-Aldrich
Anti-mouse IgG-HRP	A0168	Sigma-Aldrich

* The ERBIN antibody was kindly provided by Dr. Jean-Paul Borg (INSERM, Marseille, France) and produced in his laboratory.

2.1.2.1.5. Equipment

Table 10. The equipment list used for the experiments in the laboratory.

Instrument Name	Company
Amersham Imager 600	Dharmacon (USA)
7500 Real-Time PCR System	Applied Biosystems (USA)
AutoFlow NU-8500 Water Jacket CO2 Incubator	NuAire (USA)
PCR Thermal cycles	Applied Biosystems (USA)
NanoDrop ONE	Thermo Scientific (USA)

Instrument Name	Company
Cell Culture Hood	NuAire (USA)
Universal 320 R Centrifuge	Hettich Lab Technology (Germany)
Centrifuge 5415R Refrigerated Microcentrifuge	Eppendorf SE (Germany)

2.1.2.2. Cell Culture Materials

2.1.2.2.1. Cell Culture Reagents

Table 11. Cell culture reagents, catalog numbers, and supplier information.

Name	Catalog Number	Company
Dulbecco's Modified Eagle's Medium (DMEM), High Glucose	MCL-059	Serana Europe (Germany)
Dulbecco's Phosphate-Buffered Saline (dPBS)	BDL-001	Serena Europe (Germany)
Fetal Bovine Serum (FBS)	BI04-001-1A	Biological Industries (Israel)
Trypsin/EDTA (0.25%)	325-043 EL	Multicell (Czech Republic)
Non-Essential Amino Acids	C3240-0100	VivaCell Biosystems (China)
Penicillin/Streptomycin (xmM)	A2213	Biochrom (Germany)
Dimethyl sulfoxide (DMSO)	A3672	AppliChem (Germany)
Opti-MEM I	11058-021	Gibco (USA)
Lipofectamine RNAiMAX transfection reagent	13778075	Thermo-Fischer Scientific (USA)

2.1.2.2.2. Cell Line and Medium

For the RNA-seq and small RNA-seq study, the wild-type MDA-MB-231 cell line is used. The Medium of the cell line comprises DMEM - High Glucose, 10% FBS, 1% penicillin/streptomycin, and 1% non-essential amino acids.

2.1.2.2.3. Nucleic Acids

Table 12. The table of nucleic acids used in ERBIN Silencing.

Nucleic Acid	Catalog Number	Company	Target Sequence
siGENOME NonTargeting siRNA #2	D-001210-02- 20	Thermo Scientific (USA)	UAAGGCUAUGAAGAGAUAC
Hs_ERBB2IP_7 FlexiTube siRNA	SI00122766	Qiagen (USA)	TTGGGCAGCTTACTAACTTAA

2.2. METHODS

2.2.1. Analysis of Publicly Available Datasets

The publicly available datasets, listed in Table 1, were retrieved from online databases to explore ERBIN expression and its potential miRNA regulators in breast cancer. These datasets were processed and analyzed to identify the gene and miRNA expression patterns, correlations, candidate miRNAs, and survival associations. R packages used in gene and miRNA expression analyses are given in Table 3.

2.2.1.1. Preprocessing and Data Handling

The preprocessing, annotation, and filtering steps for Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA-BRCA) datasets were performed in R (version 4.3.2) with the involvement of Bioconductor and CRAN repositories.

The GEO datasets were downloaded using the “GEOquery” R package. To reach out and map the gene or miRNA probe IDs to their symbols, the appropriate “ExpressionSet” objects were used, and the annotations were obtained from the associated platforms (GPL). The expression and phenotype information were retrieved from the same objects. In miRNA dataset analysis, the probe IDs from only human mature miRNAs were retained. The probe IDs were converted to current miRNA names using the “miRBaseConverter” R package. The duplicate probe entry values were handled by selecting the probe with the highest average expression across samples, and missing values were cleared to increase the data quality. The phenotype information of samples, such as subtype or clinical details of each sample, in datasets accessed through “ExpressionSet” objects, was selected according to requirements, and metadata was formed. For example, patients were classified into basal and non-basal subtypes, or ER-negative and ER-positive groups.

For the analysis on the TCGA-BRCA dataset, the “TCGAbiolinks” R package was used to retrieve the mRNA expression counts and clinical information, and metadata was formed through a “SummarizedExperiment” object. The gene expressions were normalized with log-transformation for further analysis. The patient subtype information was obtained from the UCSC Xena platform using the “UCSCXenaTools” R package and combined with the ERBIN expression data based on TCGA sample ID numbers for subtype comparisons.

2.2.1.2. Expression Analyses

To explore ERBIN gene expression patterns in breast cancer and its regulation by miRNAs, gene and miRNA expression analyses were performed according to subtype and clinical characteristics of breast cancer patient samples. After obtaining the data and preprocessing, the clinical and molecular breast cancer groups were analyzed for ERBIN and candidate miRNA expressions.

In GEO datasets, ERBIN and miRNA expressions were compared across breast cancer subtypes (Basal, ERBB2/HER2 positive, Luminal A, Luminal B), according to Basal type, ER, and TP53 mutation status. In addition, miRNA expression levels were compared according to clinical parameters in different GEO datasets. The comparison groups in GEO datasets were determined according to the classifications already identified in the metadata, which are specific to each dataset. In the TCGA-BRCA dataset, the subtype determination is based on PAM50 classification. The expression data in TCGA-BRCA were normalized with the log2 transformation method to stabilize the data variance for the ERBIN expression results.

The distribution of expression data in each comparison group was determined using the Shapiro-Wilk test, histogram plots, and density plots. Based on the data distribution results, the overall significance for more than one comparison group was calculated if the data groups are typically distributed using one-way ANOVA, and the non-normally distributed ones were analyzed using the Kruskal-Wallis test. In this test, if each overall significance test provides a significant result ($p < 0.05$), it means at least the expression result of one subtype is different from the others (Field *et al.*, 2012). Post-hoc pairwise comparisons were applied to understand which specific comparison pairs have significant differences in ERBIN or miRNA expression. Their differential expressions were evaluated using the student's t-test if each pair or group followed a normal distribution, or the Wilcoxon rank-sum test was used if the normality assumptions were not met for at least one pair (Field *et al.*, 2012). The resulting group-wise comparisons were visualized with boxplots in R with “ggplot2” and “ggsignif” R packages.

The differential miRNA analysis in the volcano plot for GSE19783 data was retrieved and processed in the same way as explained above. The patients were divided into ERBIN-high and -low groups using the median expression as a cutoff. The miRNA annotations were converted into mature names with “MirBaseConverter”, and

duplicate miRNAs were eliminated. Each miRNA expressed in the data was compared using a two-sided Wilcoxon rank-sum test, and the p-values were adjusted with the Benjamini-Hochberg method. The volcano plot was generated with the “ggplot2” R package.

2.2.1.3. Correlation Analyses

The identification of miRNAs with similar expression patterns in breast cancer, the GSE19783 dataset was analyzed since this dataset has both mRNA and miRNA expression level results from the same patient tumor samples. Since the sample IDs in the mRNA expression (GPL6480) and miRNA expression (GPL8227) platforms are not the same but come from the same sources, the sample IDs were matched according to the metadata of GSE19783. After filtering, annotating the mature human miRNAs, and preparing the ERBIN expression data, the Spearman’s rank correlation coefficients for each miRNA were calculated between the ERBIN mRNA expression and all annotated miRNAs in R. The Benjamini-Hochberg false discovery rate (FDR) method was applied to the raw p-values for each correlation to correct them for multiple testing. From the results of the correlation analysis, only the miRNAs with adjusted p-values less than 0.05 were selected as statistically significant. A bar plot was generated to visualize the correlation coefficients of these miRNAs. The correlation analyses between the ERBIN gene and other genes (ESR1, ERBB2, TP53, and PGR) and between ERBIN and candidate transcription factors (TFs) were performed using the same method.

2.2.1.4. Candidate miRNA Identification

MiRNAs having a significant negative correlation with ERBIN expression across breast cancer patients were selected and intersected with the miRNA list generated from the combination of miRNA target prediction tools, in the list of Table 4, that predicted the miRNAs targeting ERBIN. The intersections were visualized using Venn diagrams using the “VennDiagram” R package (but visualized with Canva) and UpSet plots with the “UpSet” R package to summarize the miRNAs.

The positively correlated miRNAs are selected to investigate whether transcription factors are the secondary regulatory factors between ERBIN. The “dorothea” R package is used to obtain a high-confidence human TF-target interaction dataset. The “multiMiR” R package retrieves experimentally validated mRNA targets for each

miRNA, and filters these to take only TFs in the Dorothea resulting list. The resulting miRNA–TF targeting relationships with the TF–ERBIN correlations are integrated to generate a miRNA–TF–ERBIN regulatory network. The network visualizations were created in R using the “igraph”, “ggraph”, and “tidygraph” R packages.

2.2.1.5. Survival Analysis

Kaplan-Meier Plotter, an online tool, was used to explore how the expression levels of ERBIN and candidate miRNAs affected patient outcomes. The tool integrates various datasets, including GEO and METABRIC, and allows survival assessment based on expression levels. ERBIN and candidate miRNA expression effects were considered for patient clinical outcomes, only if p-values were less than 0.05.

Multiple GEO datasets in Table 1 were used to analyze the survival outcomes, such as relapse-free survival (RFS) and distant metastasis-free survival (DMFS) between patient groups with high and low ERBIN gene expression. The METABRIC breast cancer dataset explores survival outcomes in response to miRNA expression levels. The candidate miRNAs having inverse survival patterns are prioritized to identify the miRNAs regulating ERBIN.

2.2.2. Small RNA-seq Analyses

To explore the downstream effects of ERBIN downregulation on transcriptomic alterations, ERBIN expression was silenced through siRNA targeting in the MDA-MB-231 breast cancer cells. After 72 hours, the transfected cells were collected, and miRNA sequencing was performed by a RefGen Biotechnology company (Ankara, Turkey). The differentially expressed miRNAs were identified between control and ERBIN-silenced cell lines using the edgeR package. The miRNA candidates were investigated in this newly generated data to explore their potential regulatory feedback mechanisms for ERBIN expression.

2.2.2.1. Sample Preparation

The total RNA was isolated (described in Section 2.2.4.1) from MDA-MB-231 cell lines that were transfected with ERBIN-siRNA and scrambled-control siRNA. The transfection method is explained in Section 2.2.3.2. Three biological replicates were used for both control and siERBIN experiments. After transfection in the MDA-MB-231 cell line, the RNA samples were separated into aliquots, and each sample was used

for small RNA sequencing. The RNA samples were stored at -80°C and on dry ice and were used in delivery.

The initial RNA concentration and purity controls were performed with a NanoDrop ONE. The quality control of the given RNA samples was analyzed for their RNA quantity, purity, and structural integrity using fluorometric and capillary electrophoresis methods (performed by RefGen Biotechnology). The quality controls of samples are measured with Qubit 3 Fluorometer (Thermo Fisher Scientific, USA, #Q33216), 2100 Bioanalyzer (Agilent, USA, #G2939BA), or the 4200 TapeStation system (Agilent, USA, #G2991BA).

2.2.2.2. miRNA Sequencing

RefGen Biotechnology accomplished the preparation and sequencing procedures for the miRNA library. The next-generation sequencing (NGS) libraries were prepared for miRNA sequencing using NextFlex Small RNA Sequencing Kit V4 (Revvity, USA, #NOVA-5132-41). In the kit protocol, the enzymatic ligation reactions add modified adapters to the miRNA molecules' 3' and 5' ends. After adapter ligation, the first strand of cDNA is synthesized. The products were purified with a magnetic bead-based size selection kit protocol (Ambion Magnetic Stand-96, Cat #AM10027). The amplification of purified cDNAs was made with the P5 and P7 primer sets, including Unique Dual Index (UDI). The resulting library was validated with Agilent Bioanalyzer, and miRNA libraries were obtained with a target ~165 bp length. The final library was enriched by targeting single-end reading, around 10 million reads of 150 base pairs, and sequencing was performed using the Illumina NovaSeq 6000 platform.

2.2.2.3. Bioinformatics Analyses

The bioinformatics analyses, including quality control and downstream analyses of the miRNA sequencing data, conducted by RefGen Biotechnology, were utilized in this study. The quality control steps were conducted using the FASTQC tool by Babraham Bioinformatics, and the results were examined by considering the amount of data, reading qualities, GC distributions, kmer distributions, and possible adapter contaminations for each sample. The adapter/low-quality base trimming was reported to be carried out using Cutadapt (v4.9). In the trimming process of miRNA, the “quality cut off 20 adapter TGGAATTCTCGGGTGCCAAGG” and “minimum length

16” parameters were used to ensure short, low-quality reads, and 3’ adapter sequences were removed.

Trimmed reads of miRNA were aligned to the reference genome “*Homo Sapiens* Hg38” by using MiRDeep2 Mapper (v2.0.0.8.1). After alignment, the MiRDeep2 Quantifier (v2.0.0) tool was reported to quantify read counts and match the reads with the mature miRNA sequences according to the miRBase database. The upstream parameter was selected as 2, and the downstream parameter as 5.

In the normalization process of miRNA, the company reported that transcript abundance was quantified according to read counts. The result was normalized according to transcript length, and the Reads per Kilobase (RPK) values were obtained. According to RPK values, results were normalized again according to total read count, and the Transcript per Million (TPM) values were obtained.

2.2.2.4. Differential Expression Analyses

After normalization steps, to identify differentially expressed miRNAs, the “edgeR” R package and Quasi-Likelihood model were applied to calculate statistical significances of both mRNA and miRNA. From the resulting differentially expressed miRNAs, the significance value less than 0.05 and $|\log FC|$ values higher than 1 were considered. The differentially expressed miRNAs according to significance thresholds were visualized with bar plots. The expression profile of identified candidate miRNAs according to breast cancer patient analyses was examined additionally, and individual expression changes were visualized using bar plots.

2.2.3. Cell Culture-Based Techniques

2.2.3.1. Cell Culture Maintaining and Handling

The MDA-MB-231 human breast cancer cell was cultured in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum) and 1% penicillin-streptomycin. Cultures were maintained under sterile conditions at 37 °C in an incubator with 5% CO₂ and 95% humidity. Frozen stocks were taken from the laboratory's liquid nitrogen tanks. For thawing, vials containing 90% FBS and 10% DMSO were rapidly transferred into growth medium, centrifuged to remove residual DMSO, and seeded in 10 cm culture dishes. Once the cells reached 80–90% confluency, they were subcultured every 3–4 days at a 1:4 split ratio.

The medium was aspirated during passaging, and the cells were rinsed with PBS. Trypsin was added to detach adherent cells from the plate surface, and complete detachment was achieved within approximately 5 minutes. The suspension was centrifuged at 1500 RPM for 5 minutes, after which the pellet was resuspended in fresh medium and transferred into new 10 cm dishes.

A freezing solution containing 90% FBS and 10% DMSO was prepared for long-term preservation. Following trypsinization and centrifugation, pellets were resuspended in 1 mL freezing medium and aliquoted into sterile cryovials. These vials were stored at -80°C overnight in a Mr. Frosty freezing container and transferred to liquid nitrogen. Mycoplasma contamination was routinely checked throughout the study using the Mycoplasma Detection Kit (Table 7), according to the manufacturer's instructions.

2.2.3.2. siRNA Transient Transfection

For ERBIN silencing through siRNA targeting RNA and small RNA-seq, MDA-MB-231 cells were transfected with Hs_ERBB2IP_7 FlexiTube siRNA (si7-ErbB) and siGENOME non-targeting siRNA #2 (scr-RNA).

In the transfection protocol, on the first day, 250,000 cells/well MDA-MB-231 cells were seeded into 6-well plates in complete growth medium and kept overnight in the incubator. On the second day, the transfection procedure was applied using Lipofectamine RNAiMAX reagent (Invitrogen, USA). During the procedure, first, two separate solutions were prepared. One included 250 μL Optimem and 2.5 μL siRNA (si7-ErbB or scr-RNA), and the other included 250 μL Optimem and 5 μL RNAiMAX reagent. Then, two solutions were gently mixed and incubated at room temperature for 20 minutes to form transfection complexes. The cells were washed once with sterile PBS, and 1 mL of fresh growth medium without penicillin/streptomycin was added to each well. After the incubation, the transfection solution was added to each well dropwise. Each condition (control and siERBIN) was applied in triplicate. Cells were harvested after 72 hours for total RNA for further RNA, small RNA-seq study, and protein extraction for Western blot analysis.

2.2.4. Laboratory Techniques

2.2.4.1.Total RNA Isolation and cDNA Synthesis

The cells are harvested in pellet form using cold PBS and centrifuged at +4°C for RNA isolation. Total RNA was isolated from MDA-MB-231 cells using TRIpure Isolation Reagent according to the manufacturer's protocol to use in RNA and small RNA-seq study, for the validation of ERBIN expression and candidate miRNA with qRT-PCR.

The first strand cDNA, for further mRNA expression quantification with qRT-PCR, was synthesized from isolated RNA using the RevertAid First Strand cDNA Synthesis Kit, and the protocol was performed according to the manufacturer's protocols. To quantify the expression of candidate miRNAs, the miRNA cDNA was synthesized using the miRCURY LNA RT Kit, and the protocol was used according to the manufacturer's instructions.

2.2.4.2.qRT-PCR

The mRNA and miRNA qRT-PCR were performed using RevertAid First Strand cDNA Synthesis Kit and miRCURY LNA SYBR Green PCR Kit, respectively. The manufacturer's guidelines are directly applied in the experiments, and the analysis is performed according to the $2^{-\Delta\Delta C_t}$ method.

2.2.4.3.Total Protein Isolation and Protein Quantification

The cells are harvested in pellet form using cold PBS and centrifuged at +4°C for protein isolation. The RIPA buffer in Table 6 was used as the cell lysis buffer. After dissolving the cell pellets at +4°C, the RIPA buffer was added, and the mixture was incubated at +4°C in a shaker for 30 minutes. The samples were centrifuged at 13000 RPM at +4°C for 30 minutes. The resulting supernatants of total proteins were obtained and stored at -80°C for further experiments. The protein quantification was performed with the BCA Protein Assay Kit according to the manufacturer's protocol.

2.2.4.4.Western Blot

The resolving and stacking gels were prepared according to Table 13. Proteins were prepared using 4X Laemli Sample Buffer (Table 5) and 2-mercaptoethanol, then boiled at 95°C for 5 minutes. Protein samples were run on a gel and prepared in the setup in 1X Running Buffer (Table 6). The running was performed at 80V until the samples reached the resolving gel, then the voltage was changed to 120V until the samples

reached the end of the gel. The samples were transferred to a nitrocellulose transfer membrane with a wet transfer system, and the transfer was performed at 250 mA for 2.5 hours in 1X Transfer Buffer (Table 6). After staining the protein transferred membrane with Ponceau S solution, the proteins were blocked into the membrane using 5% milk powder solution for 1 hour at room temperature. The membranes were incubated overnight in the primary antibody solution at +4°C with mild shaking. The next day, the membranes were washed 3 times for 10 minutes in 1X TBS-T. Afterwards, the membranes were incubated with the secondary antibody solution for an hour at room temperature. The membranes were rewashed in the same way. For the visualization, the membranes were incubated with ECL for a minute in a dark environment. Then, the image is taken with Amersham Imager 600.

Table 13. Preparation of Resolving and Stacking Gels.

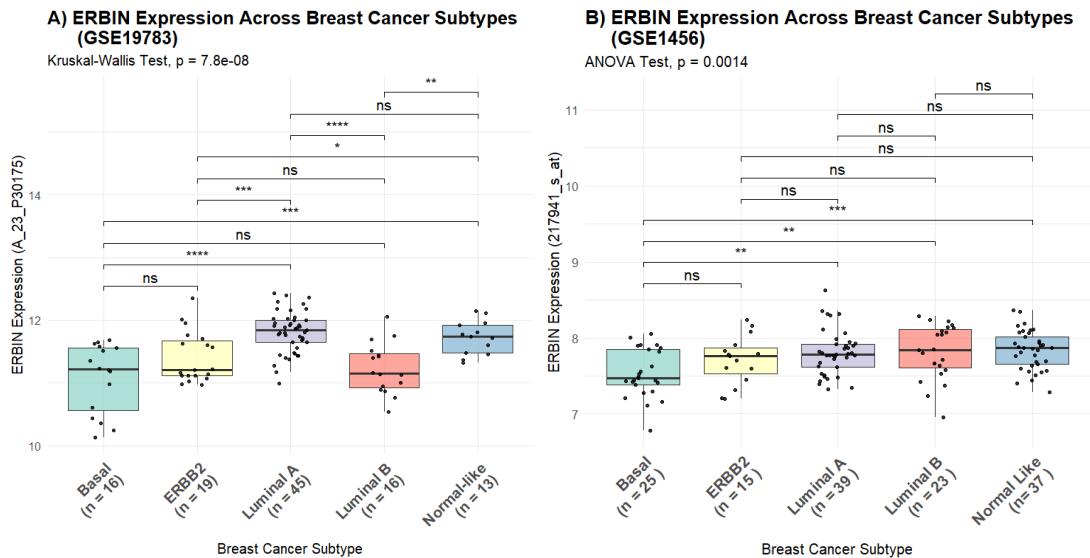
10% Resolving Gel - 10 mL	2.5 mL Resolving Buffer (Table 6), 3.3 mL 30% acrylamide/bisacrylamide mixture, 4.1 µL ddH ₂ O, 5 µL TEMED, 50 µL APS
4% Stacking Gel - 5 mL	1.26 mL Stacking Buffer (Table 6), 0.66 mL 30% acrylamide/bisacrylamide mixture, 3 mL ddH ₂ O, 5 µL TEMED, 25 µL APS.

CHAPTER 3: RESULTS

3.1. ERBIN Expression in Breast Cancer Patients in Public Datasets

Previous studies demonstrated that ERBIN functions either as a tumor suppressor or an oncogene, depending on the cancer type and cellular context. In breast cancer, it shows this dual functionality in HER2-positive breast cancer. Given that breast cancer is a heterogeneous disease and has distinct subtypes with specific molecular characteristics and clinical outcomes, ERBIN gene expression was examined across subtypes, basal and non-basal tumors, and their molecular characteristics, such as estrogen (ER), progesterone receptors (PR), HER2, and TP53 status.

The statistical analysis of ERBIN expression provided significant differences across breast cancer subtypes in publicly available independent GEO and TCGA datasets (GSE19783, GSE1456, GSE45827, and TCGA-BRCA). These microarray and RNA-seq-based datasets include well-characterized subtypes (Basal-like, HER2/ERBB2-enriched, Luminal A, Luminal B, and Normal-like) and mRNA expression results with a wide range of patient sample sizes.



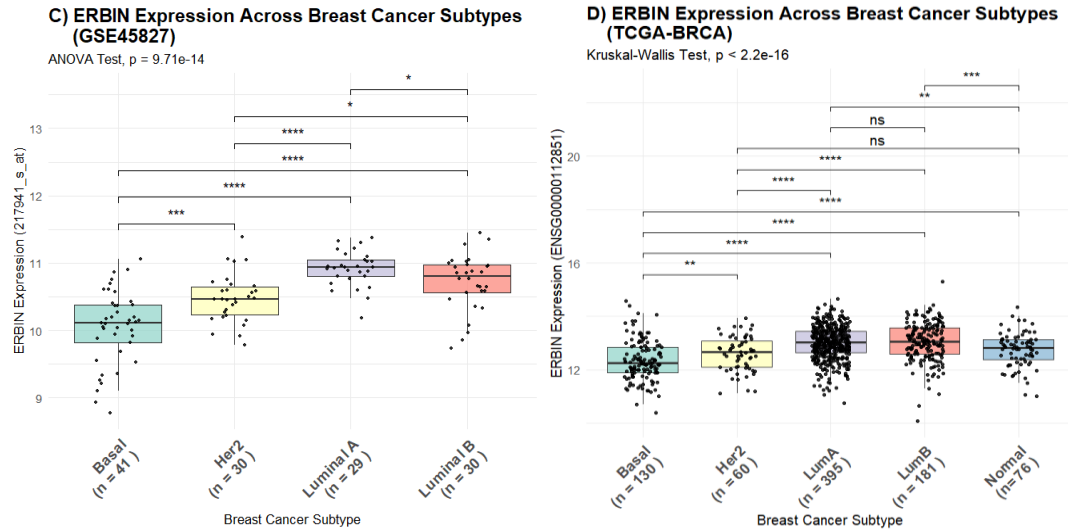


Figure 4. Boxplots presenting ERBIN expression levels across breast cancer subtypes in four independent patient datasets.

A) GSE19783, B) GSE1456, C) GSE45827, D) TCGA-BRCA. Subtypes include Basal-like, ERBB2/HER2-enriched, Luminal A, Luminal B, and Normal-like. Overall statistical significances were determined by one-way ANOVA or Kruskal-Wallis tests, followed by post-hoc pairwise tests between subtypes with t-tests or Wilcoxon rank-sum tests. Significance thresholds: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$ (ns = non-significant), n = sample size.

The pairwise comparisons in Figure 4 indicated that the ERBIN expression profile significantly differs across breast cancer subtypes, supported by different datasets. In addition, these results show that the tumor samples from breast cancer patients with the Basal-like subtype consistently have the lowest ERBIN gene expression compared to patients from other subtypes in most of the datasets and from normal-like phenotype in each dataset, supporting the statistical robustness of the results. On the other hand, the ERBIN gene has the highest expression levels in Luminal subtypes, which are the tumor types with better clinical outcomes. The patients with ERBB2/HER2-enriched subtype have intermediate levels of ERBIN expression, and the significance between comparisons of different groups with each other presents an inconsistent profile. As a result, the ERBIN expression levels suggest a potential association between ERBIN downregulation and Basal-like.

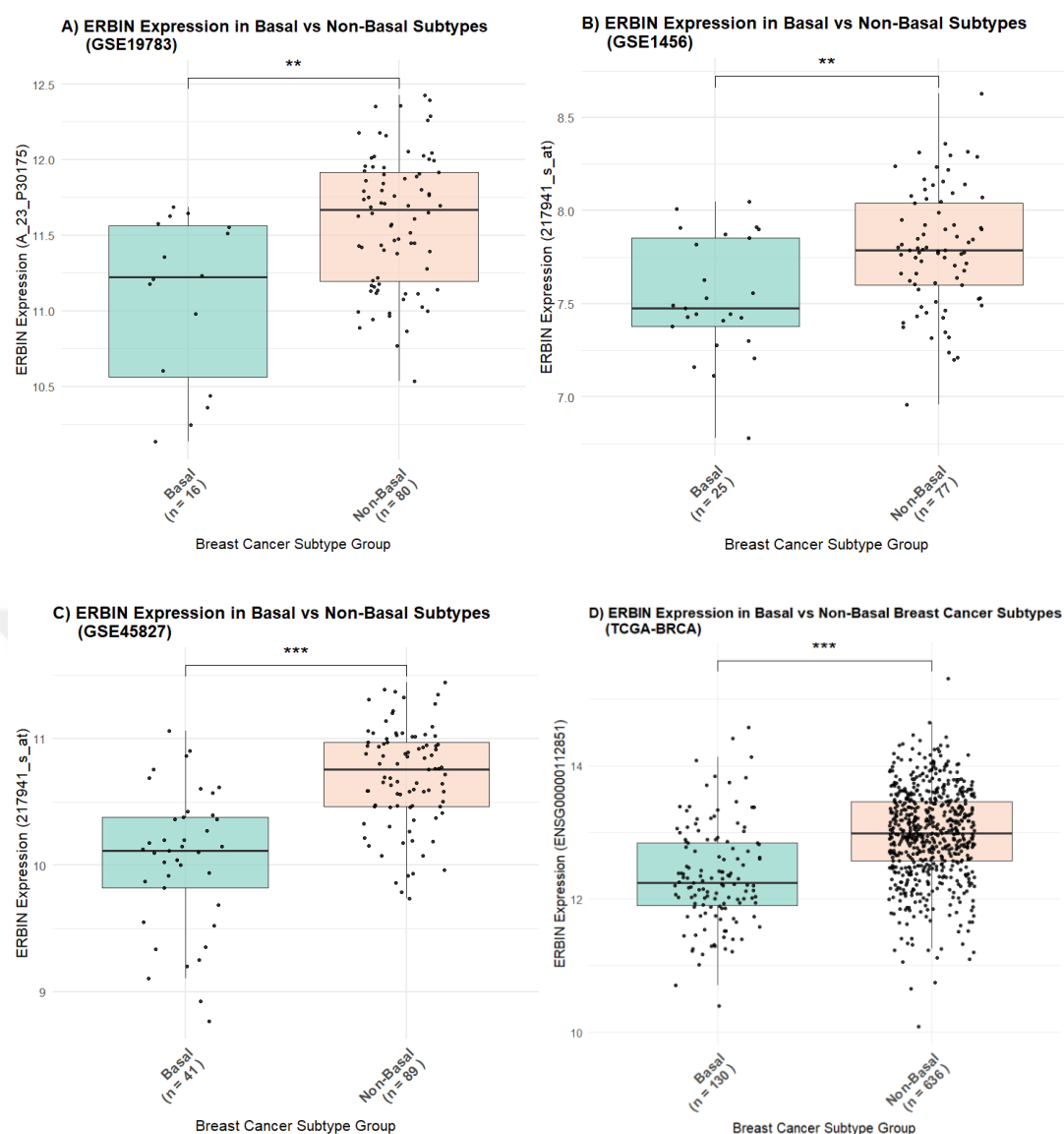


Figure 5. Boxplots presenting the ERBIN expression levels between the Basal and Non-Basal Breast Cancer patient groups.

A) GSE19783, B) GSE1456, C) GSE45827, D) TCGA-BRCA. The Non-Basal Group includes ERBB2/HER2-enriched, Luminal A, and Luminal B subtypes. The significance of analyses determined with t-tests (GSE1456, GSE45827) or Wilcoxon rank-sum tests (GSE19783, TCGA-BRCA). Significance thresholds: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$ (ns = non-significant), n = sample size.

The association between ERBIN and breast tumor aggressiveness was analyzed further by comparing ERBIN expression between Basal-like and Non-Basal subtypes. The Basal-like tumor is the most aggressive form of breast cancer, characterized by poor clinical outcomes and high proliferation rates. The patient samples with Luminal A, Luminal B, and HER2/ERBB2-enriched molecular subtypes in the non-basal category have better clinical outcomes in survival, recurrence, and therapy response.

In the same set of datasets (GSE19783, GSE1456, GSE45827, and TCGA-BRCA), after grouping the patient samples as Basal-like and Non-Basal, we evaluated the distribution of ERBIN expression values for each group. If the data show normal distribution, the t-test was performed; if not, the Wilcoxon rank-sum test was performed. The results provided significantly lower expression of ERBIN in basal-like types compared to non-basal patients in all four datasets (Figure 5), suggesting that ERBIN is downregulated in more aggressive types of breast tumors.

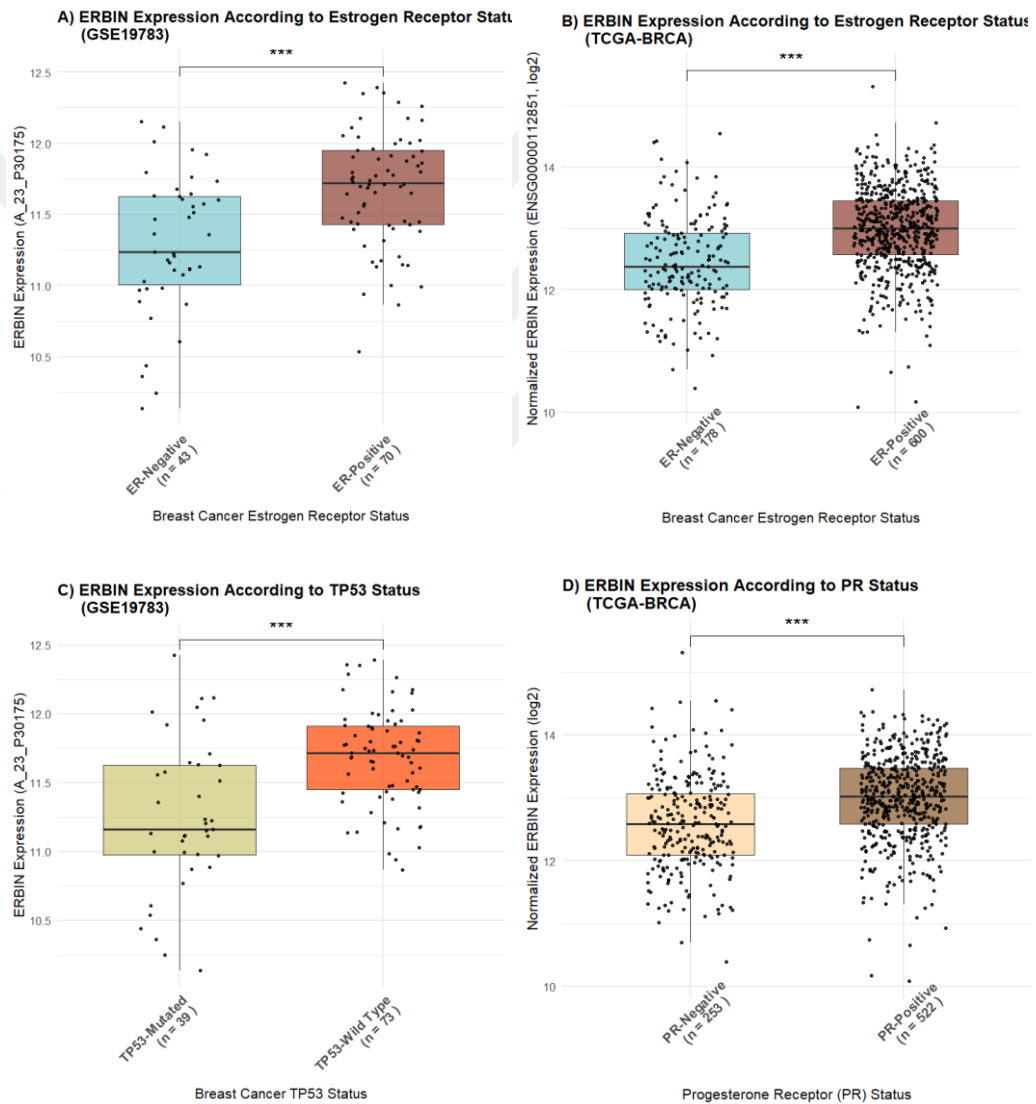


Figure 6. ERBIN expression comparisons in Estrogen Receptor Negative and Positive, and between TP53-mutated or wild-type status in breast cancer patients

A and C) GSE19783, B and D) TCGA-BRCA. The significance of analyses determined with t-tests (GSE19783) or Wilcoxon rank-sum tests (TCGA-BRCA). Significance thresholds: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$ (ns = non-significant), n = sample size.

Since estrogen receptor (ER), TP53 mutation, progesterone receptor, and status are critical to classify the breast cancer status of patients, we analyzed ERBIN expression status according to these groups of patients in different GEO datasets. The ER-negative and ER-positive status of tumor samples was well determined in GSE19783 and TCGA-BRCA datasets. When ERBIN expression is analyzed according to the patient tumors' estrogen receptor status, ERBIN is significantly lower in ER-negative than ER-positive tumors (Figure 6).

TP53 mutation status is another marker to classify breast cancer patient tumor samples in GSE19783. When we stratified patient tumors as TP53-mutated or TP53-wild type and assessed ERBIN expression, we observed that the ERBIN expression was downregulated in TP53-mutated samples compared to TP53-wild type samples ($p < 0.001$). In addition, according to the progesterone receptor status in TCGA-BRCA data, the PR negative samples had lower ERBIN gene expression than the PR positive samples ($p < 0.001$).

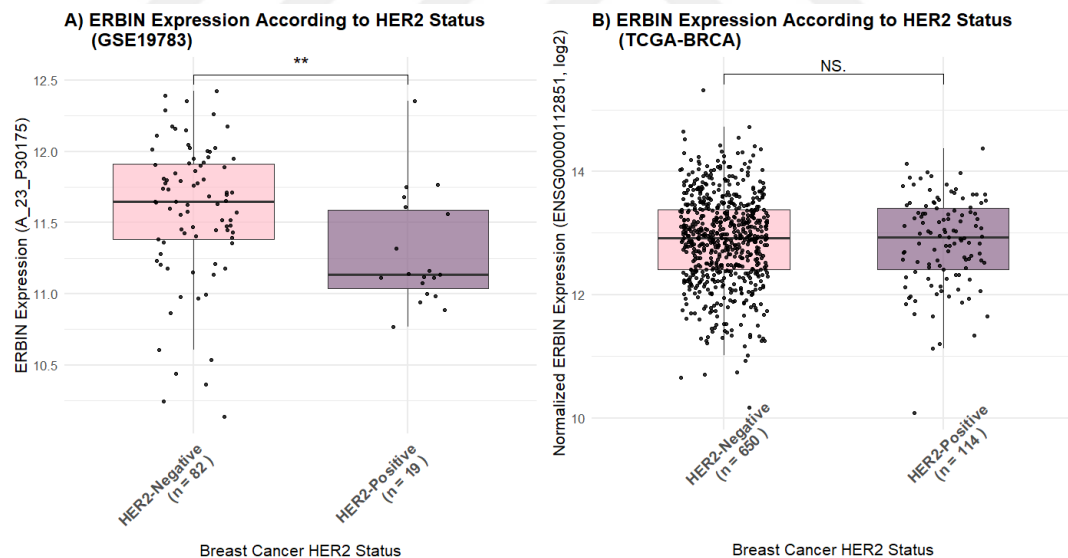


Figure 7. ERBIN Expressions according to HER2-Negative and HER2-Positive status in patient datasets.

A) GSE19783 B) TCGA-BRCA. The significance of analyses determined with Wilcoxon rank-sum tests in both datasets. Significance thresholds: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$ (NS = non-significant), n = sample size.

ERBIN gene shows a significantly higher expression level in HER2-negative status of patient tumors compared to HER2-positive status in the GSE19783 dataset ($p < 0.01$). Interestingly, in the TCGA dataset, the ERBIN gene did not present a significant difference between HER2-negative and HER2-positive status of patient tumors (Figure 7).

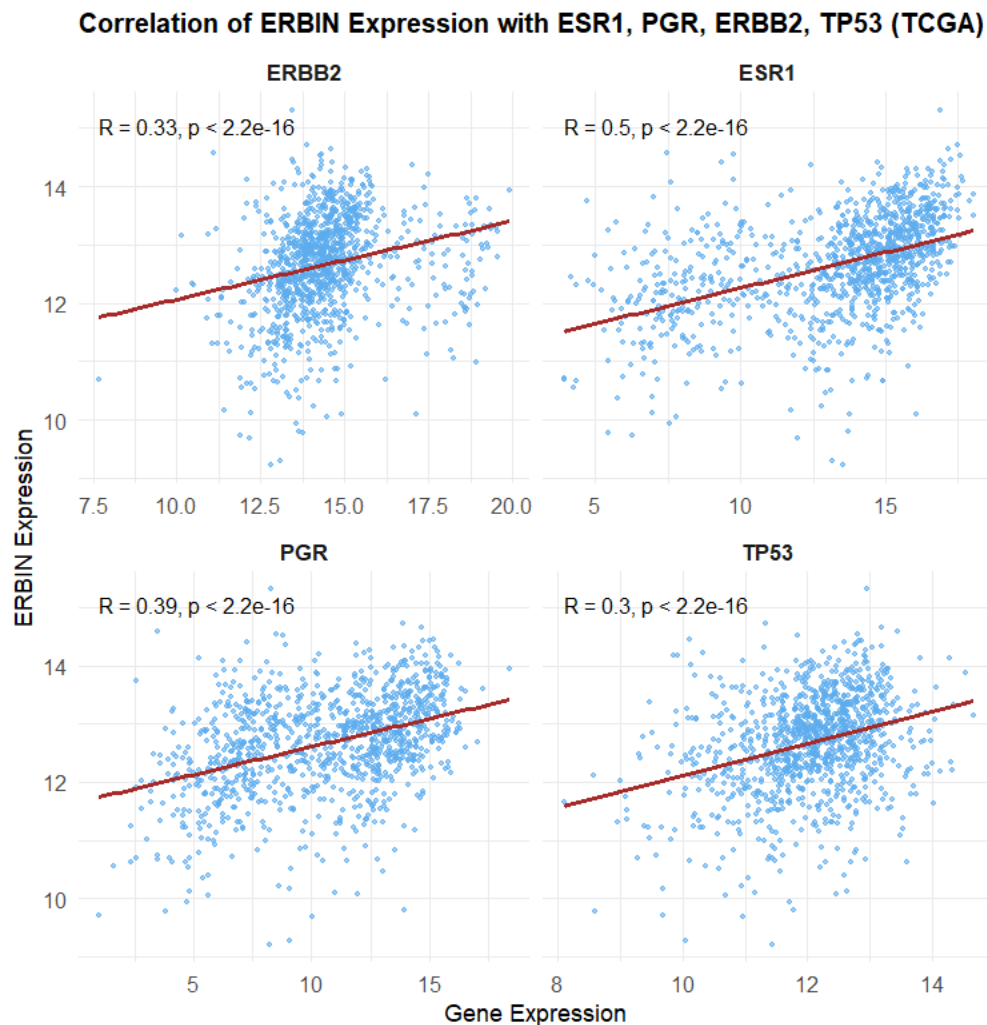


Figure 8. Correlation of ERBIN Expression with ERBB2, ESR1, PGR, TP53 gene expressions in TCGA-BRCA data.

Scatter plots show the expression correlation between ERBIN expression and the expression of four marker genes in breast cancer using TCGA-BRCA breast tumor gene expression results: ERBB2/HER2, ESR1 (Estrogen Receptor 1), PGR (Progesterone Receptor), and TP53. The Pearson correlation coefficients are given as “R” values, and the significance of the correlation analysis is shown with a p-value for each gene. The red line represents the linear regression fit.

Subtype classification was analyzed to explore further the ERBIN expression relation between well-established molecular markers associated with aggressive breast cancer. The Spearman correlation analysis was performed between ERBIN expression and the genes ERBB2, ESR1, PGR, and TP53 by using TCGA-BRCA RNA-seq data from more than 1000 breast tumors. All the correlation results were highly significant ($p < 2.2e-16$). The analysis showed a moderate expression correlation between ERBIN and ESR1 gene expression levels ($R=0.5$), Figure 8. The expression patterns of the ERBB2, PGR, and TP53 genes in these breast cancer patients showed a weaker positive correlation (coefficient values are 0.33, 0.39, and 0.3, respectively). The correlation coefficients and the significance values were calculated using Spearman's rank correlation.

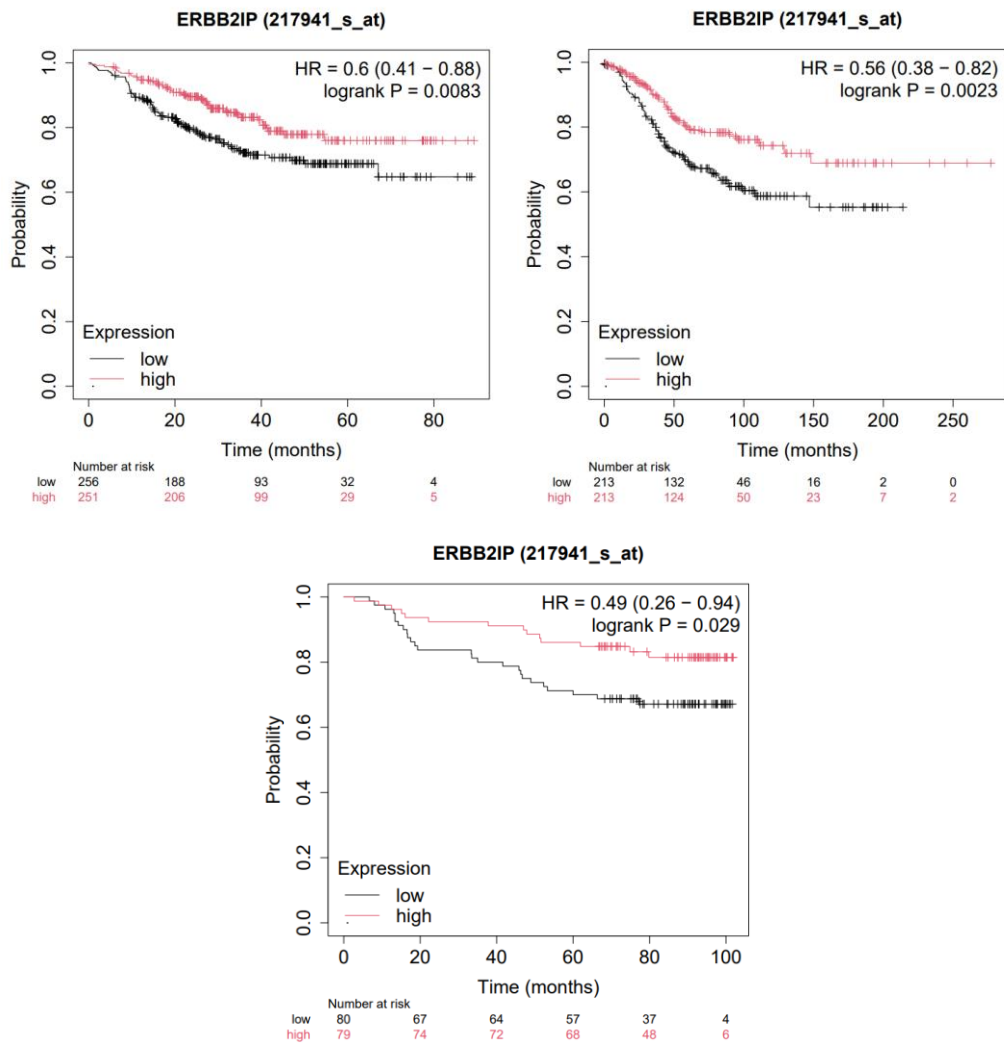


Figure 9. Relapse-free survival analyses of ERBIN (ERBB2IP) expression in breast cancer patients in three different datasets generated by the KM-Plotter tool.

The analyses were performed using GSE25066 (n = 507), E-MTAB-365 (n = 426), and GSE1456 (n = 159), respectively. Patients were grouped into high and low ERBIN expression groups based on median expression. Hazard ratios (HR), 95% confidence intervals (in parentheses next to the HR values), and log-rank p-values are shown on each plot.

We performed relapse-free survival (RFS) analysis using the KM-Plotter tool in three different breast cancer patient datasets to investigate whether ERBIN expression levels are linked to relapse-free survival in breast cancer. Among the datasets available for analysis in the KM-Plotter tool, three datasets showed statistically significant associations between ERBIN expression and relapse-free survival (Figure 9). These three datasets were GSE25066 (n = 507), E-MTAB-365 (n = 426), and GSE1456 (n = 159). In GSE25066, patients with higher ERBIN expression had a 40% lower risk of tumor relapse than those with lower expression (HR = 0.60, 95% CI = 0.41 - 0.88, log-rank p-value < 0.05). In E-MTAB-365, the high-expression group showed a 46% lower risk (HR = 0.56, 95% CI = 0.38 - 0.81, log-rank p-value < 0.05), and in GSE1456, the high-expression group showed 51% lower risk of tumor relapses (HR = 0.49, 95% CI = 0.26 - 0.94, log-rank p-value < 0.05). Overall, in each dataset, patients with higher ERBIN expression tend to live longer without tumor recurrence than those with lower expression.

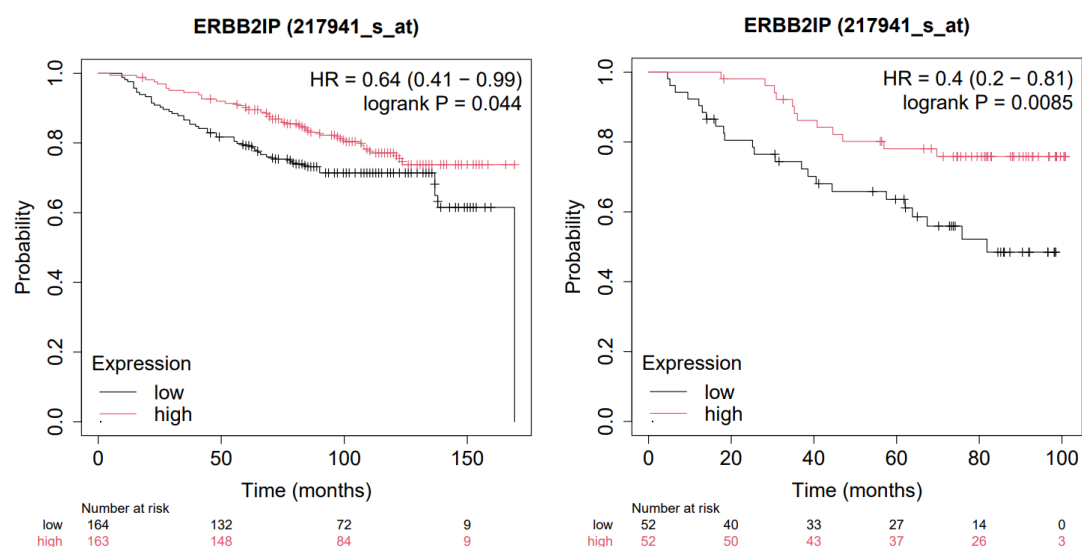


Figure 10. Overall survival analyses of ERBIN (ERBB2IP) expression in breast cancer patients in two different datasets using the KM-Plotter tool.

GSE20685 (n = 327) and GSE42568 (n = 104) datasets are used for this analysis, respectively. Patients were grouped into high and low ERBIN expression groups based on median expression. Hazard ratios (HR), 95% confidence intervals (in parentheses next to the HR value), and log-rank p-values are shown on each plot.

To assess the association between ERBIN expression levels and overall survival of breast cancer, we performed overall survival analysis utilizing the KM-Plotter tool in two different datasets. In GSE20685 (n=327), patients with high ERBIN expression showed longer overall survival with 36% lower risk of death (HR = 0.64, 95% CI = 0.41–0.99, log-rank p-value < 0.05) (Figure 10).

In the GSE42568 dataset, high ERBIN expression was associated with better overall survival (HR = 0.40, 95% CI = 0.22–0.81, log-rank p-value < 0.05), indicating a 60% reduction in the risk of death compared to the low-expression group. These results suggest that higher ERBIN expressions have the potential to be linked to better overall survival outcomes in breast cancer patients.

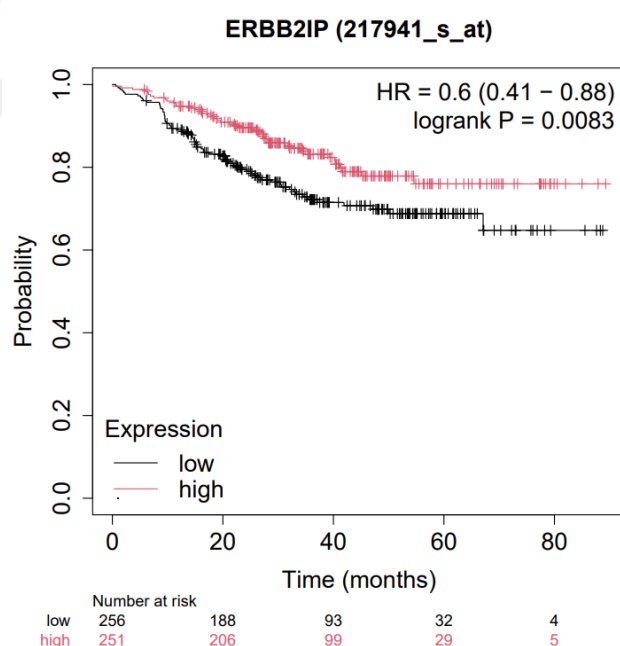


Figure 11. Distant metastasis-free survival analysis of ERBIN (ERBB2IP) expression in breast cancer patients in the GSE25066 (n = 507) dataset generated with the KM-Plotter tool.

Patients are grouped into high and low groups based on median expression. The plot shows the hazard ratio (HR), 95% confidence interval (in parentheses next to the HR value), and log-rank p-values.

We also assessed the ERBIN expression to determine whether it relates to distant-metastasis-free survival in breast cancer patients. Patients with high ERBIN expression presented significantly longer survival without distant metastasis compared to those with low expression (HR = 0.60, 95% CI: 0.41–0.88, log-rank p-value < 0.05) (GSE25066 (n = 507), Figure 11).

3.2. Identification of Potential miRNAs Regulating ERBIN Expression in Breast Cancer

To investigate the mechanism underlying the altered expression of ERBIN in breast cancer, we focused on miRNAs' regulation at the post-transcriptional level. Since miRNAs are known gene expression regulators through various mechanisms, we determined to identify the miRNAs whose expression patterns are associated with ERBIN expression in breast tumors.

To address this, we performed a correlation analysis between ERBIN and all annotated mature human miRNAs using the GSE19783 breast cancer patient dataset, which includes expression profiles at both mRNA and miRNA levels for each patient sample. This approach allowed us to narrow the number of candidate miRNAs to a focused subset that showed consistent co-expression patterns with ERBIN. From the resulting miRNAs having significant expression correlation with ERBIN expression in GSE19783 data, negatively and positively correlated miRNAs were analyzed further.

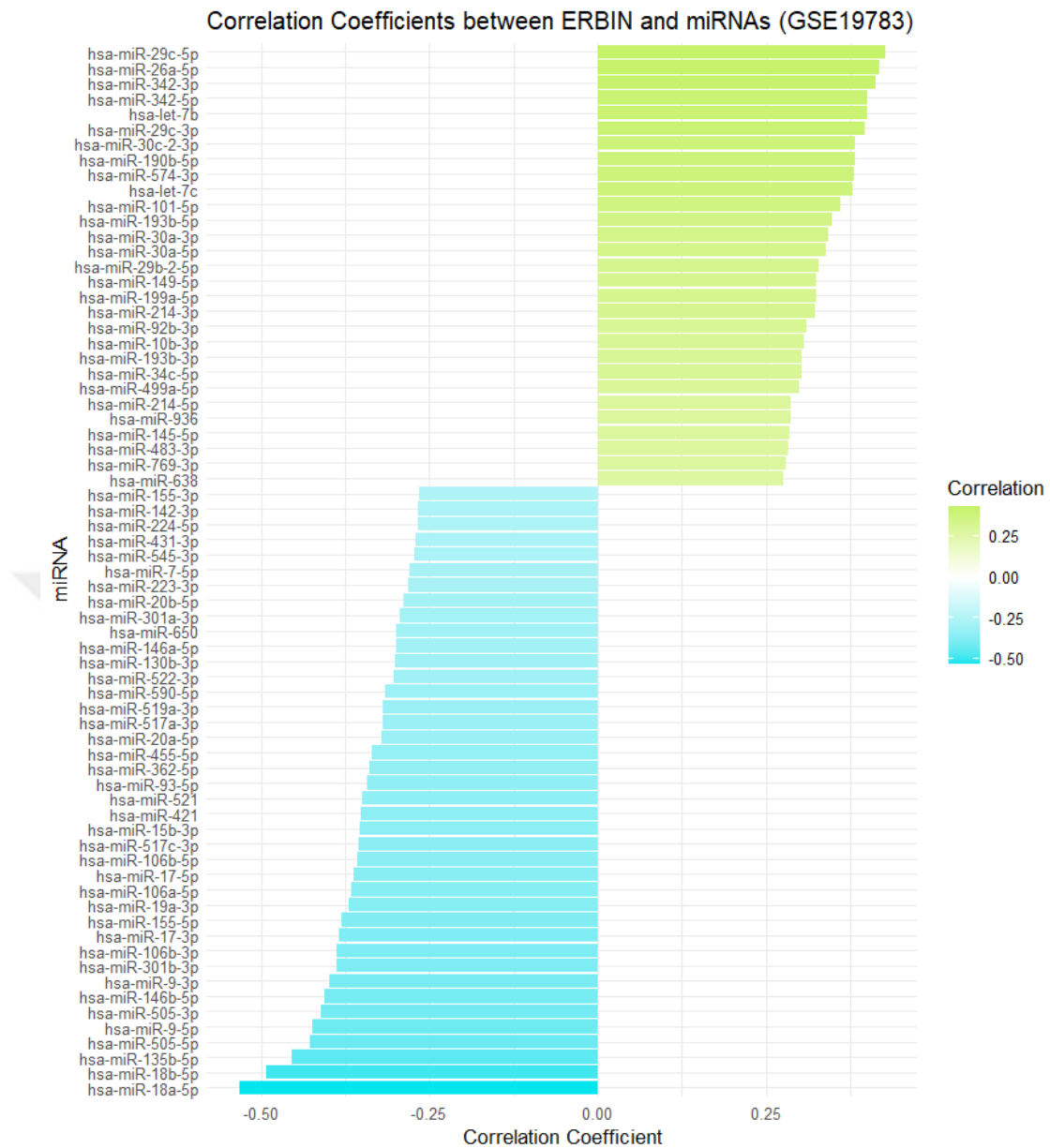


Figure 12. Bar plot of significant Pearson correlation coefficients between ERBIN expression and mature human miRNAs in breast cancer patients (GSE19783).

The miRNAs whose expression patterns are statistically associated with ERBIN expression across 101 breast cancer patients are demonstrated. The strength of their correlation sorts the miRNAs. Bar colors indicated the direction and magnitude of correlation. A threshold of adjusted p -value < 0.05 was used to determine significance, and multiple testing correction was performed using the Benjamini–Hochberg (BH) method.

The correlation analysis identified 69 miRNAs, as shown in Figure 12, having an expression profile significantly correlated with the ERBIN expression profile in breast cancer patient tumors from the GSE19783 dataset. Twenty-nine miRNAs positively correlated (correlation coefficients ranging from ~ 0.2 to ~ 0.4), while 40 miRNAs were negatively correlated (correlation coefficients ranging from ~ -0.2 to ~ -0.5). The five miRNAs that are positively correlated with ERBIN expression included hsa-miR-29c-5p, hsa-miR-26a-5p, hsa-miR-342-3p, hsa-miR-342-5p, and hsa-let-7b. On the other hand, the miRNAs with the strongest negative correlations were hsa-miR-18a-5p, hsa-miR-18b-5p, hsa-miR-135b-5p, hsa-miR-505-3p, and hsa-miR-9-5p.

The miRNAs potentially regulate ERBIN expression by binding to its target mRNA and degrading or repressing its translation. Therefore, negatively correlated miRNAs may have a higher potential to regulate ERBIN downregulation than positively correlated miRNAs. Based on this approach, we prioritized the negatively correlated miRNAs with ERBIN expression. We also evaluated miRNAs positively correlated with ERBIN expression to understand the regulatory mechanisms between miRNAs and ERBIN expression in breast cancer.

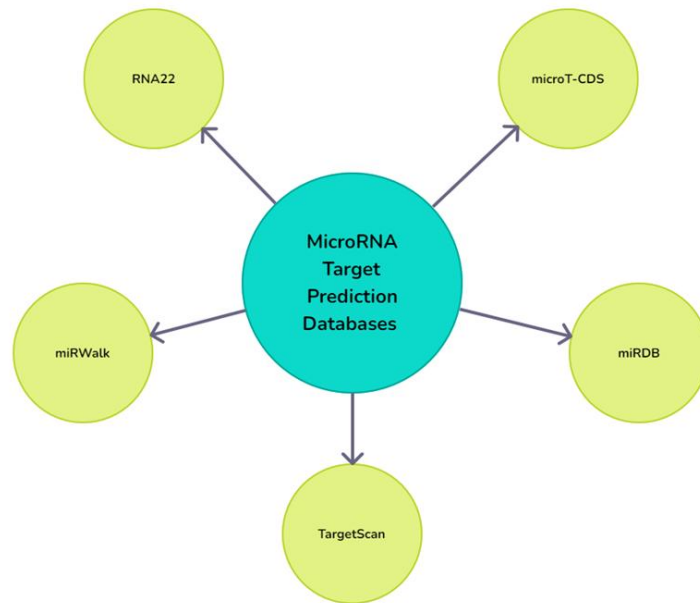


Figure 13. Diagram of miRNA target prediction databases used in this study.

The schematic representation illustrates the five miRNA target prediction databases utilized to identify candidate miRNAs potentially regulating ERBIN expression, including RNA22, miRWalk, microT-CDS, miRDB, and TargetScan.

The well-known miRNA target prediction tools; microT-CDS, miRDB, miRWalk, RNA22, and TargetScan, were used to analyze whether ERBIN mRNA 3'UTR, 5'UTR regions, and coding sequence (CDS) can be targeted by miRNAs that are negatively correlated with ERBIN expression (Figure 13).

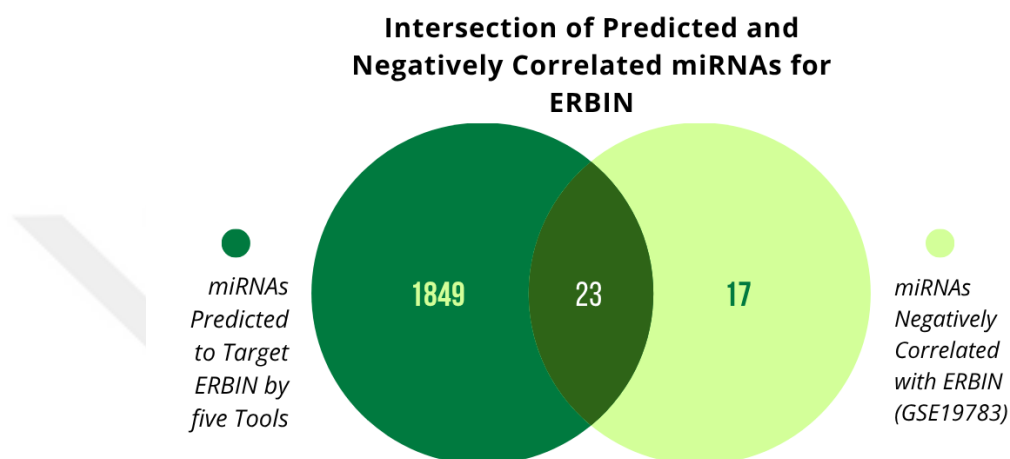


Figure 14. Intersection of predicted and negatively correlated miRNAs for ERBIN.

The Venn diagram shows the intersection between miRNAs predicted to target ERBIN by five prediction tools (miRWalk, miRDB, TargetScan, RNA22, and DIANA microT-CDS) and miRNAs negatively correlated with ERBIN expression based on patient tumor data from GSE19783.

When we combined the miRNAs that were predicted to target ERBIN mRNA with five prediction tools (miRWalk, miRDB, TargetScan, RNA22, and DIANA microT-CDS), there were 1872 miRNAs in total, including those targeting ERBIN mRNA from 3'UTR, 5'UTR, and CDS regions. As Figure 14 displays, this large miRNA set (n=1872) intersection with miRNAs negatively correlated (n=40) with ERBIN expression in the breast cancer dataset GSE19783 was identified. A total of 23 miRNAs were identified as common to both sets, predicted as targets by well-established miRNA prediction tools, and displayed a negative expression correlation with ERBIN.

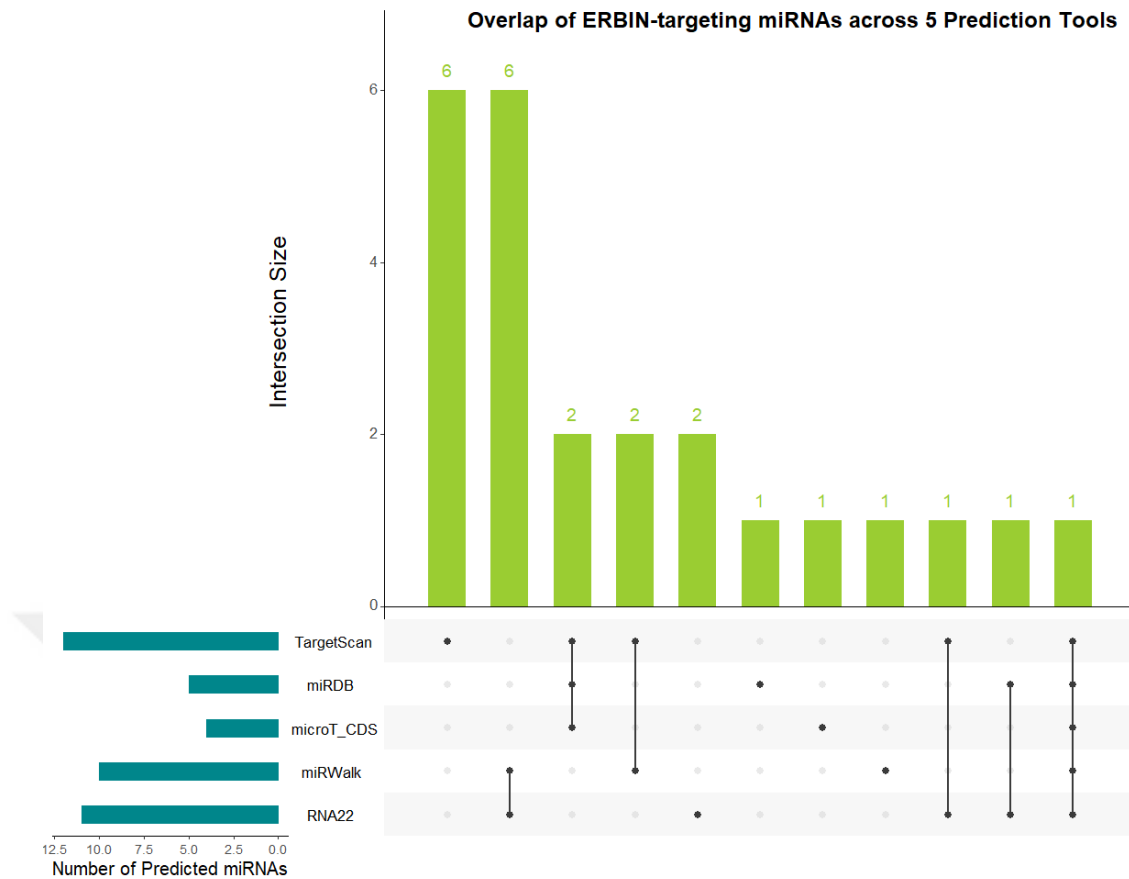


Figure 15. UpSet plot showing the numbers and overlaps of potential ERBIN-targeting miRNAs predicted by TargetScan, miRDB, microT-CDS, miRWalk, and RNA22.

Each vertical green bar represents the number of miRNAs shared by the specific combination of tools indicated by connected black dots in the matrix below. Horizontal bars on the left indicate the total number of predicted miRNAs for each tool.

The UpSet plot in Figure 15 demonstrates the overlaps and number of predicted miRNAs that potentially target the ERBIN gene in breast cancer across five prediction tools: TargetScan, miRDB, microT-CDS (DIANA), miRWalk, and RNA22. Accordingly, only TargetScan predicted six miRNAs. Both miRWalk and RNA22 commonly predicted the second set of six miRNAs. In addition, two common miRNAs are predicted by three tools: TargetScan, miRDB, and microT-CDS, two miRNAs predicted by both TargetScan and miRWalk, and two miRNAs predicted by only RNA22. Only one miRNA is predicted by miRDB, one by microT-CDS, and one by miRWalk. Moreover, the TargetScan and RNA22 together predict one miRNA, as it is in miRDB and RNA22. Only one miRNA is predicted by all the target prediction tools used in this study. The bar chart on the left summarizes the total number of miRNAs

predicted by each tool individually, with TargetScan, RNA22, and miRWalk predicting the highest numbers.

Table 14 lists those miRNAs ordered by their correlation coefficients and with the tool by which they were predicted.

Table 14. Negatively correlated candidate miRNAs targeting ERBIN were predicted by multiple tools and ordered by correlation with ERBIN.

The lists of 23 miRNAs showing a negative correlation with ERBIN expression in the GSE19783 dataset are predicted to target ERBIN using at least one computational prediction tool.

	miRNA	Correlation	Prediction Tool
1	hsa-miR-135b-5p	-0.4534720	RNA22
2	hsa-miR-505-5p	-0.4266061	miRWalk, RNA22
3	hsa-miR-9-5p	-0.4226451	miRWalk, TargetScan
4	hsa-miR-505-3p	-0.4113849	miRWalk, RNA22
5	hsa-miR-146b-5p	-0.4049938	miRWalk, RNA22
6	hsa-miR-301b-3p	-0.3879425	microT-CDS, miRDB, TargetScan
7	hsa-miR-106b-3p	-0.3869076	miRWalk, RNA22
8	hsa-miR-155-5p	-0.3801819	RNA22, TargetScan
9	hsa-miR-17-5p	-0.3624862	TargetScan
10	hsa-miR-106b-5p	-0.3574774	miRWalk, TargetScan
11	hsa-miR-421	-0.3509680	microT-CDS, TargetScan
12	hsa-miR-93-5p	-0.3428958	TargetScan
13	hsa-miR-362-5p	-0.3394213	miRWalk
14	hsa-miR-455-5p	-0.3354200	TargetScan
15	hsa-miR-20a-5p	-0.3203816	TargetScan
16	hsa-miR-519a-3p	-0.3181068	miRDB
17	hsa-miR-130b-3p	-0.3010105	microT-CDS, miRDB, miRWalk, RNA22, TargetScan
18	hsa-miR-650	-0.2991786	miRWalk, RNA22
19	hsa-miR-301a-3p	-0.2935056	microT-CDS, miRDB, TargetScan
20	hsa-miR-20b-5p	-0.2887006	TargetScan
21	hsa-miR-545-3p	-0.2727390	miRDB, RNA22
22	hsa-miR-431-3p	-0.2706181	RNA22
23	hsa-miR-142-3p	-0.2670024	miRWalk, RNA22

To investigate candidate miRNAs further and determine whether they regulate the downregulation of ERBIN in breast cancer, we observed their differential expression in patients with relatively lower and higher ERBIN expression in GSE19783.

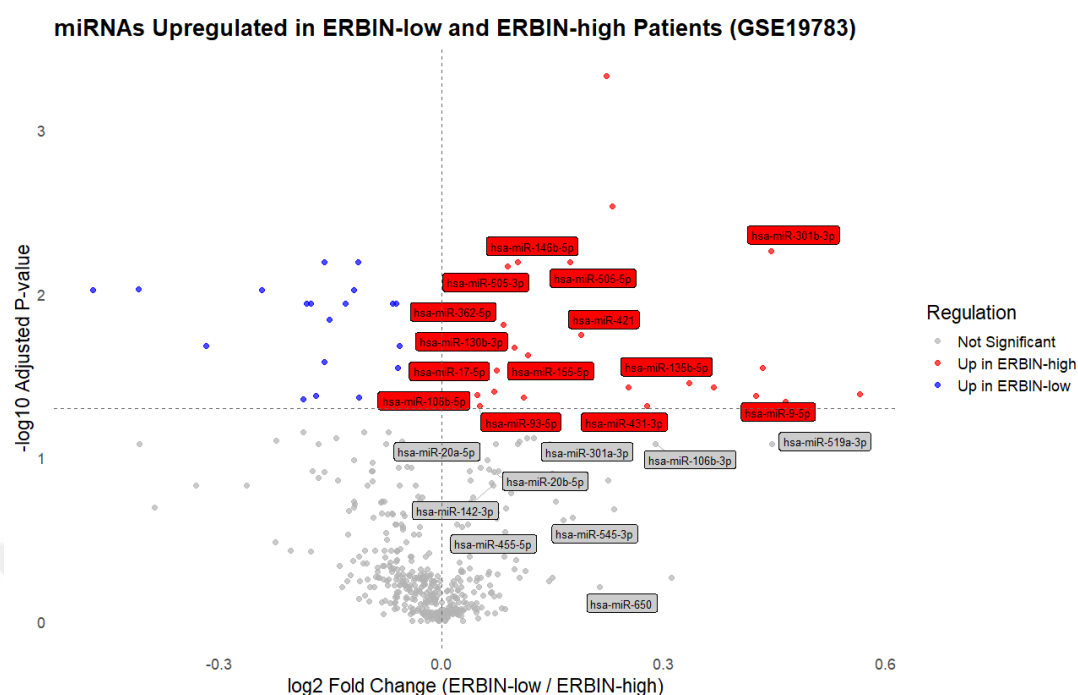


Figure 16. Volcano plot of miRNA expression between ERBIN-Low and ERBIN-High Breast Cancer Patients (GSE19783).

The x-axis represents the log₂ fold change of miRNA expression (ERBIN-low / ERBIN-high), and the y-axis represents the $-\log_{10}$ adjusted p-value. miRNAs predicted to target ERBIN and significantly upregulated in the ERBIN-low group are highlighted in red. Downregulated miRNAs are shown in blue, and non-significant ones are shown in grey. Only the 23 candidate miRNAs were labeled.

We profiled differential miRNA expression between ERBIN-high and ERBIN-low tumors in GSE19783 using Benjamini–Hochberg adjustment. The volcano plot in Figure 16 visualizes the distribution of miRNAs based on their expression and against statistical significance (y-axis). Of the 23 candidate miRNAs, only 14 showed upregulation in patients expressing ERBIN at low levels. The most significantly upregulated miRNAs in tumor samples with high ERBIN expression ($\log_2\text{FC} > 0$, adj. $p < 0.05$) are miR-301b-3p, miR-505-5p, miR-146b-5p, miR-505-3p, miR-362-5p, and miR-421, representing the candidates most compatible with inverse regulation of ERBIN.

Table 15 shows several transcripts targeted by the same miRNA from different binding sites of the ERBIN mRNA.

Table 15. Predicted binding sites of candidate miRNAs on ERBIN transcript variants.

Candidate miRNAs predicted to bind ERBIN are listed with transcript-specific binding sites, predicted binding region, and the tools they are predicted by. (Since microT-CDS does not specify which transcript is selected for target prediction, the gene ID is provided in the “ERBIN Transcript ID” column.) (PC: Poorly Conserved, C: Conserved, binding site conservation status, determined by TargetScan)

miRNA Name	ERBIN Transcript ID	Binding Site (nt)	Binding Region	Prediction Tools
hsa-miR-106b-3p	NM_001253697	3703-3724	CDS	miRWalk
	NM_001253698	3703-3724	CDS	miRWalk
	NM_001253699	3703-3724	CDS	miRWalk
	NM_001253701	3691-3712	CDS	miRWalk
	NM_018695	3703-3724	CDS	miRWalk
	NM_001006600	3703-3724	CDS	miRWalk
	NM_00100660	3609-3630	CDS	miRWalk
	ENST00000284037	5079-5102	3UTR	RNA22
	ENST00000284037	5780-5801	3UTR	RNA22
	ENST00000380943	4875-4898	3UTR	RNA22
	ENST00000380943	5576-5597	3UTR	RNA22
hsa-miR-106b-5p	NM_001253697	6807-6823	3UTR	miRWalk
	NM_001253698	6609-6625	3UTR	miRWalk
	NM_001253699	6828-6844	3UTR	miRWalk
	NM_001253701	6672-6688	3UTR	miRWalk
	NM_018695	6684-6700	3UTR	miRWalk
	ENST00000284037.5	1711-1717 (PC)	3UTR	TargetScan
hsa-miR-130b-3p	NM_001253697	1229-1248	CDS	miRWalk
	NM_001253698	1229-1248	CDS	miRWalk
	NM_001253699	1229-1248	CDS	miRWalk
	NM_001253701	1229-1248	CDS	miRWalk
	NM_018695	1229-1248	CDS	miRWalk
	ENST00000284037.5	1740-1746 (C)	3UTR	TargetScan
	ENST00000284037.5	1528-1534 (PC)	3UTR	TargetScan
	ENSG00000112851	1513-1533	3UTR	microT-CDS
	ENSG00000112851	1726-1745	3UTR	microT-CDS
	ENSG00000112851	2262-2285	3UTR	microT-CDS
	ENSG00000112851	2879-2903	3UTR	microT-CDS
	NM_001006600	1528-1740	3UTR	miRDB
	ENST00000284037	2256-2276	CDS	RNA22
	ENST00000380943	2175-2195	CDS,3UTR	RNA22
	ENST00000380938	2121-2141	CDS	RNA22
	ENST00000508515	1926-1946	CDS	RNA22
	ENST00000511297	1992-2012	CDS	RNA22
	ENST00000506030	1958-1978	CDS	RNA22
hsa-miR-135b-5p	ENST00000284037	5742-5764	3UTR	RNA22
	ENST00000380943	5538-5560	3UTR	RNA22
hsa-miR-142-3p	NM_001253697	5536-5593	3UTR	miRWalk
	NM_001253698	5338-5395	3UTR	miRWalk
	NM_001253699	5557-5614	3UTR	miRWalk
	NM_001253701	5401-5458	3UTR	miRWalk
	NM_018695	5413-5470	3UTR	miRWalk
	ENST00000284037	5670-5692	3UTR	RNA22
	ENST00000380943	5466-5488	3UTR	RNA22
hsa-miR-146b-5p	NM_001253697	5250-5293	3UTR	miRWalk
	NM_001253698	5052-5095	3UTR	miRWalk
	NM_001253699	5271-5314	3UTR	miRWalk
	NM_001253701	5115-5158	3UTR	miRWalk
	NM_018695	5127-5170	3UTR	miRWalk

miRNA Name	ERBIN Transcript ID	Binding Site (nt)	Binding Region	Prediction Tools
hsa-miR-155-5p	ENST00000284037.5 ENST00000284037.5 ENST00000284037.5 ENST00000284037	3090-3097 (PC) 3168-3174 (PC) 3332-3339 (PC) 7782-7802	3UTR 3UTR 3UTR 3UTR	TargetScan TargetScan TargetScan RNA22
hsa-miR-17-5p	ENST00000284037.5	1711-1717 (PC)	3UTR	TargetScan
hsa-miR-20a-5p	ENST00000284037.5	1711-1717 (PC)	3UTR	TargetScan
hsa-miR-20b-5p	ENST00000284037.5	1711-1717 (PC)	3UTR	TargetScan
hsa-miR-301a-3p	ENST00000284037.5 ENST00000284037.5 ENSG00000112851 ENSG00000112851 ENSG00000112851 ENSG00000112851 NM_001006600	1740-1746 (C) 1528-1534 (PC) 1513-1533 1725-1745 2267-2285 2879-2903 1528-1740	3UTR 3UTR 3UTR 3UTR 3UTR 3UTR 3UTR	TargetScan TargetScan microT-CDS microT-CDS microT-CDS microT-CDS miRDB
hsa-miR-301b-3p	ENST00000284037.5 ENST00000284037.5 ENSG00000112851 ENSG00000112851 ENSG00000112851 ENSG00000112851 NM_001006600	1740-1746 (C) 1528-1534 (PC) 1513-1533 1725-1745 2267-2285 2879-2903 1528-1740	3UTR 3UTR 3UTR 3UTR 3UTR 3UTR 3UTR	TargetScan TargetScan microT-CDS microT-CDS microT-CDS microT-CDS miRDB
hsa-miR-362-5p	NM_001253697 NM_001253698 NM_001253699 NM_001253701 NM_018695 NM_001006600	1551-1595 1551-1595 1551-1595 1551-1595 1551-1595 1551-1595	CDS CDS CDS CDS CDS CDS	miRWalk miRWalk miRWalk miRWalk miRWalk miRWalk
hsa-miR-421	ENST00000284037.5 ENSG00000112851 ENSG00000112851 ENSG00000112851 ENSG00000112851 ENSG00000112851 ENSG00000112851	189-196 (PC) 168-194 1834-1848 2508-2527 2682-2699 2516-2536 2693-2717	3UTR 3UTR 3UTR 3UTR 3UTR 3UTR 3UTR	TargetScan microT-CDS microT-CDS microT-CDS microT-CDS microT-CDS microT-CDS
hsa-miR-431-3p	ENST00000284037 ENST00000284037 ENST00000284037 ENST00000380943 ENST00000380943 ENST00000380943 ENST00000380938 ENST00000380938 ENST00000508515 ENST00000508515 ENST00000511297 ENST00000511297 ENST00000506030 ENST00000506030	2406-2425 2639-2661 5073-5093 2325-2344 2558-2580 4869-4889 2271-2290 2504-2526 2076-2095 2309-2331 2142-2161 2375-2397 2108-2127 2341-2363	CDS CDS 3UTR CDS CDS 3UTR CDS CDS CDS CDS CDS CDS CDS	RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22 RNA22
hsa-miR-455-5p	ENST00000284037.5	2083-2089 (PC)	3UTR	TargetScan
hsa-miR-505-3p	NM_001253697 NM_001253698 NM_001253699 NM_001253701 NM_018695 ENST00000284037 ENST00000380943	4985-5004 4787-4806 5006-5025 4850-4869 4862-4881 5084-5104 4880-4900	3UTR 3UTR 3UTR 3UTR 3UTR 3UTR 3UTR	miRWalk miRWalk miRWalk miRWalk miRWalk RNA22 RNA22

miRNA Name	ERBIN Transcript ID	Binding Site (nt)	Binding Region	Prediction Tools
hsa-miR-505-5p	NM_001253697	1471-1486	CDS	miRWalk
	NM_001253698	1471-1486	CDS	miRWalk
	NM_001253699	1471-1486	CDS	miRWalk
	NM_001253701	1471-1486	CDS	miRWalk
	NM_018695	1471-1486	CDS	miRWalk
	NM_001006600	666-682	CDS	miRWalk
	NM_001006600	1471-1486	CDS	miRWalk
	ENST00000284037	2411-2436	CDS	RNA22
	ENST00000380943	2330-2355	CDS	RNA22
	ENST00000380938	2276-2301	CDS	RNA22
	ENST00000508515	2081-2106	CDS	RNA22
	ENST00000511297	2147-2172	CDS	RNA22
	ENST00000506030	2113-2138	CDS	RNA22
hsa-miR-519a-3p	NM_001006600	1529-1741	3UTR	miRDB
hsa-miR-545-3p	NM_001253701	2441-2448	3UTR	miRDB
	ENST00000284037	5731-5753	3UTR	RNA22
	ENST00000380943	5527-5549	3UTR	RNA22
hsa-miR-650	NM_001006600	3712-3734	CDS	miRWalk
	NM_001253697	208-225	5UTR	miRWalk
	NM_001253698	208-225	5UTR	miRWalk
	NM_001253699	208-225	5UTR	miRWalk
	NM_001253701	208-225	5UTR	miRWalk
	NM_018695	208-225	5UTR	miRWalk
	NM_001006600	208-225	5UTR	miRWalk
	ENST00000284037	3403-3422	CDS	RNA22
	ENST00000284037	5088-5107	3UTR	RNA22
	ENST00000380943	3322-3341	CDS	RNA22
	ENST00000380943	4884-4903	3UTR	RNA22
	ENST00000380938	3268-3287	CDS	RNA22
	ENST00000508515	3073-3092	CDS	RNA22
	ENST00000511297	3139-3158	CDS	RNA22
	ENST00000506030	3105-3124	CDS	RNA22
hsa-miR-93-5p	ENST00000284037.5	1711-1717 (PC)	3UTR	TargetScan
hsa-miR-9-5p	ENST00000284037.5	1079-1085 (C)	3UTR	TargetScan
	NM_001006600	5264-5284	3UTR	miRWalk
	NM_001006600	3680-3703	CDS	miRWalk

Even though the ERBIN miRNA transcript-specific binding sites differ with different miRNA target prediction tools, the binding sequences observed can be the same or highly similar across different ERBIN transcripts and transcript isoforms. The same miRNAs can also bind to the ERBIN transcript from different binding sites with the same or similar binding sequences. As in Table 15, different miRNA target predictions might use different transcript annotations or systems for determining the binding sequences. Therefore, we focused on determining the binding sequences that are biologically relevant for each candidate miRNA by selecting the most expressed and canonical transcripts and the sequences that have the highest binding efficiencies with their miRNA, as seen mostly in target prediction tools. These sequences are presented in Table 16 and could be valuable for further studies.

Table 16. Predicted base-pairing interactions between selected miRNAs and ERBIN mRNAs.

Sequence alignments are shown with predicted interactions between the ERBIN mRNA (5'-3', upper strand) and selected mature miRNAs (3'-5', lower strand). The blue-colored nucleotides indicate positions involved in base pairing, while the red-colored nucleotides are unpaired. The vertical lines represent Watson-Crick base pairing, while green bases indicate G: U wobble interactions. The underlined regions in miRNAs indicate the seed sequences.

Heteroduplex Form of miRNA-mRNA Binding Sequences	Summary
<pre> 5' - UCCUU UG C U - 3' (ERBIN, 3UTR) GAG GCC C AGUGUG UUC UGG G UCACGC 3' - CGUCG A GU C - 5' (miR-106b-3p) </pre>	The prediction by RNA22, which is the 3'UTR binding region of miR-106b-3p on transcript ENST00000284037 (Ensembl ID) (5780-5801 site), is selected with strong base pairing ($\Delta G = -15.30$ kcal/mol) and a significant p-value (< 0.05).
<pre> 5' - UGCACUG GCAUUUUA U - 3' (ERBIN, 3UTR) ACGUGAC CGUGAAAU 3' - UAG AGU - 5' (miR-106b-5p) </pre>	The prediction by miRWalk, which is the 3'UTR binding region of miR-106b-5p on transcripts NM_001253697 (RefSeq ID) (6807-6823), is selected with strong base pairing ($\Delta G = -17.7$ kcal/mol) and significant miRWalk scoring (1.0).
<pre> 5' - AA CAAA U - 3' (ERBIN, 3UTR) GUG CU UAUUGCACU UAC GA GUAACGUGA 3' - GG AAGUA C - 5' (miR-130b-3p) </pre>	The binding site of miR-130b-3p in the 3'UTR, as predicted by microT-CDS, was selected due to a high number of conserved species supporting the site.
<pre> 5' - GAGAAUUGCU - 3' (ERBIN, 3UTR) GUGGAGAGUUUAU UACUUUUCGGUAU 3' - AGUGUAUCCU - 5' (miR-135b-5p) </pre>	The ENST00000284037 (Ensembl ID), which is the canonical transcript, is selected from RNA22 as the binding sequence from 5742-5764 side for miR-135b-5p with strong base pairing ($\Delta G = -13.10$ kcal/mol) and significant p-value (< 0.05).
<pre> 5' - G AAA G C - 3' (ERBIN, 3UTR) UU GUAAAGU AGGAAACA UACA AG UAUUUA UCCUUUGU AUGU 3' - G G - 5' (miR-142-3p) </pre>	The miRWalk prediction tool is selected to present the ERBIN mRNA and miR-142-3p binding sequence due to its longer base pairing length and significant miRWalk score (1.0).
<pre> 5' - U AC U A - 3' (ERBIN, 3UTR) GCU UGG GAUUCAGUUUUUA CGG ACC UUAAGUCAAGAGU 3' - GU AU - 5' (miR-146b-5p) </pre>	The canonical ERBIN transcript, NM_001253697 (RefSeq ID), is selected from miRWalk as the binding sequence from 5536- 5593 for miR-146b-5p with strong base pairing ($\Delta G = -22.30$ kcal/mol) and significant miRWalk score (1.0).
<pre> 5' - AA - 3' (ERBIN, 3UTR) ACUUCUA G GA GGCAUUAA UGGGGAU U CU UCGUAAAU 3' - AG G AA - 5' (miR-155-5p) </pre>	RNA22 binding sequence prediction is selected for the canonical ERBIN transcript from 7782-7802 sequence and miR-155-5p with strong base pairing ($\Delta G = -14.30$ kcal/mol) and significant p-value (< 0.05).
<pre> 5' - CAGAGUAAGUAGGCA U - 3' (ERBIN, 3UTR) GCACUUU CGUGAAA 3' - GAUGGACGUGACAUU C - 5' (miR-17-5p) </pre>	The binding sequence prediction is generated from TargetScan from 1711-1717 of the ENST00000284037.5 transcript based on only the seed sequence of miR-17-5p.
<pre> 5' - CAGAGUAAGUAGGCA U - 3' (ERBIN, 3UTR) GCACUUU CGUGAAA 3' - GAUGGACGUGAUAAU U - 5' (miR-20a-5p) </pre>	The binding sequence prediction is generated from TargetScan from 1711-1717 of the ENST00000284037.5 transcript based on only the seed sequence of miR-20a-5p.

Heteroduplex Form of miRNA-mRNA Binding Sequences	Summary
5'- CAGAGUAAGUAGG U - 3' (ERBIN, 3UTR) CAGCACUUU GUUCGUGAAA 3'- GAUGGACGUGCUU C -5' (miR-93-5p)	The binding sequence prediction is generated from TargetScan from 1711-1717 of the ENST00000284037.5 transcript based on only the seed sequence of miR-93-5p.
5'- ACUUGG GAA C - 3' (ERBIN, 3UTR) AGCUAG ACCAAAG UCGAUC UGGUUUC 3'- AGUAUG UAU U -5' (miR-9-5p)	The binding sequence prediction is used from TargetScan from 1079-1085 of the ENST00000284037.5 transcript, based on only the seed sequence matching of miR-9-5p, determined as a highly conserved region.

Candidate miRNAs Grouped by Family (TAM 2.0)

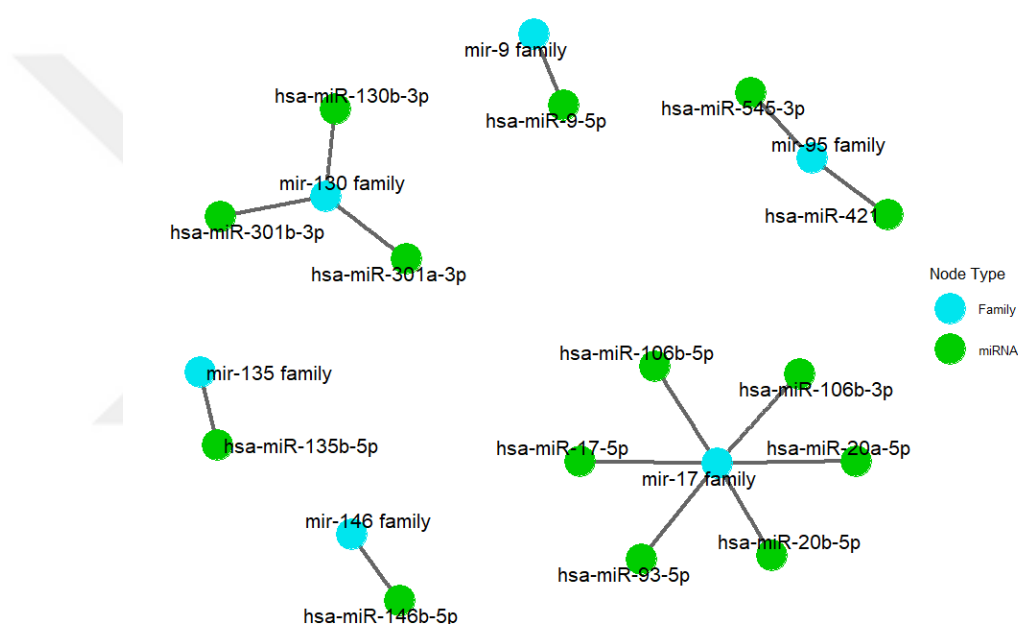


Figure 17. The network diagram illustrates the classification of candidate miRNAs into families using the TAM 2.0 database.

Green nodes show candidate human miRNAs, while blue nodes indicate the miRNA families they belong to.

Figure 17 illustrates candidate miRNAs (n=23) grouped according to the miRNA families they belong to by using the TAM 2.0 tool, which classifies human miRNAs according to the seed sequence similarity and the miRBase database. In total, six families were identified among the candidate miRNAs, including mir-130, mir-95, mir-17, mir-146, and mir-135 families. Some of the families contain multiple candidate miRNAs, such as the mir-130 family (hsa-miR-130b-3p, hsa-miR-301a-3p, hsa-miR-301b-3p) and the mir-17 family (hsa-miR-106b-5p, hsa-miR-106b-3p, hsa-miR-20a-

5p, hsa-miR-20b-5p, hsa-miR-93-5p, and hsa-miR-17-5p), while some of them (e.g., mir-9, mir-135, and mir-146) include only one miRNA among candidate miRNAs.

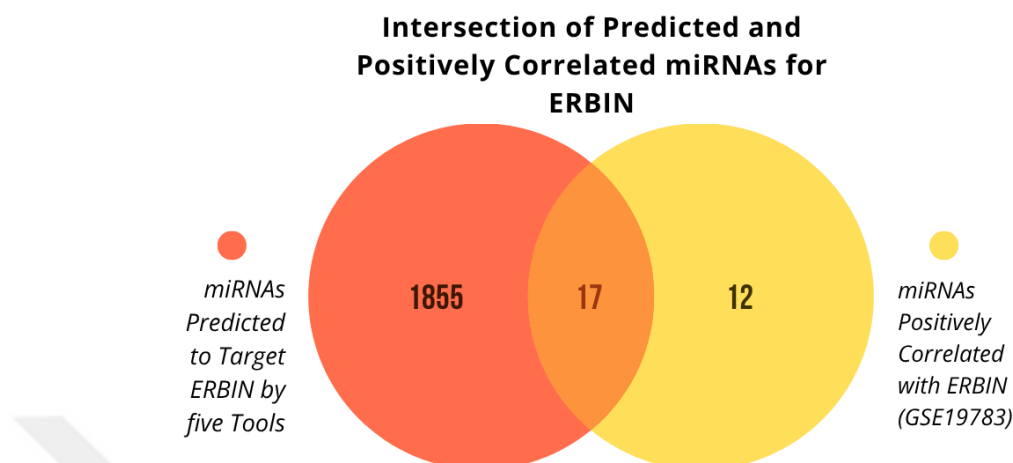


Figure 18. Intersection of predicted and positively correlated miRNAs for ERBIN.

The Venn diagram shows the intersection between miRNAs predicted to target ERBIN by five prediction tools (miRWalk, miRDB, TargetScan, RNA22, and DIANA microT-CDS) and miRNAs positively correlated with ERBIN expression based on patient tumor data from GSE19783.

In addition to negatively correlated miRNAs that may directly reduce ERBIN expression, we also evaluated positively correlated miRNAs, which might regulate ERBIN indirectly. The Venn diagram in Figure 18 shows the overlap of 1872 miRNAs predicted to target ERBIN by five different prediction tools, with 29 positively correlated miRNAs. As a result, 17 miRNAs were positively correlated with ERBIN expression in GSE19783 breast cancer patient data and target ERBIN transcripts. This filtering approach was applied to reduce the dataset to produce a list of transcription factors that may be more biologically relevant to ERBIN regulation.

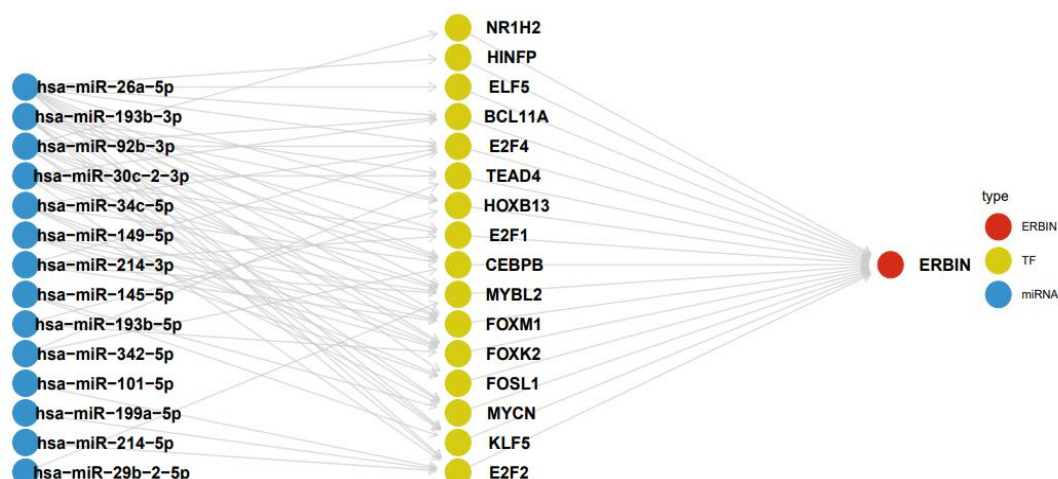


Figure 19. Network graph shows miRNAs, positively correlated with ERBIN expression, and their transcription factor targets that are negatively correlated with ERBIN in the GSE19783 dataset.

The miRNAs are ordered by the number of targeted transcription factors (TF). Blue nodes are representatives of positively correlated miRNAs, yellow nodes are TFs, and the red node is the node that shows the ERBIN node.

We further explored how the 17 miRNAs, which were found to be positively correlated with ERBIN in the patient dataset, might influence ERBIN expression indirectly. MiRNAs positively correlated with ERBIN, and their experimentally validated transcription factors identified in the GSE19783 dataset were selected. These transcription factors are also negatively correlated with ERBIN expression. The transcription factors targeted by these miRNAs are determined according to the current knowledge of experimental validations by the multiMiR R package.

Among all the TFs targeted by positively correlated miRNAs, we focused on those negatively correlated with ERBIN expression in the GSE19783 breast cancer dataset using Dorothea Database. This approach provided a regulatory framework illustrated in Figure 19, including the positively correlated miRNAs and their validated target TFs, which are negatively correlated with ERBIN expression. The complete list of these interactions is presented in Table 17.

Table 17. List of miRNAs positively correlated with ERBIN expression and their experimentally validated transcription factor targets negatively correlated with ERBIN in GSE19783.

The number of targeted TFs orders the miRNAs.

miRNAs	Transcription Factors
hsa-miR-26a-5p	BCL11A, CEBPB, E2F2, E2F4, ELF5, FOSL1, FOXK2, FOXM1, HINFP, HOXB13, KLF5, MYBL2
hsa-miR-193b-3p	CEBPB, E2F1, E2F2, FOXK2, HOXB13, KLF5, MYCN, TEAD4
hsa-miR-92b-3p	BCL11A, CEBPB, E2F4, FOXM1, KLF5, MYBL2, MYCN, NR1H2
hsa-miR-30c-2-3p	BCL11A, E2F1, E2F2, FOXK2, FOXM1, MYBL2, TEAD4
hsa-miR-34c-5p	CEBPB, E2F4, FOSL1, FOXK2, MYCN
hsa-miR-149-5p	FOSL1, FOXK2, FOXM1, MYBL2
hsa-miR-214-3p	E2F1, E2F4, FOXM1, MYBL2
hsa-miR-145-5p	KLF5, MYCN,
hsa-miR-193b-5p	FOXK2, HOXB13
hsa-miR-342-5p	CEBPB, TEAD4
hsa-miR-101-5p	E2F2
hsa-miR-199a-5p	E2F2
hsa-miR-214-5p	E2F2
hsa-miR-29b-2-5p	MYBL2

Overall, Figure 19 and Table 17 show that among 17 miRNAs, 14 miRNAs target at least one TF that is negatively correlated with ERBIN. Among the analyzed miRNAs, miR-26a-5p, miR-193b-3p, and miR-92b-3p targeted the highest number of TFs negatively correlated with ERBIN expression.

The most frequently targeted transcription factors by these miRNAs are E2F2, FOXK2, MYBL2, CEBPB, and FOXM1. It is worth noting that E2F2, FOXK2, and CEBPB are each targeted by five to six different miRNAs, such as miR-26a-5p and miR-193b-3p, which are the miRNAs that target many TFs, as seen in Figure 19 and Table 17.

3.3. Expression Analysis of Candidate miRNAs Across Breast Cancer Subtypes and Clinical Parameters

To investigate whether miRNAs regulate ERBIN downregulation in breast cancer subtypes, we focused on candidate miRNAs identified through expression correlation and target binding analyses. The GSE19783 breast cancer patient dataset was used primarily because ERBIN downregulation is observed in basal subtypes, estrogen receptor-negative status, and TP53 mutation status within the same tumor samples from the same patients. In addition to the GSE19783 dataset, other miRNA expression datasets of breast cancer patients are analyzed to observe candidate miRNA expression patterns in breast cancer characteristics.

In GSE40267, the expression of candidate miRNAs was compared in basal subtypes and non-basal subtypes, in node-positive and node-negative situations, and according to the tumor size. The GSE37963 breast cancer patient dataset was also included to evaluate candidate miRNAs expression in high and low grades of breast cancer, and in early and late stages of breast cancer. In addition to the subtype and molecular characteristics of breast cancer patients, the impact of candidate miRNAs' expression on survival in patients is also assessed by including the METABRIC dataset.

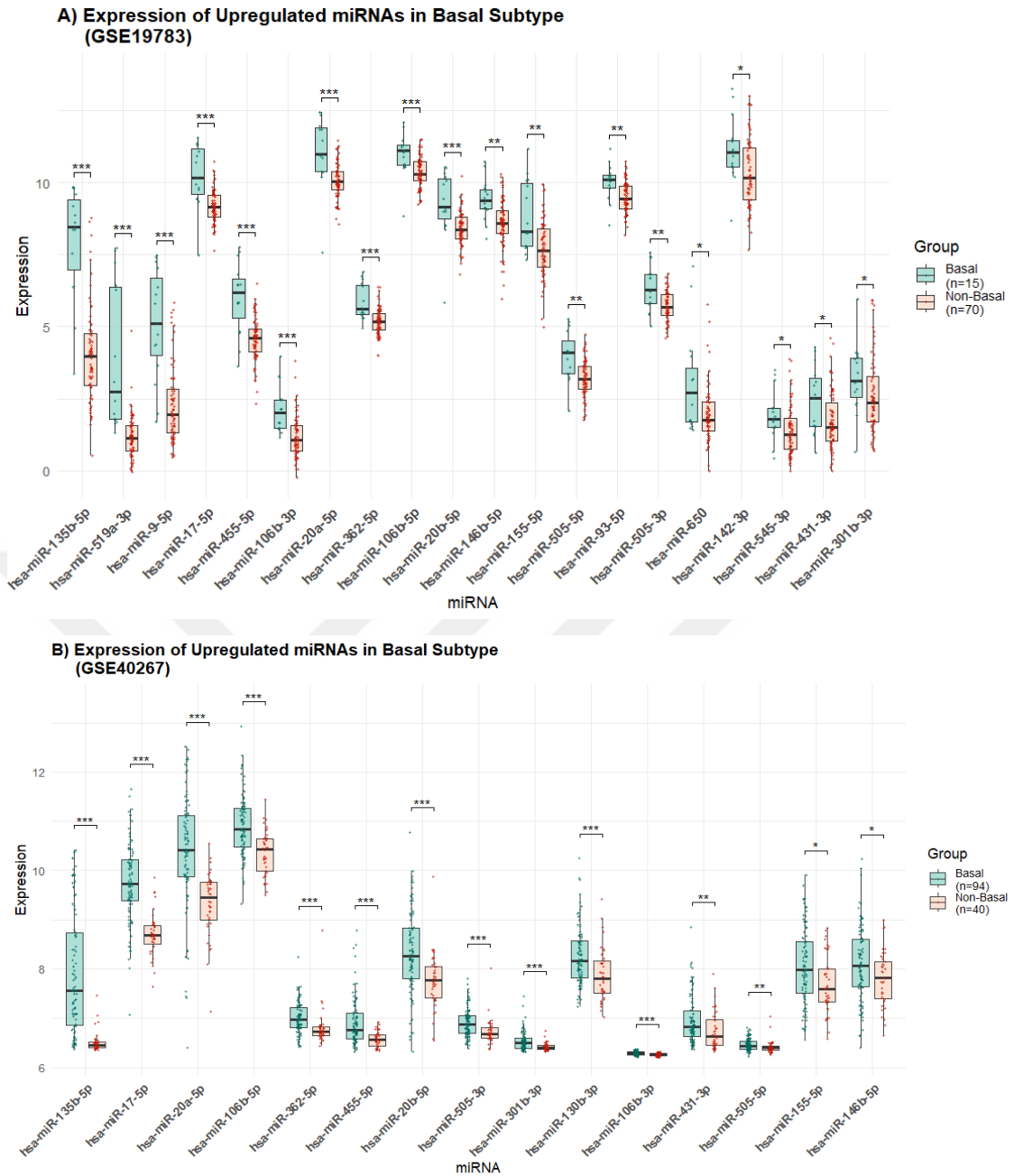


Figure 20. Expression levels of significantly upregulated miRNAs in basal-like breast cancer across two independent datasets (GSE19783, GSE40267), ordered by their statistical significance.

Boxplots show the expression levels of 20 miRNAs for the GSE19783 (A) and 15 miRNAs for the GSE40267 (B) that are significantly upregulated in basal-like breast cancer compared to non-basal subtypes, based on the microarray GSE19783 dataset. The miRNA expression values are normalized. Statistical significance was assessed using the Wilcoxon rank-sum test, and determined thresholds are as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$ (n = sample size).

In the GSE19783 dataset (Figure 20A), in which we observed lower ERBIN expression levels in the basal type of breast cancer compared to the non-basal type, 20 candidate miRNAs out of 23 candidate miRNAs are found significantly lower in basal subtypes. Of these, 10 miRNAs, including miR-135b-5p, miR-9-5p, miR-17-5p, and miR-362-5p, show strong statistical significance ($p < 0.001$, ***). The GSE40267 (Figure 20B) breast cancer patient dataset presents 15 miRNAs that are upregulated in basal-type patients, with 11 highly significant ($p < 0.001$, ***). This dataset supported the findings in GSE19783. 8 miRNAs show the highest significant upregulation in basal type in both GSE19783 and GSE40267 datasets. These are as follows: miR-135b-5p, miR-17-5p, miR-455-5p, miR-106b-3p, miR-20a-5p, miR-362-5p, miR-106b-5p, miR-20b-5p.

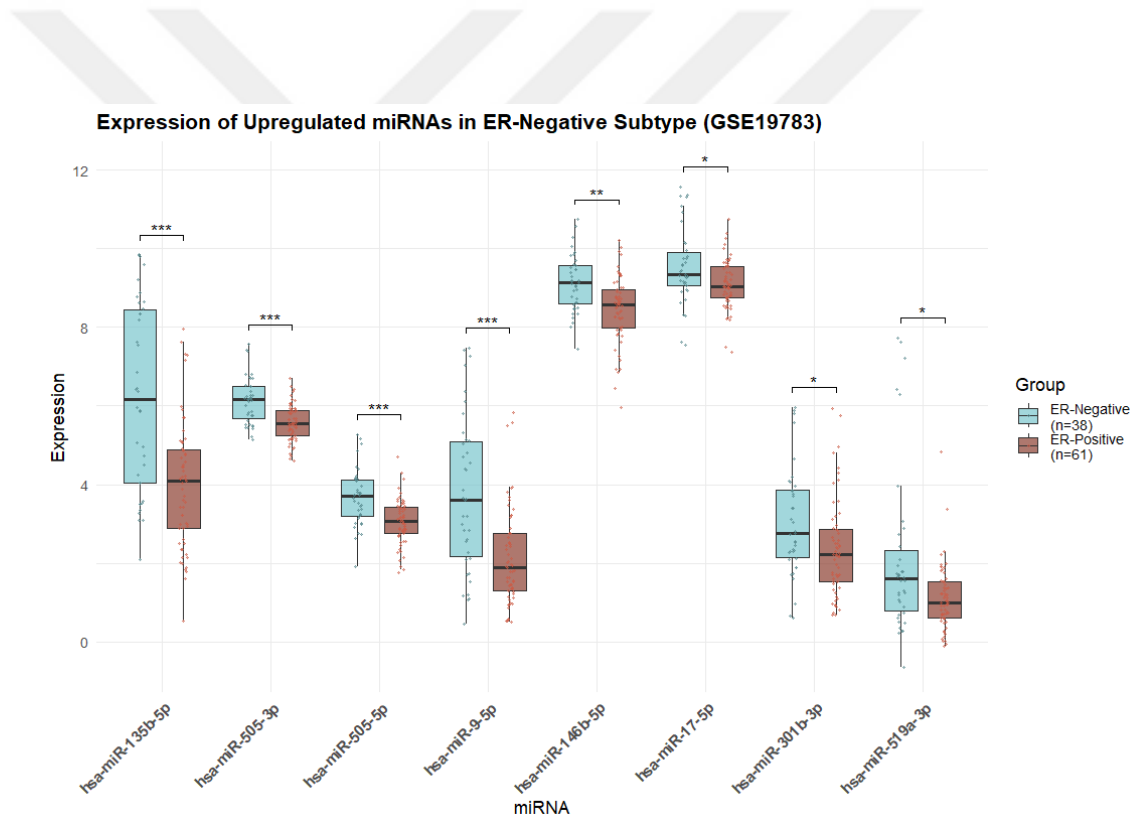


Figure 21. Expression levels of candidate miRNAs, ER-negative versus ER-positive breast cancer samples (GSE19783), ordered by their statistical significance.

Box plots showing the expression of 8 candidate miRNAs that were significantly upregulated in ER-negative samples compared to ER-positive samples, obtained from the GSE19783 dataset, which includes miRNA microarray profiles of breast cancer patients. Statistical comparisons were performed using the Wilcoxon rank-sum test. Significance levels are indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$.

We grouped the breast cancer patients in GSE19783 data according to their estrogen receptor status, which shows lower ERBIN expression when estrogen receptor status is defined as negative. We assessed whether 23 candidate miRNAs show higher expression in estrogen-negative status. The result, in Figure 21, indicates that eight miRNAs among all the candidate miRNAs show significantly high expression in the ER-negative situation compared to the ER-positive situation. Among these significantly upregulated eight miRNAs in ER-negative patients, four miRNAs showed the highest significance, including miR-135b-5p, miR-505-3p, miR-505-5p, and miR-9-5p.

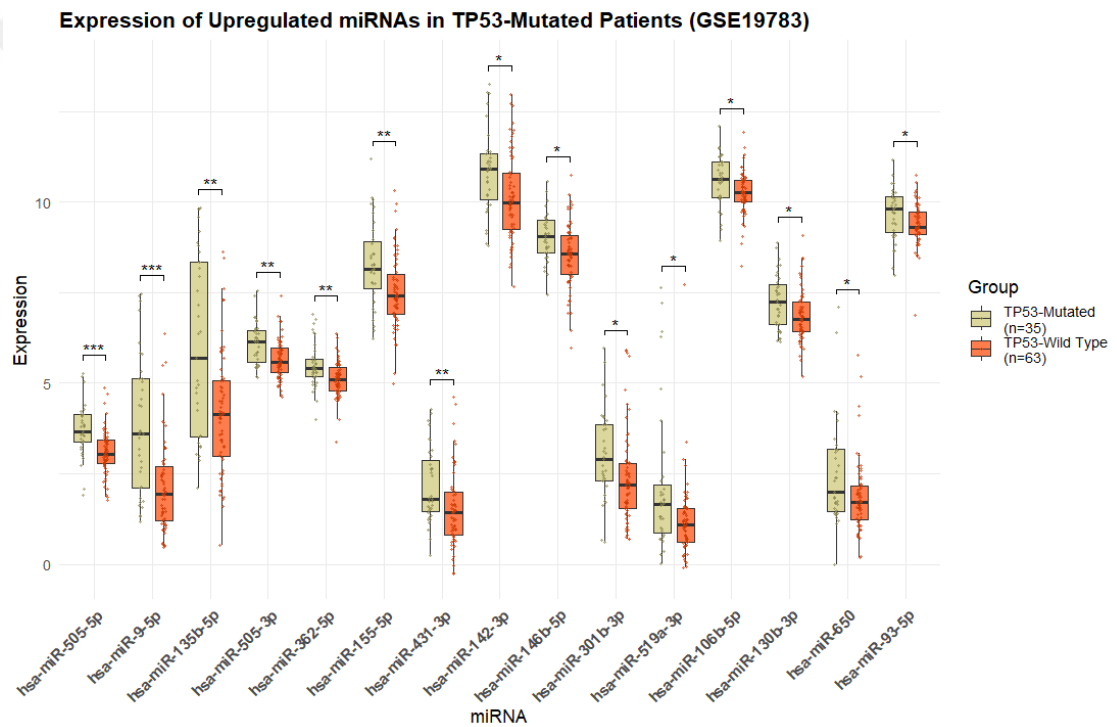


Figure 22. Expression levels of candidate miRNAs in TP53-mutated and wild-type breast cancer samples (GSE19783), ordered by their statistical significance.

Boxplots representing the expression levels of 15 miRNAs significantly upregulated in TP53-Mutated compared to TP53-wild-type (WT) breast cancer samples, from the GSE19783 microarray dataset. Statistical significance was assessed using the Wilcoxon rank-sum test. Significance is shown as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$.

The expression of candidate miRNAs was also evaluated in patients stratified by TP53 mutation status in the GSE19783 dataset (Figure 22). miRNAs showing significantly higher expression levels in TP53-mutated breast cancer were considered, as the

ERBIN expression was lower in these patients. Among 23 candidate miRNAs, 15 showed higher expression in TP53-mutated breast cancer patients. Two miRNAs, miR-505-5p and miR-9-5p, presented the highest significance, and 5 miRNAs, including miR-135b-5p, miR-505-3p, and miR-362-5p, indicated relatively moderate significance in this comparison.

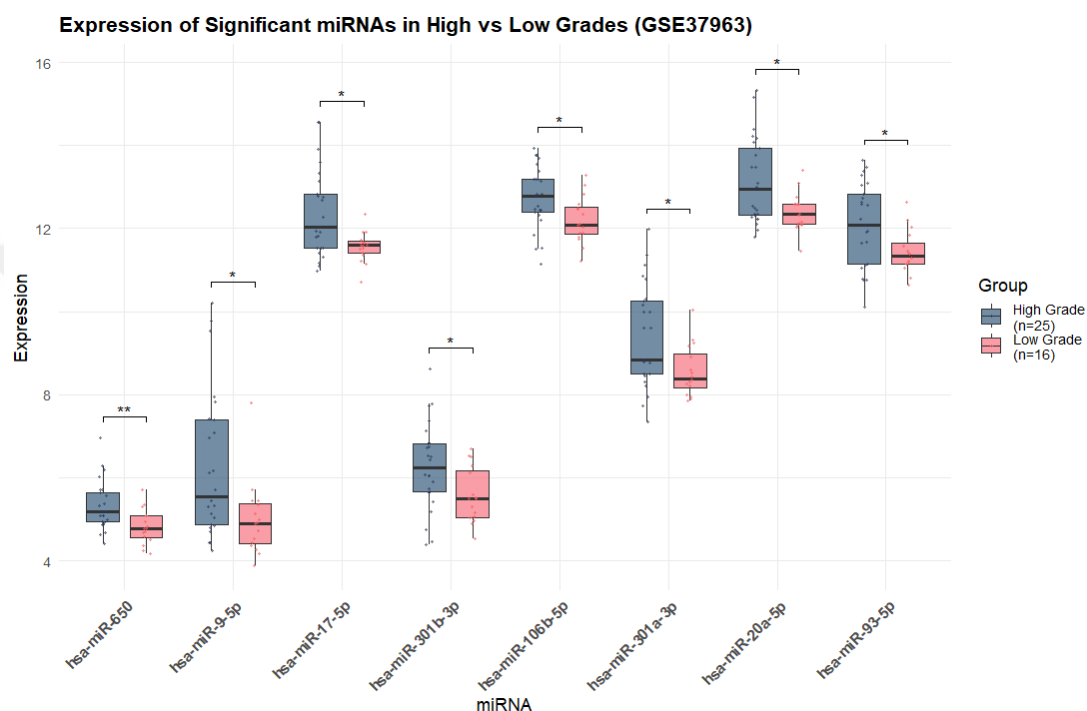


Figure 23. Significantly upregulated miRNAs in high-grade breast cancer samples compared to low-grade samples (GSE37963), ordered by their statistical significance.

Boxplots of the expression levels of selected miRNAs that are significantly upregulated in high-grade (n = 25) compared to low-grade (n = 16) breast cancer samples, based on microarray data from the GSE37963 dataset. Statistical significance was assessed using the Wilcoxon rank-sum test. Significance is shown as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$.

To understand the expression patterns of candidate miRNAs in aggressive breast cancer, we used another set of breast cancer patient data, GSE37963, which has miRNA expression values and tumor grade characteristic information of tumor samples. Tumor grades are determined by the dataset providers based on histopathological evaluation. For our analysis, we classified patients with tumor grade 1 or 2 as low grade, which are less aggressive, and those with tumor grade 3 as high grade, which are more aggressive tumors. In this comparison, as Figure 23 shows, 8 miRNAs, among 23 potential regulators of ERBIN, were expressed at a significantly

higher rate in patients with high tumor grade. These miRNAs are as follows: miR-650, miR-9-5p, miR-17-5p, miR-301b-3p, miR-106b-5p, miR-301a-3p, miR-20a-5p, and miR-93-5p.

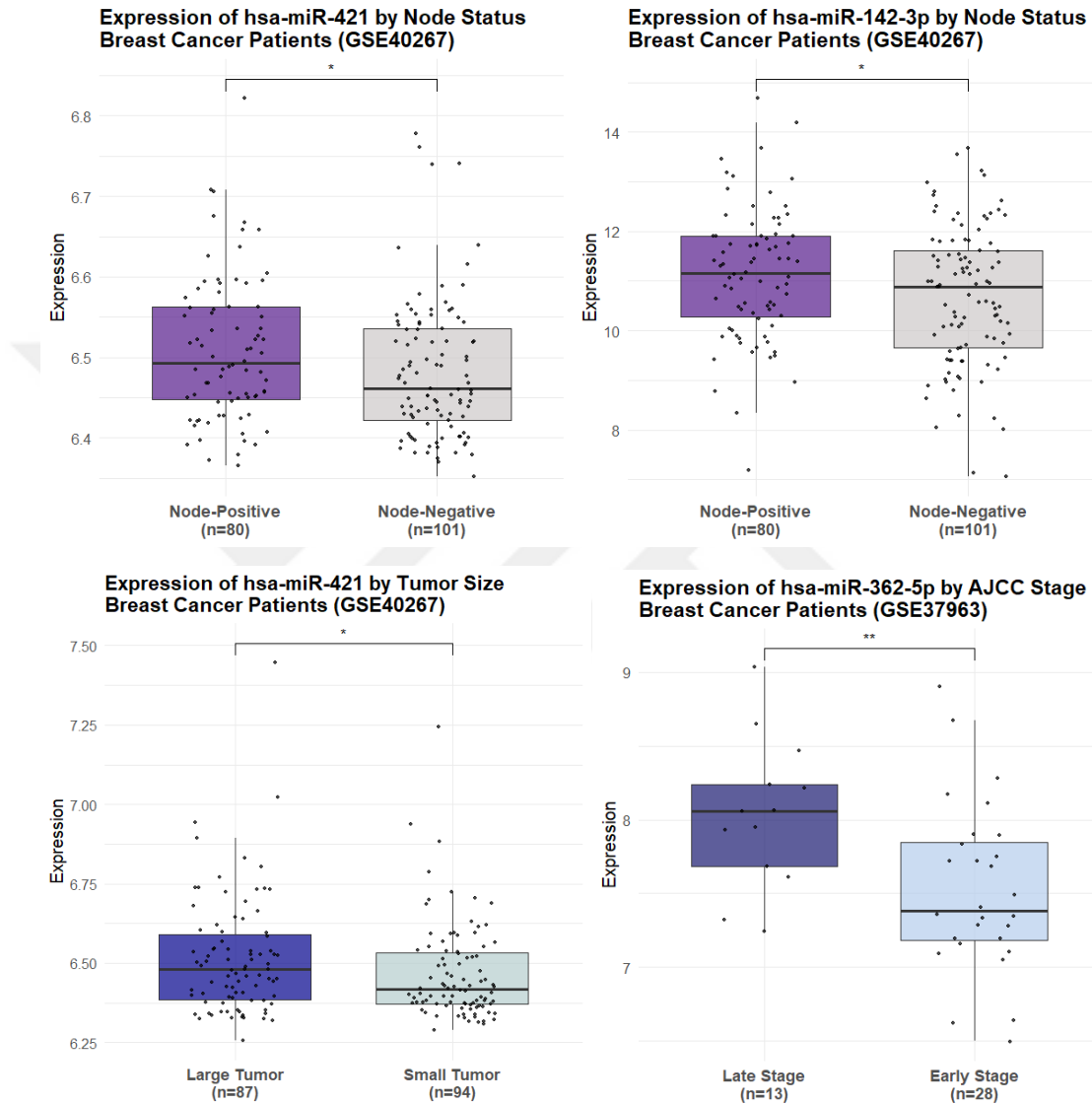


Figure 24. Significantly Upregulated Candidate miRNAs according to Node Status, Tumor Size, and Cancer Stage in Breast Cancer Patients.

hsa-miR-421 expression is significantly higher in node-positive compared to node-negative patients (GSE40267). hsa-miR-142-3p is upregulated in node-positive versus node-negative samples (GSE40267). hsa-miR-421 shows elevated expression in patients with large tumors compared to those with small tumors (GSE40267). hsa-miR-362-5p is significantly upregulated in late-stage compared to early-stage breast cancer patients (GSE37963). Significance is shown as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), $p > 0.05$.

We used the node status, tumor size, and stage status characteristics defined in the GSE40267 and GSE37963 datasets to evaluate the potential miRNA regulators of ERBIN in aggressive types of breast cancer. In the GSE40267 (Figure 24), when we classified patients based on their lymph node status, which indicates the spreading of tumor cells beyond their primary site and their aggressiveness and metastatic features, we identified only two miRNAs that were significantly upregulated in node-positive breast cancer: miR-421 and miR-142-3p.

The GSE40267 (Figure 24) dataset also provides the tumor size of breast cancer in patients with the maximum diameter of the primary tumor at diagnosis. When we grouped the patients by their tumor size, we used the median tumor size of the data. When we evaluated all the candidate miRNAs in the small and large tumor sizes, we found that only miR-421 was upregulated in the large tumor size comparison group, which indicates the spreading capacity and aggressiveness of the tumor.

In GSE37963 (Figure 24), we grouped patients according to their stages determined according to the AJCC cancer staging system developed by the American Joint Committee on Cancer (AJCC). We grouped patients at stages 0, 1, and 2 as early stage and stages 3 and 4 as late stage, and explored the upregulated expression of candidate miRNAs in late stages. Among 23 candidate miRNAs, only miR-362-5p was found to be significantly upregulated in this group.

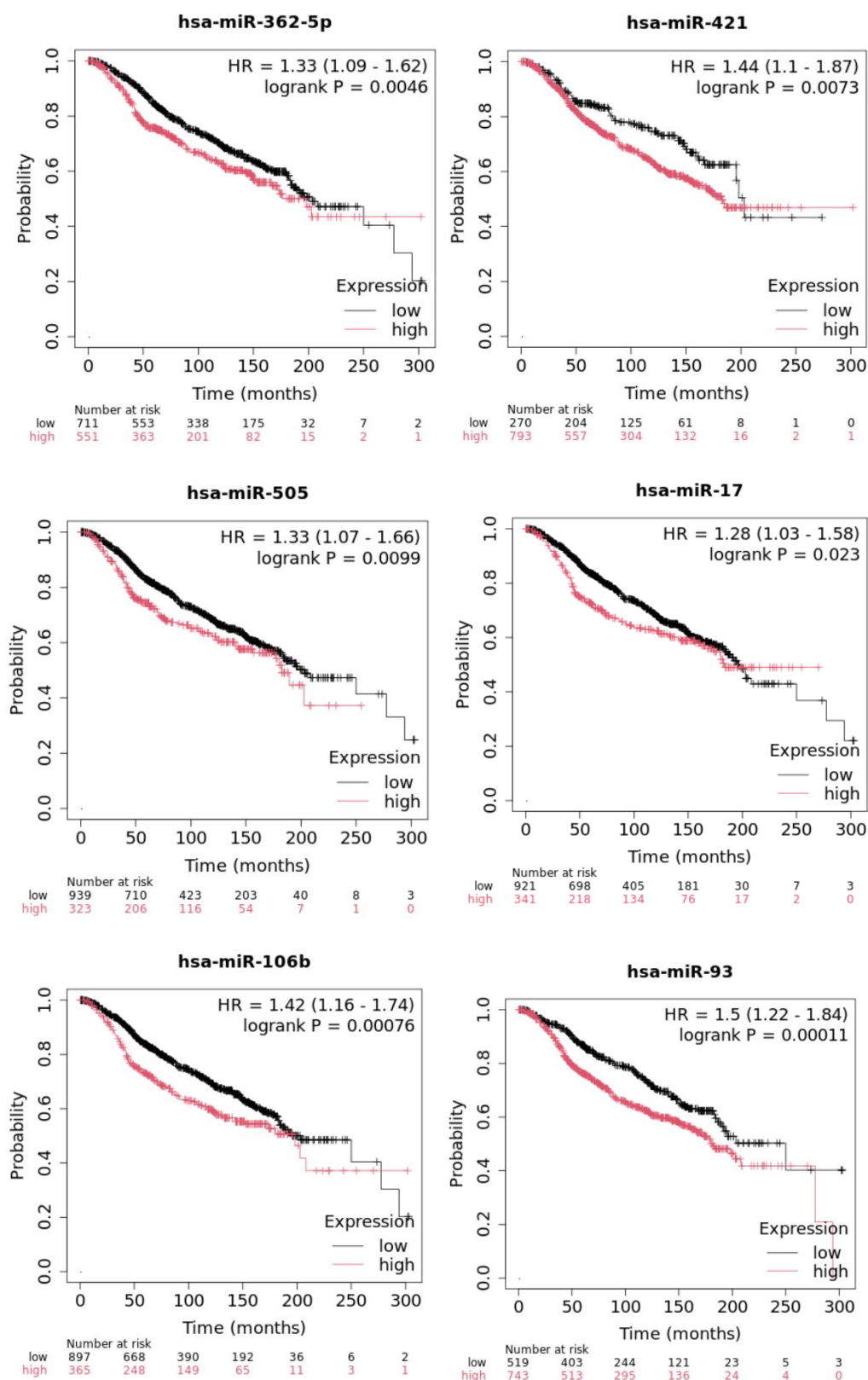


Figure 25. Overall survival of candidate miRNAs (miR-362-5p, miR-421, miR-505, miR-17, miR-106b, and miR-93) (METABRIC) in all subtypes of breast cancer. Patients were divided into high- and low-expression groups based on optimal percentile cutoff. Hazard ratios (HR) with 95% confidence intervals, and log-rank p-values are shown for each miRNA.

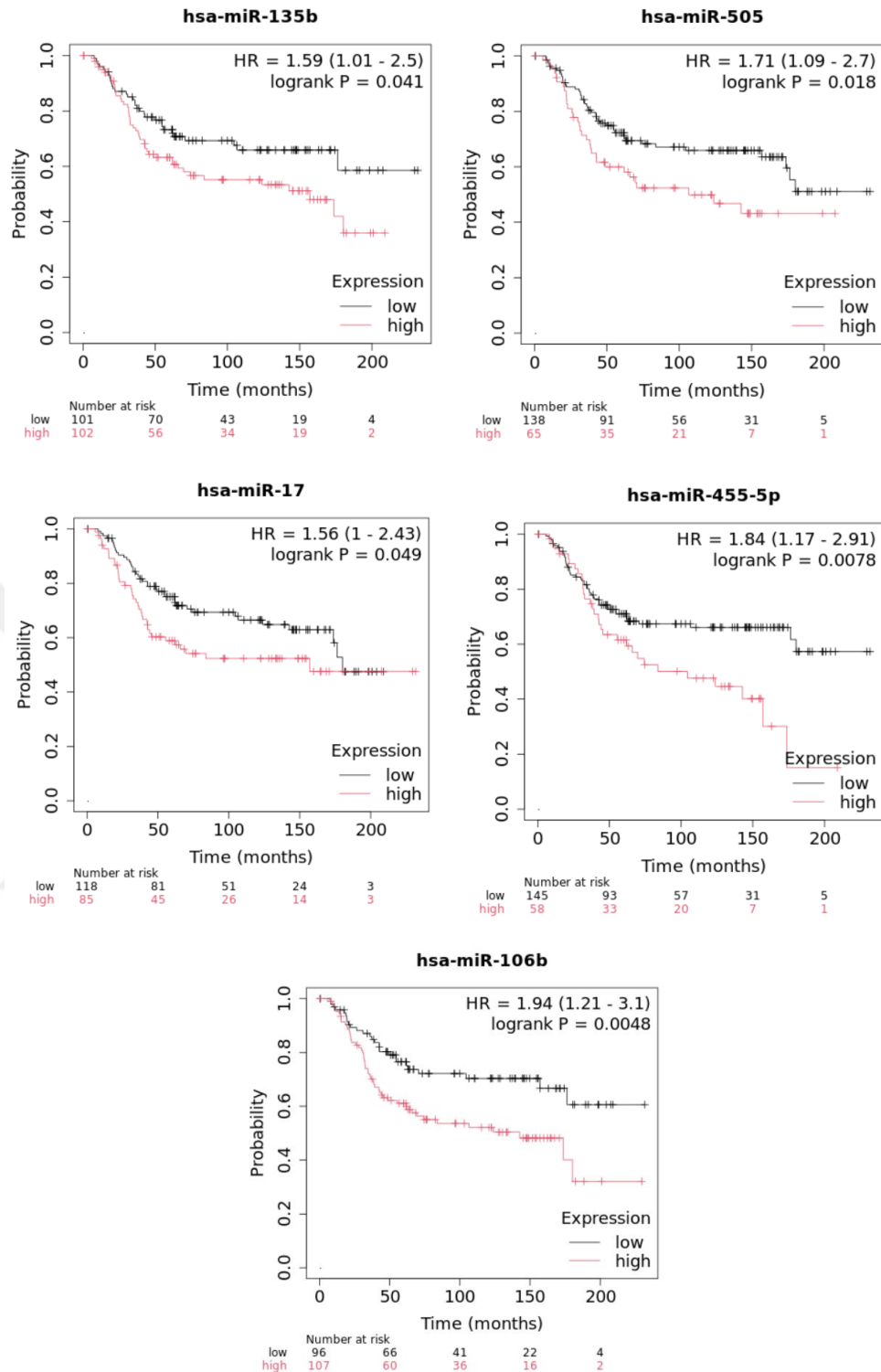


Figure 26. Overall survival of candidate miRNAs (miR-135b, miR-505, miR-17, miR-106b, and miR-455-5p) (METABRIC) in only basal-like breast cancer patients.

Patients were divided into high- and low-expression groups based on optimal percentile cutoff. Hazard ratios (HR) with 95% confidence intervals, and log-rank p-values are shown for each miRNA.

To understand whether the expression levels of the candidate miRNAs were associated with survival outcomes in breast cancer, we performed Kaplan-Meier survival analyses using the KM-Plotter tool using the METABRIC breast cancer patient dataset. A total of the low expression of 8 miRNAs among 23 candidate miRNAs showed significantly better survival when we included all subtypes (Figure 26). When we analyzed only patients with the basal-like subtype in the same dataset, we found 5 miRNAs having an association between their low expression and longer overall survival times.

Overall, these results indicate that low expression of several candidate miRNAs is related to longer overall survival, which is the opposite trend observed for ERBIN expression in different datasets than METABRIC.

Table 18. Summary of Candidate miRNAs Correlated with ERBIN and Their Association with Aggressive Breast Cancer Features and Patient Survival.

The table presents 23 candidate miRNAs that negatively correlated with ERBIN expression in breast cancer patients. MiRNAs ordered based on correlation strength with ERBIN expression, prediction of direct targeting using various miRNA–mRNA interaction tools, expression trends across aggressive clinical subtypes (including basal-like, ER-negative, TP53-mutated, high-grade, node-positive), and survival impact.

	miRNA	Correlation	Prediction Tool	Aggressive Types Status
1	hsa-miR-135b-5p	-0.4534720	RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783) Lower Expression Predicted Better Survival in Basal Subtypes (METABRIC)
2	hsa-miR-505-5p	-0.4266061	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783) Lower Expression Predicted Better Survival in All and Basal Subtypes (METABRIC)
3	hsa-miR-9-5p	-0.4226451	miRWalk, TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783) Upregulated in High Grade (GSE37963)
4	hsa-miR-301b-3p	-0.3879425	microT-CDS, miRDB, TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783) Upregulated in High Grade of Breast Cancer (GSE37963)

	miRNA	Correlation	Prediction Tool	Aggressive Types Status
5	hsa-miR-362-5p	-0.3394213	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in TP53-Mutated Samples (GSE19783) Upregulated in Later Stages of Breast Cancer (GSE37963) Lower Expression Predicted Better Survival in All Subtypes (METABRIC)
6	hsa-miR-17-5p	-0.3624862	TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in High Grade of Breast Cancer (GSE37963) Lower Expression Predicted Better Survival in All and Basal Subtypes (METABRIC)
7	hsa-miR-93-5p	-0.3428958	TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in TP53-Mutated Samples (GSE19783) Upregulated in High Grade of Breast Cancer (GSE37963) Lower Expression Predicted Better Survival in All Subtypes (METABRIC)
8	hsa-miR-505-3p	-0.4113849	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783)
9	hsa-miR-146b-5p	-0.4049938	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783)
10	hsa-miR-155-5p	-0.3801819	RNA22, TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in TP53-Mutated Samples (GSE19783)
11	hsa-miR-106b-5p	-0.3574774	miRWalk, TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Lower Expression Predicted Better Survival in All Subtypes and Basal Subtypes (METABRIC)
12	hsa-miR-519a-3p	-0.3181068	miRDB	Upregulated in Basal Subtype (GSE19783) Upregulated in ER-Negative Samples (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783)
13	hsa-miR-650	-0.2991786	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783) Upregulated in High Grade of Breast Cancer (GSE37963)
14	hsa-miR-142-3p	-0.2670024	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783) Upregulated in TP53-Mutated Samples (GSE19783) Upregulated in Samples with Node-Positive (GSE40267) Higher Expression Predicts Better Survival in Basal Subtypes (METABRIC)
15	hsa-miR-421	-0.3509680	microT-CDS	Upregulated in Samples with Node-Positive and Larger Tumor Size (GSE40267) Lower Expression Predicted Better Survival in All Subtypes (METABRIC)

	miRNA	Correlation	Prediction Tool	Aggressive Types Status
16	hsa-miR-455-5p	-0.3354200	TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267) Lower Expression Predicted Better Survival in Basal Subtype (METABRIC)
17	hsa-miR-130b-3p	-0.3010105	microT-CDS, miRDB, miRWalk, RNA22, TargetScan	Upregulated in Basal Subtype (GSE40267) Upregulated in TP53-Mutated Samples (GSE19783)
18	hsa-miR-431-3p	-0.2706181	RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267) Upregulated in TP53-Mutated Samples (GSE19783)
19	hsa-miR-20a-5p	-0.3203816	TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267)
20	hsa-miR-301a-3p	-0.2935056	microT-CDS, miRDB, TargetScan	Upregulated in High Grade of Breast Cancer (GSE37963)
21	hsa-miR-20b-5p	-0.2887006	TargetScan	Upregulated in Basal Subtype (GSE19783, GSE40267)
22	hsa-miR-545-3p	-0.2727390	miRDB, RNA22	Upregulated in Basal Subtype (GSE19783)
23	hsa-miR-106b-3p	-0.3869076	miRWalk, RNA22	Upregulated in Basal Subtype (GSE19783, GSE40267)

3.4. Analysis of miRNA Expression Following ERBIN Silencing

Given that ERBIN is significantly downregulated in some of the breast cancer subtypes in patient datasets, which include basal and estrogen receptor-negative tumors, we aimed to investigate the downstream regulatory effects of low ERBIN expression in MDA-MB-231 breast cancer cells. We silenced ERBIN gene expression at the mRNA and protein levels (Figure 27) with ERBIN siRNA targeting and performed small RNA-seq studies. We identified differentially expressed miRNAs (Figure 28) when ERBIN is downregulated. We then evaluated the candidate miRNAs to determine whether they are differentially expressed in response to lower ERBIN expression. Additionally, we validated the expression of one candidate miRNA, miR-362-5p, by qRT-PCR to support the sequencing results.

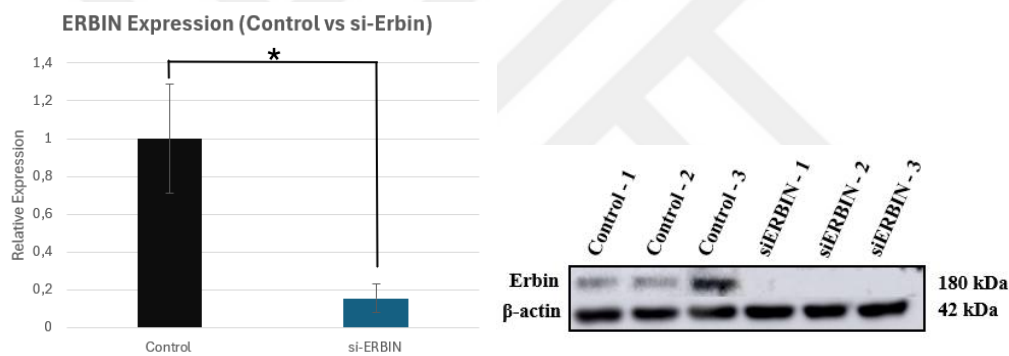


Figure 27. qRT-PCR and Western Blot results after ERBIN silencing (MDA-MB-231).

Real-time quantitative PCR (qRT-PCR) and Western blot analysis confirming ERBIN knockdown in MDA-MB-231 breast cancer cells following siRNA-mediated silencing. qRT-PCR results showed an 83.53% decrease in ERBIN mRNA expression compared to the scrambled control. Western blot analysis further validated the reduction at the protein level, with β -actin used as an internal control. Significance is shown as “*”, meaning $p < 0.05$.

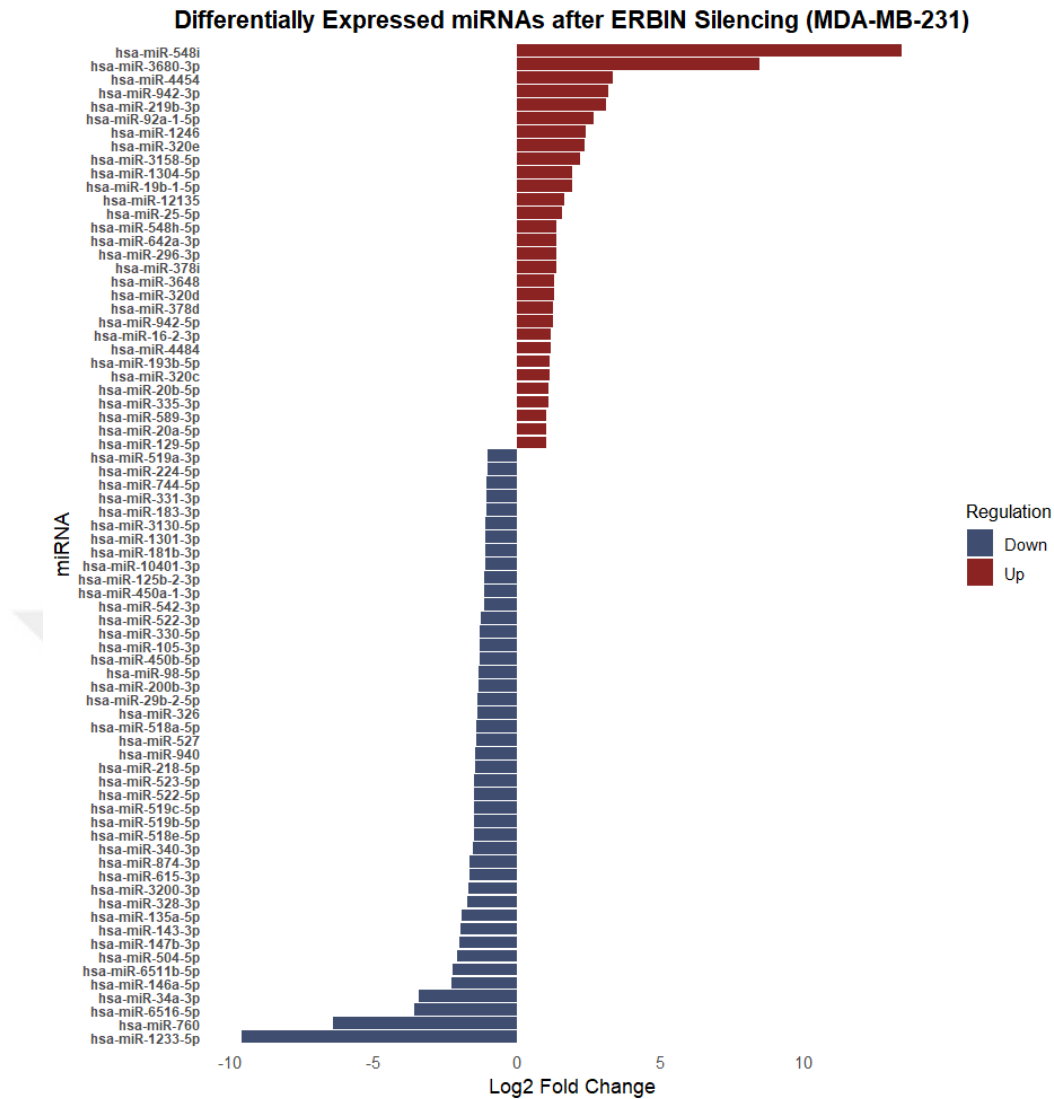


Figure 28. Differentially Expressed miRNAs ($|\log_2FC| > 1$).

Following ERBIN Silencing in MDA-MB-231 Cells. Bar plot showing miRNAs (miRNAs) with absolute $\log_2$ fold change greater than one after ERBIN knockdown in MDA-MB-231 breast cancer cells. miRNAs upregulated upon ERBIN silencing are shown in red, while downregulated miRNAs are in blue. The results are based on small RNA-seq analysis and represent the top differentially expressed miRNAs filtered by $|\log_2FC| > 1$.

As the bar plot in Figure 28 displays the top differentially expressed all the known miRNAs with absolute $\log_2$ fold change values greater than one, 75 miRNAs were found to be significantly differentially expressed following ERBIN knockdown. Among these, 30 miRNAs were upregulated, and 45 were downregulated following ERBIN knockdown. The miRNAs such as miR-548i, miR-3680, and miR-4454 were the ones that were mainly upregulated with ERBIN silencing, while miR-123-5p, miR-760, and miR-6516-5p were the ones that were most downregulated.

These results support that ERBIN silencing in MDA-MB-231 cells substantially impacts miRNA expression regulation and potentially alters at least some of the post-transcriptional regulatory pathways.

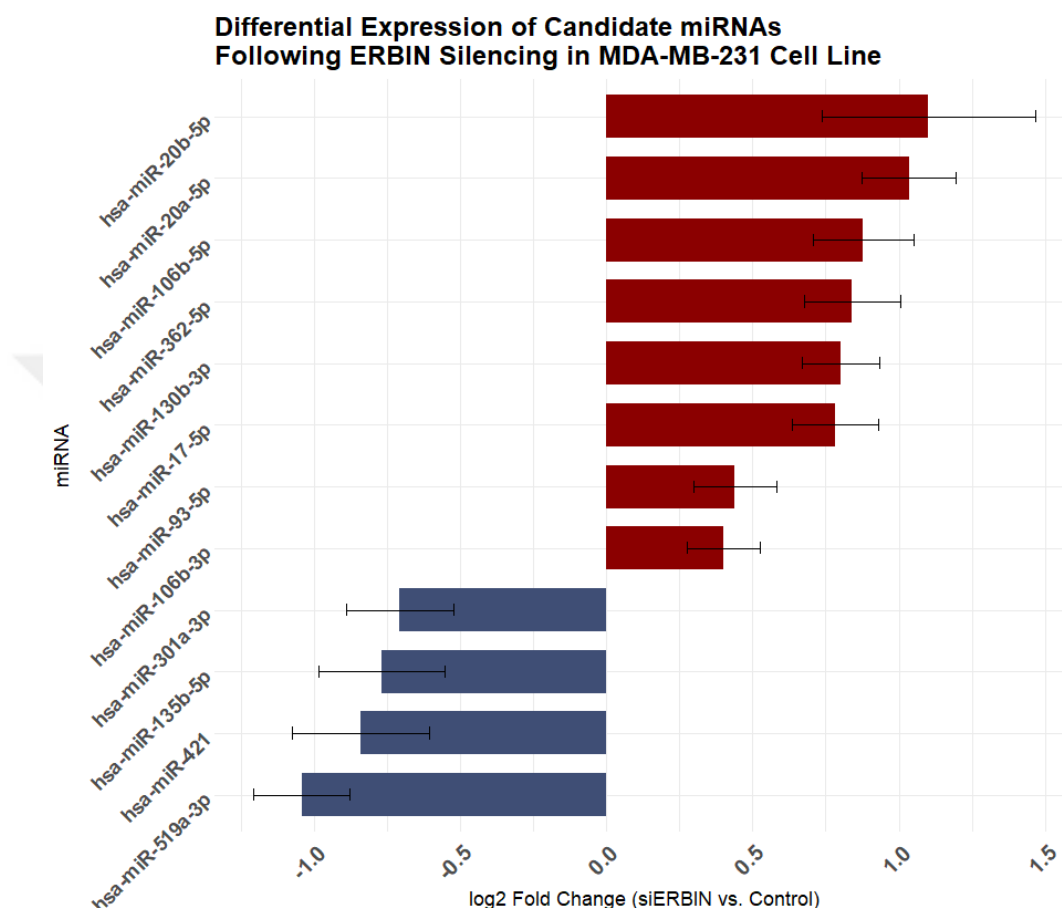


Figure 29. Expressions of Candidate ERBIN-Regulating miRNAs Following ERBIN Silencing in MDA-MB-231 Cells.

Bar plot showing the differential expression ($\log_2$ fold change) of selected candidate miRNAs following ERBIN knockdown in MDA-MB-231 breast cancer cells, based on Small RNA-seq. These miRNAs were previously identified as potential regulators of ERBIN in breast cancer patients through correlation, target prediction, and clinical parameter-based analyses. Error bars represent standard error across biological replicates ($n = 3$). Negative values indicate downregulation, and positive values indicate upregulation in siERBIN samples compared to controls.

We analyzed the differential expression of candidate miRNAs in ERBIN-silenced MDA-MB-231 cells from small RNA-seq analysis. Figure 29 shows that 12 out of 23 miRNAs were differentially expressed following ERBIN knockdown, with eight upregulated and four downregulated miRNAs. The miR-20b-5p and miR-20a-5p show the highest upregulation with fold change greater than 1, while only miR-519a-3p

exhibits the strongest downregulation with a fold change less than -1. Additionally, other miRNAs such as miR-106b-5p, miR-362-5p, miR-130b-3p, miR-17-5p, miR-93-5p, and miR-106b-3p also give significant upregulation, and miR-421, miR-135b-5p, and miR-301a-3p provide significant downregulation, even though fold changes are moderate. These results suggest that ERBIN expressions may modulate miRNAs targeting ERBIN expression in breast cancer, suggesting a potential feedback mechanism.

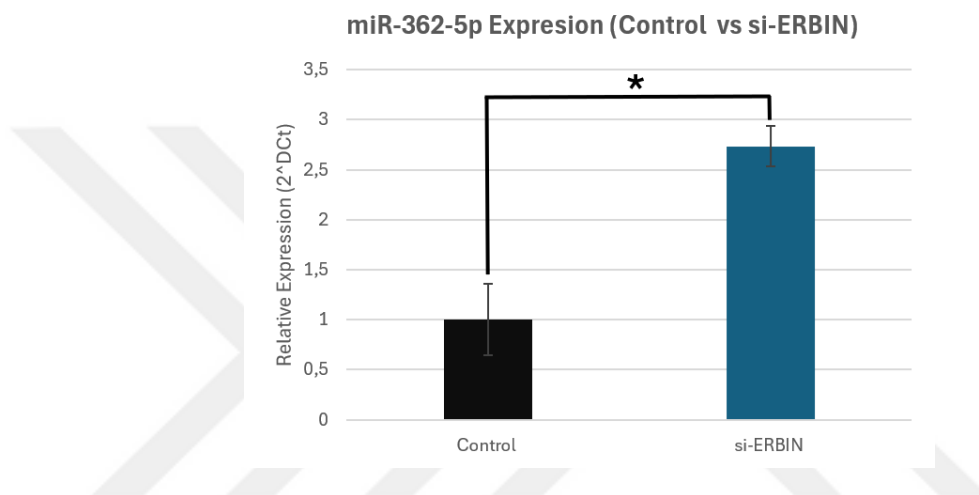


Figure 30. miR-362-5p qRT-PCR expression result when ERBIN is silenced in MDA-MB-231 cells.

Bar plot showing the relative expression of miR-362-5p in control and ERBIN-silenced (si-ERBIN) samples. Three biological replicates are used, and upregulation is determined as 174%. Significance is shown as “*”, meaning $p < 0.05$.

Among differentially expressed candidate miRNAs, miR-362-5p was experimentally validated by qRT-PCR using the same set of samples. As demonstrated in Figure 30, the result confirmed the expression pattern observed in small RNA-seq data with the significant upregulation of miR-362-5p after ERBIN knockdown. Therefore, the upregulation of miR-362-5p is reproducible with different methods, and the association between ERBIN and miR-362-5p expression supports the role of this miRNA in the regulatory feedback network in breast cancer.

CHAPTER 4: DISCUSSION

ERBIN expression is dysregulated in a context-dependent manner in breast cancer, while the factors underlying this remain poorly understood. In line with this, this study analyzed the association of ERBIN expression with molecular subtypes, TP53 mutation status, and estrogen receptor (ER) status, and assessed potential upstream regulators, particularly miRNAs, that may contribute to its altered expression. We found that ERBIN was significantly downregulated in aggressive breast cancer subtypes (basal-like, ER- and PGR-negative, TP53-mutated). ERBIN expression was correlated with breast cancer-specific gene markers. Additionally, ERBIN high expression predicted better patient survival outcomes, such as relapse-free, distant-metastasis-free, and overall survival.

The combined analyses of ERBIN-miRNA correlations and target prediction tools identified 23 candidate miRNAs potentially involved in ERBIN downregulation in breast cancer. These candidate miRNAs were further analyzed based on their predicted binding sequences, expression patterns across patient subtypes and clinical groups, survival outcomes, and their potential roles as regulators in feedback loops between ERBIN, using our small RNA-seq data from ERBIN-silenced MDA-MB-231 cells.

To understand ERBIN expression in breast cancer subtypes and whether its expression changes are related to the characteristics of breast cancer, we analyzed the large-scale transcriptomic patient datasets. Additionally, we compared patient groups based on molecular subtypes, hormone receptor, and TP53 mutation status. These results revealed that ERBIN expression shows distinct expression patterns across different breast cancer types, and is significantly lower in aggressive breast cancer types, such as basal-like, ER-negative, PR-negative, and, to some extent, HER2-negative and TP53-mutated tumors, consistent with previous studies reporting decreased ERBIN expression in invasive breast carcinoma, especially in HER2-positive breast cancer. (Jeong *et al.*, 2025; Liu *et al.*, 2013). The absence of ER and PR (hormone receptor) molecular markers, and the TP53 mutations, with worse survival of patients and highly aggressive features of breast cancer, which are defined by metastasis and relapse of cancer and lower responsiveness to treatment (Afifi & Barrero, 2023; Shahbandi *et al.*, 2020). These suggest that lower ERBIN expression levels in these subtypes indicate

its potential role in more malignant phenotypes of breast cancer, such as increased proliferation, invasion, and migration, and it can be a marker for prognosis.

The survival analyses demonstrated that patients with higher ERBIN expression had better survival, including more prolonged relapse-free survival, fewer distant metastases, and longer overall survival. These expression patterns of ERBIN in breast cancer suggest its potential therapeutic target to increase the survival of patients.

Correlation analysis between ERBIN and established breast cancer gene markers, ESR1, ERBB2, PGR, and TP53, displayed positive expression patterns. ESR1, which encodes the estrogen receptor α (ER α), showed the strongest correlation with ERBIN, suggesting potential co-regulation and association between ERBIN expression and better outcomes in ER-positive patients.

ERBIN also showed moderate positive correlations with ERBB2, PGR, and TP53 genes, which may indicate shared regulatory relationships in breast cancer. ERBIN and ERBB2 were proposed to be regulated together by transcription factors and growth factors, miRNAs that regulate ERBB2 gene expression (Cheng, 2024). In addition, ERBIN with PGR is suggested to be regulated together by the same transcription factors, such as ER α (Z. Li *et al.*, 2022), miRNAs, such as miR-135 (Gilam *et al.*, 2017), and miR-155, which mediate the mTOR signaling leading to dysregulation of ER α signaling and PGR expression (Martin *et al.*, 2014). We found that miR-135b-5p and miR-155-5p negatively correlate with ERBIN expression and are predicted to bind the ERBIN transcripts. The miR-135b-5p, found to belong to the miR-135 family in our results, suggests that our candidate miR-135b-5p may regulate ERBIN and PGR expression together. Although the literature studies miR-155 and does not state which mature form is investigated, our results suggest that miR-155-5p may participate in ERBIN regulation while influencing PGR expression via mTOR signaling.

TP53 mRNA expression differs from TP53 mutation status, as it is associated with basal-like and HER2-enriched breast cancer subtypes compared to luminal types (Williams *et al.*, 2018). The significant moderate correlation suggests ERBIN expression might be influenced by transcriptional programs activated under cellular stress, DNA damage, or oncogene signaling, regulating TP53 expression (Borrero & El-Deiry, 2021). Among our candidate miRNAs that regulate ERBIN expression, miR-421 is found to decrease ATM expression (H. Hu *et al.*, 2010), and miR-106b family

targets CDKN1A and miR-155-5p targets TP53INP1, which both ATM and TP53INP1 regulate p53 protein, but neither affects TP53 mRNA expression (Espina-Ordoñez *et al.*, 2024; Ivanovska *et al.*, 2008). Even though correlation does not imply functional relevance, the correlation between EBRIN and these marker genes provides a preliminary analysis to investigate ERBIN expression upstream regulation relevance with key breast cancer marker genes in further studies.

Since miRNAs are key post-transcriptional regulators, we explored the factors that contribute to the downregulation of ERBIN in aggressive breast cancer. The correlation analysis performed on the publicly available patient dataset identified 23 miRNAs negatively correlated with ERBIN mRNA and predicted to target it. Among these candidates, ten miRNAs were found highly relevant to ERBIN expression and upregulated in aggressive subtypes, including basal-like, ER-negative, TP53-mutated, high-tumor-grade, and late-stage tumors, summarized in Table 18. These suggest that miRNAs potentially mediate ERBIN expression loss and contribute to more aggressive biological behaviors of breast tumors.

Given that the highest expression correlation and association with worse outcomes of breast cancer patients, miR-135b-5p has strong potential to reduce ERBIN expression. The previous studies also support the role of miR-135b-5p in aggressive breast cancer; for instance, its overexpression is found to be correlated with high proliferation, invasion, and metastasis in the basal-like type of triple-negative breast cancer (TNBC) (Lv *et al.*, 2019; Uva *et al.*, 2018), while its downregulation leads to increased breast cancer cell growth and EMT, migration, and invasion in the early stage of ductal carcinoma (Pu *et al.*, 2019). In addition to the role of miR-135b-5p in metastatic pathways, miR-135b is found to regulate estrogen receptor alpha (ER α) and affect breast and prostate cancer growth (Aakula *et al.*, 2015), and its expression is found to be upregulated in doxorubicin-resistant breast cancer cells (Zhang *et al.*, 2019). Our findings from patient data analyses, which show upregulation of miR-135b-5p, downregulation of ERBIN in ER-negative patients, and positive correlation of ERBIN with ESR1 altogether, suggest their co-regulation by miR-135b-5p in ER-negative phenotypes of breast cancer.

Among other strong miRNA candidates that may regulate ERBIN downregulation in invasive breast cancer phenotypes, miR-301b-3p, miR-362-5p, and miR-17-5p were

also reported to promote aggressive breast cancer in previous studies. The miR-301b-3p expression is significantly increased in breast cancer tissues, particularly in ER- and PR-negative, invasive ductal carcinoma, and non-luminal subtypes (Zhou *et al.*, 2023), aligning with our findings in patient data analysis. In addition, its overexpression is correlated with poor patient outcomes according to clinical results (Zhou *et al.*, 2023). The functional studies have been performed on breast cancer cell lines, and the enhancing role of miR-301b-3p is shown on proliferation, migration, invasion, and apoptosis, by targeting NR3C2, HOXA5, TGFBR2, and EBF3. It affects JAK-STAT signaling through TGFBR2 and glycolysis and stem-like features of cells through EBF3 in breast cancer (Fan *et al.*, 2021; Lu *et al.*, 2021; Lou *et al.*, 2024; J. Huang *et al.*, 2024).

The miR-362-5p association with aggressive breast cancer is stated with its upregulation in TNBC (Chang *et al.*, 2015; Aarthiy *et al.*, 2021). Its promoting role for the cell proliferation, migration, and invasion by targeting CYLD, and leading to NF- κ B pathway activation and reduced apoptosis, is studied in the MCF7 cell line (Ni *et al.*, 2015). The findings in this thesis, as expressed results of miR-362-5p in clinical results, negative correlation between ERBIN and miR-362-5p expression in the patient dataset and in ERBIN-silenced and control MDA-MB-231 samples, which have aggressive characteristics, support the miR-362-5p upregulation in invasive types of breast cancer and present preliminary results to investigate miR-362-5p interaction with ERBIN mRNA further.

Interestingly, miR-17-5p was lower in TNBC cell lines and clinical tissues than in normal tissue (Li *et al.*, 2017). On the other hand, our findings showed its upregulation in the basal type of breast cancer compared to non-basal cancer subtypes, in line with previous studies that showed high expression of miR-17-5p in MDA-MB-231 (basal) cell line but low in MCF7 (luminal) cell line (H. Li *et al.*, 2010), suggesting a subtype-specific expression pattern of miR-17-5p in breast cancer. In addition, Wang *et al.* (2019) showed upregulation of miR-17-5p in TCGA breast cancer patient data and found that it increases migration and invasion in breast cancer cells by targeting NTN4. As a result, the consistent upregulation of strong miRNA candidates, having a negative correlation with ERBIN expression and predicted to target the mRNA of ERBIN, in aggressive breast tumors and their roles in promoting cancer progression, according to

our results and current literature, supports their potential role in tumor progression by influencing ERBIN loss.

The consistent upregulation of these miRNAs, especially miR-135b-5p, miR-301b-3p, miR-362-5p, and miR-17-5p in aggressive tumors in our results, supported by the current studies, together with their negative correlation with ERBIN and predicted bindings to ERBIN mRNA, suggests their roles in regulating ERBIN expression downregulation in breast tumor progression.

The expression analysis results of candidate miRNAs in ERBIN-silenced MDA-MB-231 cells provide further networks between ERBIN and its post-transcriptional regulators, such as potential feedback mechanisms or shared regulatory pathways. Among miRNAs that showed strong potential to regulate ERBIN expression in aggressive types of breast cancer, miR-362-5p, miR-17-5p, and miR-93-5p were significantly upregulated upon ERBIN knockdown. This expression alteration suggests that these miRNAs decrease ERBIN expression, and ERBIN may also have a role in decreasing their expression. In this proposed regulatory negative feedback mechanism, ERBIN downregulation may lead to activation of signaling cascades or alteration in gene expression mechanisms, leading to increased expression of these miRNAs, leading to further ERBIN downregulation.

The only known regulator of miR-362-5p is long non-coding RNA XIST, acting as a competing endogenous RNA and modulating UBAP1, which is the target of miR-362-5p, and affects breast cancer progression (B. Liu *et al.*, 2020). Even though ERBIN's role in regulating non-coding RNAs has not been studied yet, its role in signaling pathways might affect lncRNAs or other transcriptional and epigenetic regulators that modulate miR-362-5p.

The upstream regulation of miR-17-5p has been studied more compared to miR-362-5p. The miR-17-92 cluster, including miR-17-5p and miR-20a-5p from our candidate miRNAs, is regulated by transcription factors such as c-Myc, E2F transcription factors (especially E2F1), Sonic Hedgehog, and Notch (Fujiwara & Kimura, 2015). Our results also showed the negative correlation between E2F1 and the ERBIN gene (Figure 19 and Table 17). In addition, MAPK signaling positively regulates miR-17-92 cluster miRNAs through the transcriptional activator Elk-1, which binds to the promoter sequence of miR-17-92 cluster miRNAs (Fiedler & Thum, 2016). The

studies demonstrated that ERBIN inhibits the MAPK pathway, supporting the potential feedback mechanism that ERBIN downregulation leads to enhanced MAPK signaling pathway (D. Liu *et al.*, 2010), which can lead to miR-17-5p upregulation, and further miR-17-5p targeting ERBIN mRNA and leading to its downregulation. Therefore, based on the literature and our findings, ERBIN's regulatory role is on the MAPK/ERK-E2F1-miR-17-92 (miR-17-5p and miR-20a-5p) regulatory axis.

Interestingly, two of the strong candidate miRNAs, such as miR-135b-5 and miR-421, that may regulate ERBIN expression were found to be downregulated in response to ERBIN downregulation. This result indicates that the expression of these miRNAs is directly or indirectly dependent on ERBIN expression or signaling pathways affected by the decrease in ERBIN expression in breast cancer. As an example of an indirect mechanism, other molecular factors, such as transcription factors or signaling mechanisms modulated by ERBIN, downregulate these miRNAs.

In this study, we identified 14 miRNAs that target transcription factors (TFs) negatively correlated with ERBIN expression. In this potential indirect regulatory mechanism, miRNAs increase ERBIN expression by repressing their target TFs, because these TFs would suppress ERBIN expression otherwise. The TFs targeted by many miRNAs (E2F2, FOXK2, MYBL2, CEBPB, and FOXM1), and the miRNAs targeting many TFs (miR-26a-5p, miR-193b-3p, miR-92b-3p), have the potential to be in a regulatory network. Previous studies showed that E2F2, FOXM1, and MYBL2 regulate cell-cycle progression, which induces tumor cell proliferation (Johnson *et al.*, 2016; Katzenellenbogen *et al.*, 2023). These TFs, together with CEBPB and FOXK2, induced EMT through transcriptional reprogramming that enhances migratory and invasive phenotypes in breast cancer (Marada *et al.*, 2024; D. Tao *et al.*, 2014; Dey *et al.*, 2020; Qi *et al.*, 2023; Shan *et al.*, 2016).

The miR-193b-3p acts as a tumor-suppressor for metastatic features by inducing apoptosis, suppressing migration, and regulating stem cell markers in TNBC cells, and it is significantly associated with the regulation of WNT/ β -catenin, c-MET, and integrin signaling (Giacomelli *et al.*, 2021). The miR-26a-5p also functions as a tumor-suppressive in TNBC cells and induces chemosensitivity (Y. Zhang *et al.*, 2023), and its downregulation contributes to trastuzumab resistance in HER2+ breast cancer (Tormo *et al.*, 2017). Together with our former findings that ERBIN downregulation

promotes drug resistance in breast cancer cell lines (Sunar, 2021), miR-26a-5p lower expression is related to drug resistance, suggesting their indirect regulatory relation in breast cancer.



CHAPTER 5: CONCLUSION

This study analyzes the expression patterns, clinical relevance, and potential upstream regulation of ERBIN in breast cancer. ERBIN expression is found to be significantly downregulated in distinct subtypes of breast cancer. These subtypes include basal-like, ER-negative, PR-negative, and TP53-mutated breast tumors, which have more aggressive characteristics. High expression of ERBIN is associated with better patient survival outcomes, such as longer relapse-free, distant-metastasis-free, and overall survival. There are also positive correlations between ERBIN and several key breast cancer marker genes (ESR1, ERBB2, PGR, TP53), suggesting these genes can share similar regulatory mechanisms.

A combination of patient datasets, target prediction tools, and small RNA-seq data identified 23 candidate miRNAs potentially regulating ERBIN. Several of these miRNAs, including miR-135b-5p, miR-301b-3p, miR-362-5p, and miR-17-5p, were consistently upregulated in aggressive phenotypes of breast cancer in different datasets, while also negatively correlated with ERBIN expression. Previous studies also support the roles of these miRNAs in enhancing proliferation, invasion, and metastasis, aligning with the hypothesis that these specific miRNAs play roles in ERBIN downregulation during tumor progression.

The positively correlated miRNAs with ERBIN expression also provided a regulatory feedback framework, including ERBIN, miRNAs (miR-26a-5p, miR-193b-3p, miR-92b-3p), and transcription factors (E2F2, FOXK2, MYBL2, CEBPB, FOXM1) that are targeted by positively correlated miRNAs and negatively correlated with ERBIN. These TFs are known to drive cell cycle progression and EMT in breast cancer, and these processes are associated with low ERBIN expression. In this context, downregulation of the TFs by specific miRNAs could result in higher ERBIN expression and suppression of cell-cycle and EMT mechanisms.

To conclude, the study provides a comprehensive regulatory analysis of ERBIN in breast cancer, reveals its consistent downregulation in aggressive subtypes and association with poorer patient outcomes, and provides insights into potential miRNA-mediated mechanisms through integrated expression profiling, clinical correlations, and regulatory-network analysis.

CHAPTER 6: FURTHER PERSPECTIVES

In this study, we revealed that several miRNAs potentially target ERBIN expression in aggressive breast cancer based on miRNA and ERBIN gene expression results from patient datasets and miRNA target prediction tools, and some of them suggest their role in regulatory feedback loops. However, further research is required to validate these analysis results in both breast cancer cell lines and tumor samples, as well as in other cancer models, by including xenograft models, in addition to the investigation of the therapeutic aspects of the identified miRNA-mRNA regulatory pairs.

The functional validation of direct binding between the strong candidate miRNAs, including miR-135b-5p, miR-301b-3p, miR-362-5p, miR-17-5p, and other candidate miRNAs and ERBIN mRNAs is necessary to reveal their regulatory axis in aggressive breast cancer. Transfection of miRNA mimics and miRNA inhibitors, as well as subsequent control of ERBIN mRNA expression changes across different breast cancer and other cancer cell lines, is required to present the functional influence of each candidate miRNA. Luciferase reporter assays using wild-type and mutated ERBIN 3', 5' UTR or CDS regions would present the direct interaction from predicted sites of candidate miRNAs, as depicted in Table 16.

Functional studies would also be performed to determine whether the altered expression of ERBIN, due to miRNA regulation, has a functional role in cancer-related mechanisms. In these functional assays, ERBIN expression can be silenced or overexpressed by using miRNAs, and the effects on proliferation, cell growth, metastasis, and therapy resistance in different breast cancer subtypes can be observed. These experiments would provide evidence that ERBIN expression alterations impact aggressive types of breast cancer.

Investigation of the upstream regulators of the candidate miRNAs, ERBIN, and miRNAs positively correlated with ERBIN would provide insight into how ERBIN is regulated both in breast cancer and other cancer types. The upstream regulations include transcription factors, long non-coding RNAs, epigenetic modifications, and signaling pathways, especially those with strong potential to regulate candidate miRNAs and ERBIN in aggressive breast cancer.

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