Investigating miRNA signatures in metastatic breast cancer: An *in-silico* analysis.

Lakshminarasimhan Harini[1], Lekshmi Madhav[2], Sweta Srivastava[3], Gopalakrishna Ramaswamy[2], Rakesh Ramesh[4*].

1. ICMR DHR Women scientist, St. Johns National Academy of Health sciences, Bangalore.
2. Theracues Innovations Private Limited, #1436, 2nd Floor, B-Block, Sahakar nagar, Bangalore-560092, India
3. Associate Professor, Division of Molecular Biology and Genetics, St. Johns National Academy of Health Sciences, St John's Medical College, Bangalore.
4. Professor, Department of Surgical Oncology, St. Johns National Academy of Health Sciences, St John's Medical College, Bangalore.

* Corresponding Author

ABSTRACT

miRNAs are small non-coding RNAs that regulate most cellular processes. Tumorigenesis disrupts the normal balance in the cell, which leads to changes in the cell cycle, cell signalling, activation of growth factors, miRNA deregulation, etc. Thus, the variations in miRNA expression between normal and tumor stages can be used to predict, diagnose, detect and identify different cancer stages, thereby suggesting the potential use of the miRNAs as biomarkers or potential therapeutic targets for cancer.

In this study we aim to identify differentially expressed miRNAs which could serve as potential biomarkers or therapeutic targets for breast cancer.

Microarray-based expression analysis was performed on tissue samples from patients with early and locally advanced breast cancer and nCounter analysis was performed to detect differentially expressed miRNA. Additionally, functional enrichment analysis, miRNA linked gene prediction, and in silico analysis for miRNA expression in cancer databases were carried out. This analysis revealed increased expression of Hsa-miR-199a and b. Further these miRs had increased expression levels across various cancer. Importantly, genes such as CALR, SSR2, and YWHAZ predicted as miR's target were highly expressed in breast cancer. However, these genes lack therapeutic drugs targeting them. Overall, our study identified previously unexplored miRNAs and their gene targets, which could be exploited as potential targets for future therapeutic interventions.

INTRODUCTION:

MicroRNAs (miRNAs) are small, non-coding RNA molecules that play a crucial role in gene regulation [1]. These 22-nucleotide-long RNA strands are highly conserved across viruses, plants, and animals, [1] and they are involved in various cellular processes, including development, differentiation, apoptosis, and homeostasis [2]. Dysregulation of miRNAs has been linked to numerous diseases, particularly cancer. In the case of breast cancer, miRNA signatures have emerged as potential clinical markers, diagnostic tools, and therapeutic targets [3]. miRNAs are generated through a two-step cleavage process. Initially, primary microRNAs (Pri-miRNAs) are transcribed from miRNA genes and cleaved to form precursor miRNAs (pre-miRNAs). The pre-miRNAs then undergo further cleavage in the cytoplasm, resulting in the formation of mature miRNAs [4]. These mature miRNAs can regulate gene expression through post-transcriptional and translational mechanisms. They can repress translation by deadenylating or decapping the mRNA, leading to degradation. Additionally, miRNAs can upregulate transcription by binding to specific regions of the mRNA, resulting in increased expression of the target gene [5]. These regulatory mechanisms make miRNAs key players in fine-tuning gene expression and maintaining cellular homeostasis.

The dysregulation of miRNAs in breast cancer has provided a basis for utilizing miRNA signatures as therapeutic targets. By identifying specific miRNA signatures in early and late-stage breast cancer patient samples, researchers can gain insights into the underlying molecular mechanisms of metastasis. In silico analyses can be conducted to predict miRNA-regulated genes, which can then be investigated as potential therapeutic targets. Such an approach holds promise for designing targeted therapies that modulate miRNA expression or function, thereby inhibiting cancer progression and improving treatment outcomes. Understanding the miRNA signatures associated with metastatic breast cancer and identifying miRNA-regulated genes as therapeutic targets can pave the way for more personalized and effective treatments.

METHODS:

Samples:

Blood and tissue samples were collected from seven breast cancer patients across different stages including 2A, 2B, 3A, 3B and 4. The samples were collected following ethical consent obtained from the patient.

miRNA Isolation, Quantitation and NanoString miRNA Expression assay

RNA isolation was performed using Qiagen miRNeasy Mini Kit (1038703). Tissue samples were homogenized using TOMY homogenizer in the presence of liquid nitrogen.700 µl of Qiazol was added and vortexed briefly, this mixture was incubated at RT for 5 minutes followed by the addition of 150µl of chloroform for phase separation. Samples were centrifuged at 12000*g for 15 minutes. The upper aqueous phase was transferred to a new microcentrifuge tube, and 1.5 volumes of 100% ethanol were added and mixed well by pipetting. The mixture was loaded onto the RNeasy mini column and the rest of the protocol was followed as per the manufacturer's instructions. Elution was performed in 30µl of RNase-free water.

Quality assessment and quantitation of the RNA samples were carried out using Agilent 2100 bioanalyzer and Qubit RNA HS assay (Invitrogen Cat # Q32852). The eluted RNA was stored at -80. Quality check and quantitation of the RNA samples were performed using Agilent 2100 bioanalyzer and Qubit RNA HS assay (Invitrogen Cat # Q32852). Eluted RNA was stored at -80. Equimolar concentrations of total RNA from different samples were pooled as per the sample details provided in **Table 1**. The samples were categorised as early breast cancer (EBC) and locally advanced breast cancer (LABC).

For the nanoString miRNA expression assay, a 3ul aliquot was taken from the pooled sample and the assay was performed as per the nCounter miRNA Expression Assay (User Manual-MAN-C0009-07). The miRNA analysis was conducted by Theracues Innovations Pvt Ltd, Bangalore, India, using the nanoString nCounter SPRINT machine.

miRNA expression analysis

miRNA analysis was performed on the pooled samples using the nCounter Analysis System and the nCounter Human v3a miRNA Panel which includes 798 unique clinically relevant miRNA barcodes along with appropriate positive and negative controls. The raw miRNA data in a . RCC (Reporter Code Count) format was analyzed using nSolver analysis software (NanoString technologies) version 4.0. Quality control metrics of Imaging, Binding density, positive control, and the limit of detection and ligation were checked before proceeding with the analysis. Normalization of the raw data was performed using the geometric mean of positive controls and the geometric mean of the top 100 highly expressed miRNAs as per the instructions provided in the manual.

The differential expression between the two groups (EBC and LABC) as mentioned in **Table 1,** was calculated using the build ratio module in nSolver. The EBC group was considered as the baseline for comparison. To identify the significantly differentially miRNAs, the following criteria should be fulfilled: DE call should be predicted as 'YES' and the geometric mean expression should be >= 40.

In silico Analysis of the data:

The fold change data (Table 2) was subjected to in silico analysis. Primarily the data were sorted based on their values and segregated into upregulated and downregulated categories. Further, FUNRICH was used to understand the molecular function, biological pathway, and biological process associated with the enriched miRNAs. Target genes for each DE miRNA were obtained from mirDB and mirTARDB. Genes exhibiting 95-100% sensitivity were chosen from miRDB while all the genes were selected from mirTARDB. These genes were further analyzed using the http://ualcan.path.uab.edu/ tool.

Further, the STRING database was used to investigate the potential interactions between the identified genes. These same genes were also analyzed for mutation and copy number variation across breast cancer datasets from CBioPortal.

RESULTS:

Differential expression of miRNA in LABC as compared to EBC:

A comparison of the miRNA profile of EBC and LABC revealed a distinct difference between EBC and LABC. A differential gene expression analysis was performed on the two categories using nSolver. In this analysis, a negative fold change value indicates downregulation of the respective miRNA while a positive fold change value indicates upregulation. The counts in any of the samples >= 40 were considered significant. Based on these criteria, the following miRNAs were identified as differentially expressed: hsa-miR-1910-5p, hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-4454, hsa-miR-7975, hsa-miR-451a, hsa-miR-514a-5p, hsa-miR-522-3p, hsa-miR-1299, hsa-miR-135b-5p, hsa-miR-144-3p, hsa-miR-517c-3p, and hsa-miR-519a-3p. The miRNA was segregated based on their fold change value into downregulated and upregulated categories as listed in Table 1. Amongst these, the miRs, hsa-miR-451a, hsa-miR-144 3p, hsa-miR-522 3p, hsa-miR-135b-5p, and hsa-miR-1299 were found to be downregulated while hsa-miR-4787-3p, hsa-miR-204-5p, hsa-miR-4454+hsa-miR-7975, and hsa-miR-601 were found to exhibit positive fold change values, signifying significant upregulation.

LABC has a distinct molecular landscape as compared to EBC.

miRNAs are vital regulators of gene expression and thus participate in diverse biological processes including disease progression. In the present study, we have identified a list of miRNAs which may play an essential role in the progression of breast cancer. It is important to identify the genes regulated by these miRNAs. To analyse the genes and biological processes plausibly regulated by the altered miRNAs, we employed FUNRICH tool. FUNRICH is an open software for OMICS data analysis [6].

In terms of biological processes, the altered expression of miRNA resulted in the enrichment of several beta-oxidation pathways in LABC as compared to EBC. These include beta-oxidation of palmitoyl-CoA to myristoyl-CoA, Beta oxidation of hexanoyl-CoA to butanoyl-CoA, Beta oxidation of octanoyl-CoA to hexanoyl-CoA and Beta oxidation of decanoyl-CoA to octanoyl-CoA-CoA. These processes showed a high fold change with 386.51, 386.51, and 290.12 as respective values (Supplement data).

In the biological process category, cellular morphogenesis was highly enriched with a 196.77-fold value (Supplement data). Other enriched processes are the Transmembrane receptor protein tyrosine kinase signalling pathway, Intercellular junction assembly and/or maintenance, Negative regulation of enzyme activity, Regulation of cell shape, G-protein signalling, coupled to cyclic nucleotide second messenger, Peroxisome organization and biogenesis, Extracellular structure organization and biogenesis, Coenzyme and prosthetic group metabolism.

Several biological processes were also downregulated. These processes include Vesicle organization and biogenesis, Cellular defence response, Glycoprotein metabolism, Synaptic vesicle transport, Plasma membrane organization and biogenesis, nuclear organization and biogenesis, Reproduction, Antigen receptor-mediated signalling pathway, Xenobiotic metabolism and Neurotransmitter metabolism.

In molecular functions, Dipeptidase activity, Glucosyltransferase activity, Polysaccharide binding functions were enhanced and Sterol transporter activity, Channel or pore class transporter activity, Catalase activity, B cell receptor activity, Coreceptor activity, Oxidative phosphorylation uncoupler activity, Peptide transporter activity were downregulated.

Expression analysis of miRNA using TCGA database

Comparison of miRNA between LABC and EBC revealed the enrichment and downregulation of some important miRNAs. We find that these miRNAs regulate important biological processes and may be thus instrumental in disease progression. We, therefore, decided to undertake a study to identify their expression in other tumours and their significance in tumour progression.

The Cancer Genome Atlas (TCGA) is a database which has genomic, transcriptomics, epigenomics and clinical data from various cancer types. It provides a vast and comprehensive dataset that enables the exploration of molecular characteristics associated with various types of cancer. OncomiR and UALCAN were thus used to analyse the TCGA dataset for expression analysis of the miRNAs chosen in this study.

OncomiR [7] provided the expression data for the chosen miRNAs across various cancer types (Fig. 2a). Amongst the DE miRNAs, hsa-miR-199a-3p and hsa-miR-199b-3p was expressed highly in almost all of the cancers except low-grade glioblastomas and ovarian cancers. These two miRNAs showed an overlap in the expression graph (Fig. 2a). The miRNA that came after them in terms of expression level was hsa-miR-451a.hsa-miR-517c-3p and hsa-miR-522-3p are expressed higher in thymus cancer and TGCT, the rare cancers on the tendon sheath. However, we did not find data for hsa-miR-1299, hsa-miR-4454+hsa-miR-7975, hsa-miR-519a-3p, hsa-miR-514a-5p, hsa-miR-4454, and hsa-miR-7975 using OncomiR. While the miRNAs, hsa-miR-1910 and hsa-miR-135b are not highly expressed in any of the cancers.

A comparative analysis of these miRNAs across breast cancer stages was performed using UALCAN [8]. Figure 2b illustrates the variation in differentially expressed miRNAs among different stages of breast cancer. This analysis indicates hsa-miR-199a-3p and hsa-miR-199b-3p exhibited higher expression in cancer samples compared to the normal samples. Although a decreasing trend was observed from stages I to III, the expression of these miRNAs was slightly upregulated in stage IV. The miRs hsa-miR-144 and hsa-miR-514 were downregulated in cancer samples compared to normal. While hsa-miR135b-3p, hsa-miR-144, hsa-miR522, hsa-miR-4454, hsa-miR-451a, hsa-miR-514a-3p, and hsa-miR19a-3pwere expressed at levels lower than 500 reads per million in both cancer samples and normal samples.

Plausible genes enriched due the miRNA.

The miRNA-associated genes were identified using tools such as miRDB and mirTARBASE. miRDB contains predicted miRNA target genes based on computational algorithm while miTARBASE focuses on experimentally validated miRNA-target interactions. Thus, while using miRDB, the genes with scores greater than 95% were chosen in the analysis. However, for miTARBASE all the genes were chosen for the analysis. Table 3 and 4 presents the genes identified using miRDB and miTARBASE . Table 3 presents genes for the differentially expressed downregulated miRNAs while Table 4 presents genes for upregulated miRNA. The expression of these genes in normal and different breast cancer stages was analyzed using UALCAN and heatmaps weres generated for each gene. Fig. 3 displays the heat map of genes

with significant expression level changes between normal and cancer samples MIF, YWHAZ, CALR, P4HB, TIMP1, and PSB8 were upregulated.

A further analysis of the expression of the genes between normal and cancer was performed and the genes targeted by downregulated miRs whose expression varied from normal to cancer samples are presented as listed in Table 5. Genes like MIF, YWHAZ, CALR, P4HB, TIMP1, and PSB8 were upregulated in cancer compared to normal samples. Similarly, the genes predicted to be downregulated in cancer due to overexpression of miRNAs include ETS2, JUNB, SOD2, CCDC80, and TSPAN3, and are presented in Table 5b.

Functional enrichment analysis of the predicted miRNA target genes to reveal the biological status of the cell.

The gene lists from Table 5 a and b was further subjected to STRING analysis to predict protein-protein interactions. A full-string network with all active interaction networks and a medium confidence interaction score was chosen. Fig. 4a and 4b presents the protein interaction network for upregulated and downregulated genesets. The upregulated gene network Fig. 4a consists of 54 nodes and 153 edges with a PPI enrichment p-value of 1.0e-16. The examination of molecular functions associated with this protein network revealed that steroid hormone receptor binding, integrin binding, nuclear receptor binding, chaperone binding, nuclear hormone receptor binding, and hormone receptor binding displayed higher strength. Importantly the proteins that were involved in most of these functions were CALR, RAN, APP, APOE, FN1, ITGB1, and STAT3. The down regulated gene network consists Fig. 4b consists of 24 nodes and 13 edges with a PPI enrichment p-value of 0.0099. The examination of functions enrichment analysis for this protein network revealed Growth hormone synthesis, secretion and action has higher strength in KEGG pathways.

Genomic variations associated with the miRNA target genes.

It is important to note that genetic variations within the miRNA target genes can affect miRNA binding sites and alter gene expression levels. Single Nucleotide Polymorphisms, insertions, deletions, copy number variations and other structural variations can affect gene expression patterns. Thus, an analysis of the genomic variation was undertaken for the genes listed in Tables 5a and 5b.

Mutation and copy number variation in the genes was analysed using CBioPortal. The analysis focussed on different types of breast cancer, including invasive breast carcinoma, adenoid cystic breast carcinoma (2015), and metastatic breast cancer 2021. The data was from studies from MSK cancer cells 2018, MSK nature cancer 2022, MSK cancer discovery 2022, HTAN2022, MSK clinical cancer research 2022, MSK NPJ breast cancer 2019, SMC 2018, proteogenomic landscape breast cancer CPTAC 2020, and metastatic breast cancer project 2020. A total of 7063 samples were queried. The analysis revealed that gene amplification is the major alteration observed in most of the genes. TMEM183A exhibited the highest amplification rate of 25%. SSR2 and YWHAZ genes also displayed significant amplification rates, with 21% and 19%, respectively. Other genes, such as APH1A (18%), RBM8A (17%), TPD52 (17%), and PARP1 (14%), were also found to be amplified in the samples.

Drug target prediction miRNA-associated genes.

It is imperative to study the possibility of therapeutic possibility using the identified targets. To explore this possibility, a study was performed to identify drugs targeting the genes associated with miRNA expression. Drug availability for CALR, RAN, APP, APOE, FN1, ITGB1, STAT3, SSR2, TMEM183A, RBM8A, APH1A, TPD52, and PARP was retrieved from the Therapeutic Target Database [9]. Table 6 lists the drugs for these targets. It is to be noted that not all the genes identified in this study have a drug suited for therapy, however, APOE, FN1, ITGB1 and STAT3 show a targeted drug available.

The availability of targeted drugs for these genes opens possibilities for further research and development of therapies that aim to modulate their expression or function. Overall, the study provides valuable insights into potential therapeutic targets and highlights the importance of ongoing research and development to uncover new drugs and therapies for various conditions associated with miRNA expression.

DISCUSSION:

miRNA (microRNA) analysis is an important tool in cancer research and diagnosis [10]. They are small non-coding RNA molecules and play a significant role in gene regulation by binding to messenger RNA (mRNA) and consequently regulating various cellular processes such as cell growth and proliferation. miRNA expression variation can be used as Biomarkers for cancer diagnosis, subtyping of tumours, prognostic indicator, and therapeutic targets [11]. In the present study, miRNA analysis was conducted to understand the difference between LABC and EBC with respect to miRNA expression.

A comparison of upregulated and downregulated miRNAs reveals enhancement of long and medium-chain fatty acid beta-oxidation, changes in cellular morphogenesis, and downregulation of B cell-mediated immune reaction. These findings correlate with previous studies indicating that beta-oxidation of fatty acids in cancer relates to proliferation, survival, stemness, drug resistance, and metastatic progression, which drives breast cancer cells to EMT leading to metastasis [12, 13]. Another important aspect of cancer progression is alteration in immune response. Enrichment analysis of EBC vs. LABC in biological processes and pathways confirms the downregulation of B cell receptor activity and cellular defence mechanisms. Recent studies on the immune system adaptation of metastatic cancer cells have discovered that these cells adapt to or evade the immune response and survive [14].

A TCGA based analysis of the expression patterns of the identified miRNAs revealed that hsa-miR-199a and -b are highly upregulated in most cancers compared to normal samples while hsa-miR-451a and hsa-miR-144-3p are downregulated in breast cancers compared to normal cells.

Whereas, Results from the miRBase and RNA databases suggest that hsa-miR-199a along with hsa-miR-199b negatively regulate matrix metalloproteinase and positively regulate endothelial migration. Another study on hsa-miR-199a-3p suggests its targeting of BRCA gene and sensitisation of Triple negative breast cancer (TNBC) to therapy [15]. We also observe that hsa-miR-451a was significantly downregulated in the LABC sample. Hsa-miR-451a has been reported to target CCND2, contributing to breast cancer progression [16]. Another miRNA that was downregulated in LABC tissue was hsa-miR-144a. Downregulation of hsa-miR-144a correlates to a poor prognosis in colorectal cancers [17].

Analysis of genes targeted by the differentially expressed miRNAs revealed CALR, RAN, APP, APOE, FN1, ITGB1, and STAT3 as plausible targets. These genes play a key role in cancer progression. Interestingly, APOE, FN1, ITGB1 and STAT3 have available inhibitors and can be used for treatment of cancers. Calreticulin protein is involved in the invasiveness and metastasis of breast cancer [18] and has been identified as a potential biomarker in various cancers [19]. However, there isn’t a good inhibitor of CALR which can be used as a therapeutic molecule. Similarly, RAN is also a biomarker for cancer progression and overexpression of RAN indicates invasiveness and metastasis in breast cancer [20]. Unfortunately, similar to CALR, there is no effective inhibitor for RAN. Our analysis also revealed that genes including SSR2, TMEM183A, RBM8A, YWHAZ, APH1A, PARP1, and TPD52 showed higher genetic alterations. SSR2 is an endoplasmic reticulum transmembrane protein that plays a role in ER stress in HCC patients [21], however, its role in breast cancers is yet to be explored. Additionally, there is no available information on SSR2 in the drug target database.

Analysis reveals that the most downregulated miRNA in advanced cancers is hsa-miR-451a and it regulates the expression of genes including PSMB8, MIF, ERH, and YWHAZ. STRING analyses of the gene set revealed that YWHAZ, a transmembrane protein, is a common node for mitochondrial matrix permeabilization and transcriptional regulation. as it interacts with proteins like PAK4, YWHAG, YWHAQ, PFN1, ITGB, etc. Interestingly, the CBioPortal analysis Fig. 6 indicates the YWHAZ gene has a higher level of genetic alteration.

The miRNA signature identified from the metastatic breast cancer patient tissues in this study shows that hsa-miR199a/b are upregulated while hsa-miR-451a/3p and hsa-miR-144-3p are downregulated.

The availability of targeted drugs for some genes opens possibilities for further research and development of therapies that aim to modulate their expression or function. Additionally, the study identified novel targets to develop novel inhibitors. Overall, the study provides valuable insights into potential therapeutic targets and highlights the importance of ongoing research and development to uncover new drugs and therapies for various conditions associated with miRNA expression.

CONCLUSION:

This study identifies miRNA signatures in early and late breast cancer tissue. This was further scrutinized *in silico* for the prediction of the potential therapeutic target. The role of proteins CALR, SSR2 and YWHAZ could be further explored as a therapeutic target.

Figures and Tables:

Fig. 1: Functional enrichment of upregulated and Downregulated miRNA in FUNRICH A) Enriched beta oxidation of large and medium chain fatty acid in Biological pathway, B) Downregulated B cell receptor activity and enriched dipeptidase activity in molecular function C) Upregulated cellular morphogenesis in Biological process.

Fig. 2: TCGA analysis of DE miRNA. A) Pan cancer analysis of DE miRNA (data from oncoMIR database) indicating the higher expression of hsa-miR-199a hsa-miR-199b and hsa-miR-451a was highly expressed in most of the cancers , B) expression of DE miRNA in different stages of breast Invasive carcinoma (data from UALCAN database). Indicates the change in expression level of miRNAs from stage I to stage IV of breast cancer patients.

Fig. 3: Heatmap of downregulated miRNA targeted genes expression variation in normal vs breast cancer from UALCAN database. A) Heatmap of validated upregulated target genes B) Heatmap of predicted target genes.

Fig. 4: Heatmap of upregulated miRNA targeted genes expression variation in normal vs breast cancer from UALCAN database.

Fig. 5: STRING interaction of genes A) upregulated gene targets and B) downregulated gene targets selected based on expression analysis from UALCAN. Genes like YWHAZ, RAN, ITGB1, STAT3, FN1 etc were found to be the common genes involved in most of the molecular function in enrichment analysis.

Fig. 6: Genetic alteration in target genes: CBioPortal images of mutations and amplification of selected genes from STRING interactions in breast cancer patients.

Table 1: Sample details

Sample#	Sample ID	Sample Name	Pool Details	Sample Type
1	SAM1245	DNA 4036090	Pool 1	**Early Breast Cancer (EBC)**
2	SAM1250	TM 4044468 17/02/18 TM DNA R-1		
3	SAM1260	4073267/ 1335214 T DNA	Pool 2	**Locally Advanced Breast Cancer (LABC)**
4	SAM1278	4069989/ 1331112 4069989 DNA		

Table 2 List of top ten downregulatd miRNA and upregulated miRNA

a) List of downregulated miRNA

Downregulated miRNA		
miRNA		Fold change value
hsa-miR-451a	MIMAT0001631	-70.47
hsa-miR-144-3p	MIMAT0000436	-42.15
hsa-miR-522-3p	MIMAT0002868	-9.99
hsa-miR-135b-5p	MIMAT0000758	-8
hsa-miR-521	MIMAT0002854	-5.19
hsa-miR-1299	MIMAT0005887	-4.68
hsa-miR-514a-5p	MIMAT0022702	-4.46
hsa-miR-510-5p	MIMAT0002882	-4.4
hsa-miR-517c-3p+hsa-miR-519a-3p	MIMAT0002866	-4.37

b. List of upregulated miRNA

Upregulated miRNA		
miRNA	miRBase Accession	Fold Change
hsa-miR-4787-3p	MIMAT0019957	13.52
hsa-miR-204-5p	MIMAT0000265	4.37
hsa-miR-4454+hsa-miR-7975	MIMAT0018976	3.32
hsa-miR-601	MIMAT0003269	2.82
hsa-miR-1249-5p	MIMAT0032029	2.78
hsa-miR-503-3p	MIMAT0022925	2.58
hsa-miR-193a-5p+hsa-miR-193b-5p	MIMAT0004614	2.56
hsa-miR-200b-3p	MIMAT0000318	2.53
hsa-miR-199a-3p+hsa-miR-199b-3p	MIMAT0000232	2.51
hsa-miR-205-5p	MIMAT0000266	2.51

Table 3: List of selected gene targets (Predicted and Validated) for downregulated miRNA

	Score	Predicted gene targets	Validated Target	
hsa-miR-135b-5p	99	BEND4	hsa-miR-144-3p	PLAG1
	99	DOCK7		FGG
	99	LZTS1		FCA
	99	SYT2		FGB
	99	SMIM13		NOTCH1
hsa-miR-1299	100	GPR3	hsa-miR-1910-5p	PRRC2B
	100	RALGAPB		YWHAQ
	100	EGLN3		MYL6
hsa-miR-1910-3p	98	cAMSAP2		DNAJA1
	98	APBB2		TMEM43
	97	MB21D2	hsa-miR-522-3p	SOX2
hsa-miR-1910-5p	97	BTBD7		FXN
	96	SCN2B		AKAP1
	95	SOS1	hsa-miR-1299	VAMP3
hsa-miR-514a-5p	97	SLC22A3		GNAS
	95	STOX2		SESN2
	95	CAMTA1		

Table 4 : Selected list of predicted and validated gene targets for upregulated miRNA

		Score	**Predicted gene list**			**Validated gene list**
hsa-miR-199a-3p+hsa-miR-199b-3p		100	ADAMTSL3	hsa-miR-199a-3p		ETS2
		99	KATNBL1			MECP2
		99	CELSR2			MED6
hsa-miR-4454		90	BAG5	hsa-miR-199b-3p		JUNB
hsa-miR-7975		95	SLC35F1	hsa-miR-4454		TRAM2
		94	BAG5			MON1B
		94	MPP2			CASP16
		94	ANKRD42	hsa-miR-7975		KBTBD8
		94	ATG10			NFATC3
		93	PDE5A			LRAT
		93	STRN3			

Table 5. List of gene targets for differentially expressed miRNA.

Table 5 a: List of predicted and validated target genes for the differentially expressed downregulated miRNAs.

miRNA	**Validated**	**miRNA**	**Predicted**
hsa-miR-451a	MIF	hsa-miR-451a	PSMB8
	ERH	hsa-miR-144-3p	ZFP36L2
	YWHAZ		HTRA3
hsa-miR-144-3p			FN1
	APP		VKORC1L1
	SLC39A6		CDV3
	HNRNPAB		EFEMP1
hsa-miR-1910-5p	YWHAQ		PNRC1
	CALR		MBNL1
	ITGB1	hsa-miR-135b-5p	YWHAG
	HNRNPA1		SSR2
	ESR1		DDX3X
hsa-miR-514a-5p	EEF2		KLF4
	P4HB	hsa-miR-1299	STAT3
	TIMP1		CAST
hsa-miR-517c-3p			TM9SF3
	APH1A		MMD
	RPS27A	hsa-miR-514a-5p	ZFAND5
hsa-miR-519a-3p	HSPA8	hsa-miR-519a-3p	TGFBR2
	PARP1		FBXL5
	RAN		APCDD1
	MFAP2		NFIB
	CSDE1		DPYSL2
	SYNM		JAK1
	PSMA7		BLCAP
	PFN1		HBP1
	TPD52		
	NCL		
	ANXA5		
	TMEM59		
	APP		

Table 5 (b) List of predicted and validated target genes for the differentially expressed upregulated miRNAs

miRNA	Validated	miRNA	Predicted
hsa-miR-199a-3p+hsa-miR-199b-3p	ETS2	hsa-miR-199a-3p	PAK4
	JUNB		GALNT7
	SMARCA2	hsa-miR-7975	TMEM183A
	CAV2		
	APOE		
	PNRC1		
	QKI		
	IGF1		
	SOD2		
	HLA-B		
	TSPAN3		
	CCDC80		
	FGF1		
	STAT3		
	CD151		
	PNRC1		
	OCIAD2		
	CCDC80		
	SH3D19		
	RBM8A		
	MYADM		
	ITPR1		
	NCOA7		

Table 6: List of genes and its available drugs from Drug Target Database

Target	Drug
CALR	No drug available
RAN	No information
APP	No drug available
APOE	AEM-28
FN1	Ocriplasmin
ITGB1	131I-radretumab
STAT3	Acitretin, ISIS-STAT3
SSR2	No information
TMEM183A	No information
RBM8A	No information
APH1A	No information
TPD52	No information
PARP	Nicotinamide
YWHAZ	No information

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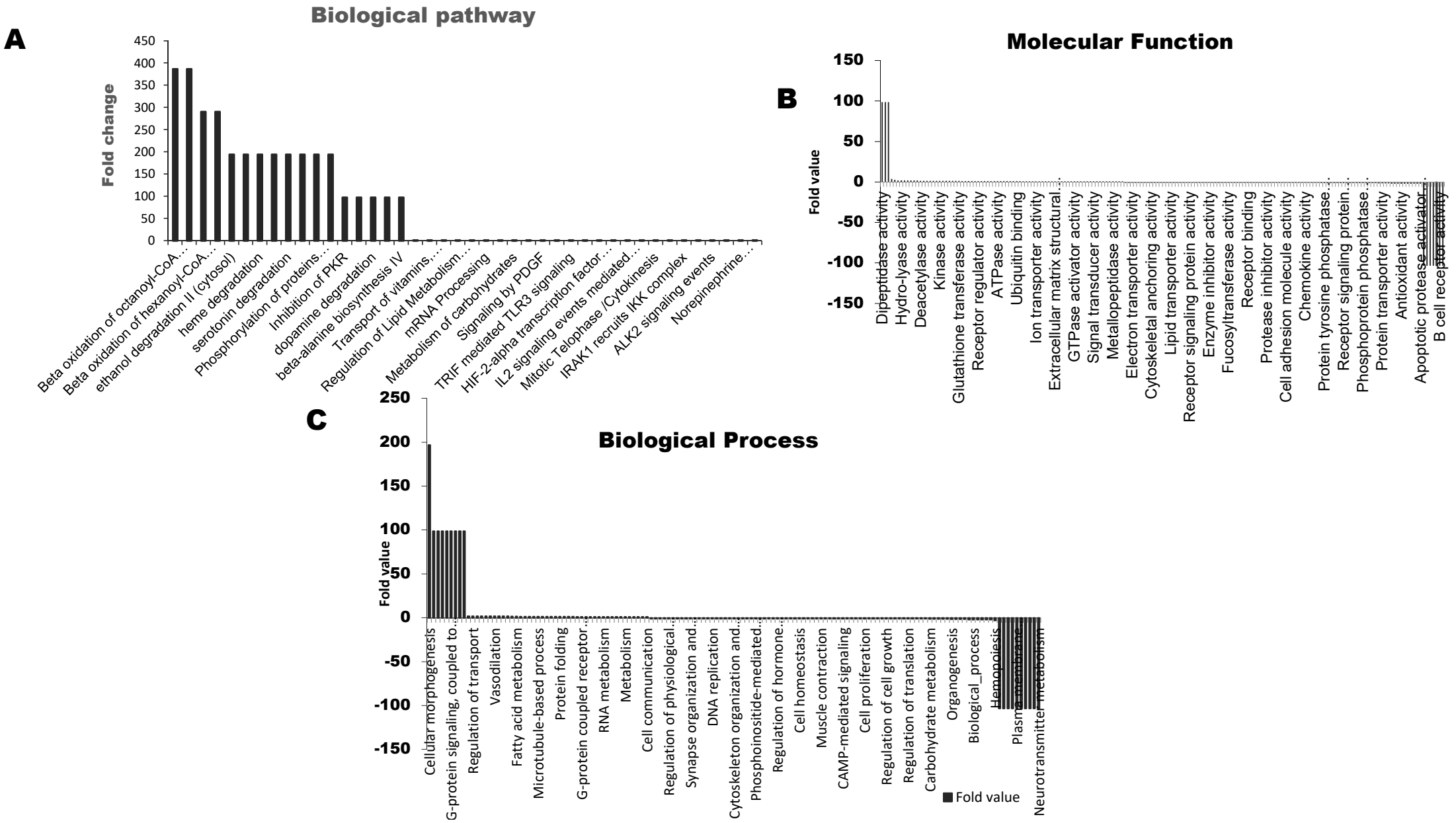
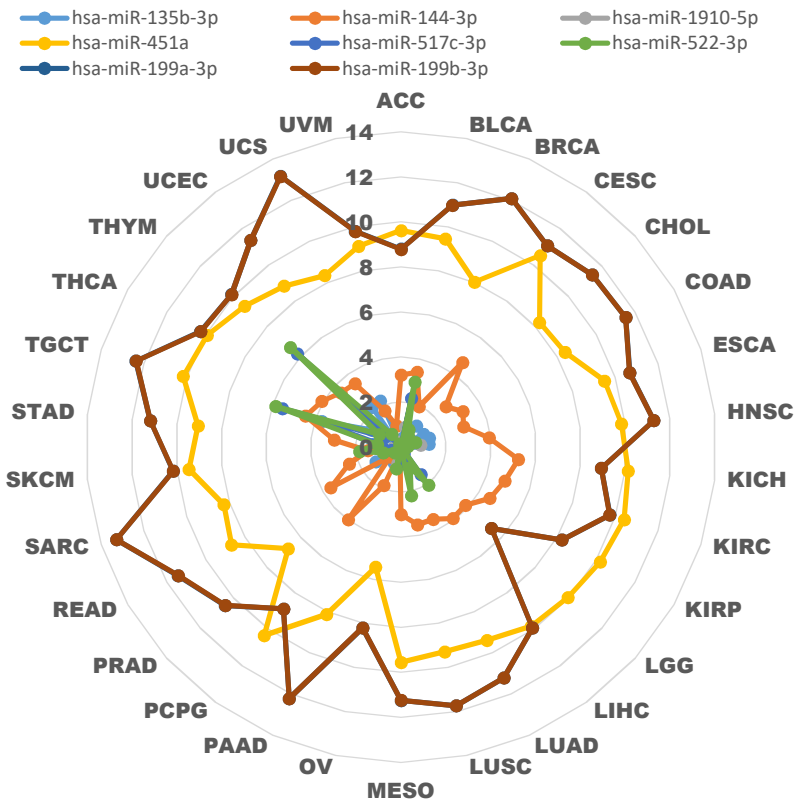
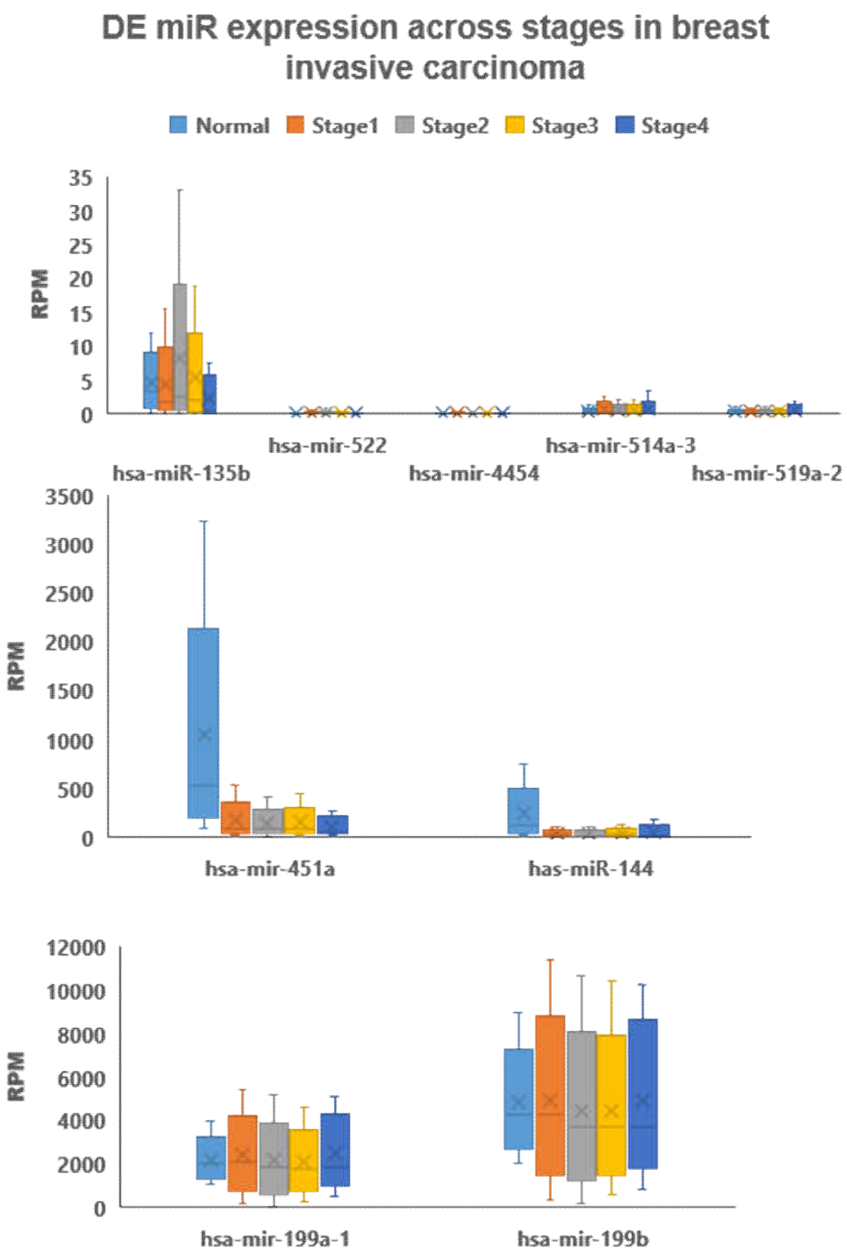

A
Biological pathway
Fold change
450
400
350
300
250
200
150
100
50
0
Beta oxidation of octanoyl-CoA…
Beta oxidation of hexanoyl-CoA…
ethanol degradation II (cytosol)
heme degradation
serotonin degradation
Phosphorylation of proteins…
Inhibition of PKR
dopamine degradation
beta-alanine biosynthesis IV
Transport of vitamins…
Regulation of Lipid Metabolism…
mRNA Processing
Metabolism of carbohydrates
Signaling by PDGF
TRIF mediated TLR3 signaling
HIF-2-alpha transcription factor…
IL2 signaling events mediated…
Mitotic Telophase /Cytokinesis
IRAK1 recruits IKK complex
ALK2 signaling events
Norepinephrine…
B
Molecular Function
Fold value
150
100
50
0
-50
-100
-150
Dipeptidase activity
Hydro-lyase activity
Deacetylase activity
Kinase activity
Glutathione transferase activity
Receptor regulator activity
ATPase activity
Ubiquitin binding
Ion transporter activity
Extracellular matrix structural…
GTPase activator activity
Signal transducer activity
Metallopeptidase activity
Electron transporter activity
Cytoskeletal anchoring activity
Lipid transporter activity
Receptor signaling protein activity
Enzyme inhibitor activity
Fucosyltransferase activity
Receptor binding
Protease inhibitor activity
Cell adhesion molecule activity
Chemokine activity
Protein tyrosine phosphatase…
Receptor signaling protein…
Phosphoprotein phosphatase…
Protein transporter activity
Antioxidant activity
Apoptotic protease activator…
B cell receptor activity
C
Biological Process
Fold value
250
200
150
100
50
0
-50
-100
-150
Cellular morphogenesis
G-protein signaling, coupled to…
Regulation of transport
Vasodilation
Fatty acid metabolism
Microtubule-based process
Protein folding
G-protein coupled receptor…
RNA metabolism
Metabolism
Cell communication
Regulation of physiological…
Synapse organization and…
DNA replication
Cytoskeleton organization and…
Phosphoinositide-mediated…
Regulation of hormone…
Cell homeostasis
Muscle contraction
CAMP-mediated signaling
Cell proliferation
Regulation of cell growth
Regulation of translation
Carbohydrate metabolism
Organogenesis
Biological_process
Hemopoiesis
Plasma membrane
Neurotransmitter metabolism
Fold value

A

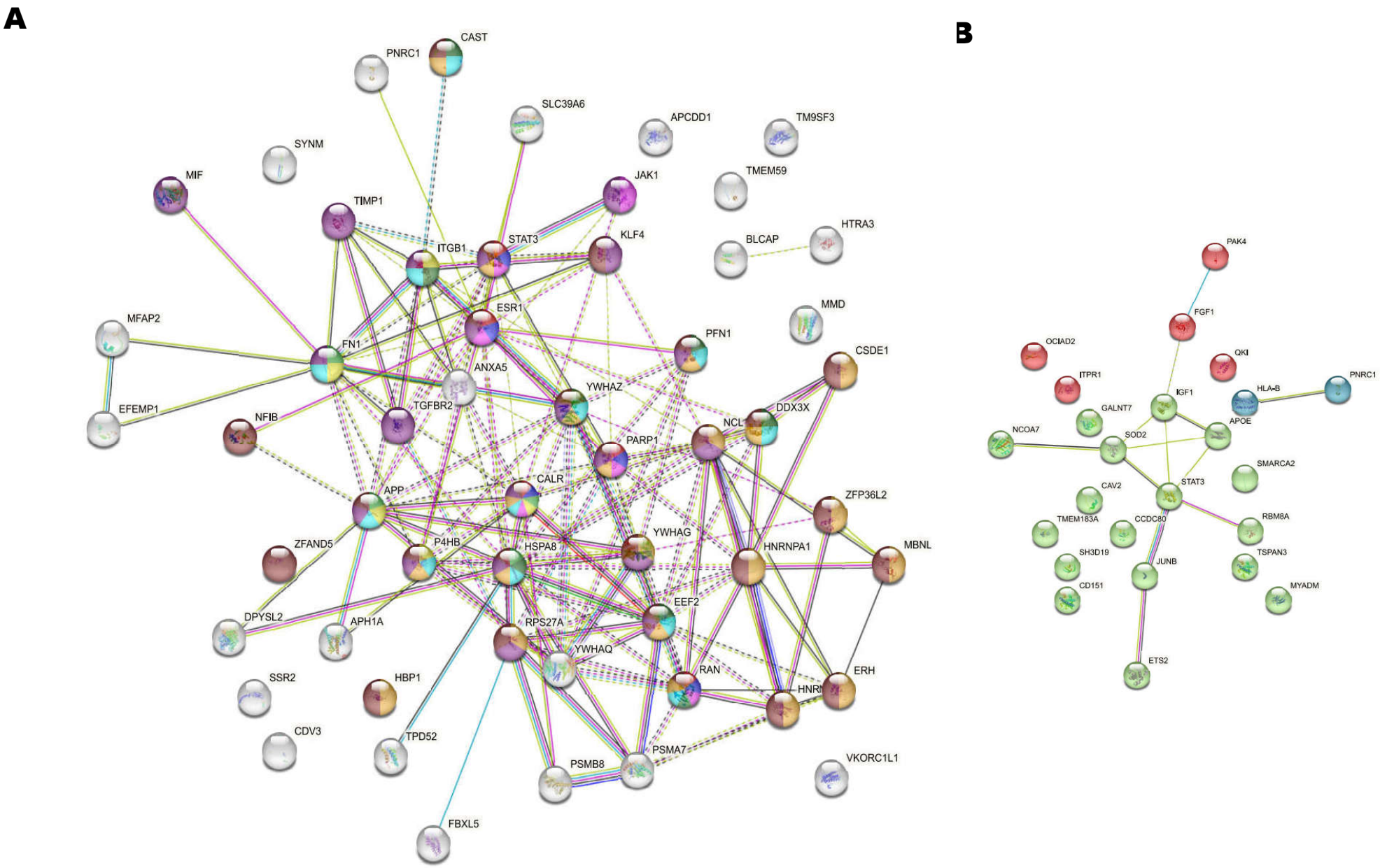

hsa-miR-135b-3p
hsa-miR-144-3p
hsa-miR-1910-5p
hsa-miR-451a
hsa-miR-517c-3p
hsa-miR-522-3p
hsa-miR-199a-3p
hsa-miR-199b-3p
ACC
BLCA
BRCA
CESC
CHOL
COAD
ESCA
HNSC
KICH
KIRC
KIRP
LGG
LIHC
LUAD
LUSC
MESO
OV
PAAD
PCPG
PRAD
READ
SARC
SKCM
STAD
TGCT
THCA
THYM
UCEC
UCS
UVM
0
2
4
6
8
10
12
14

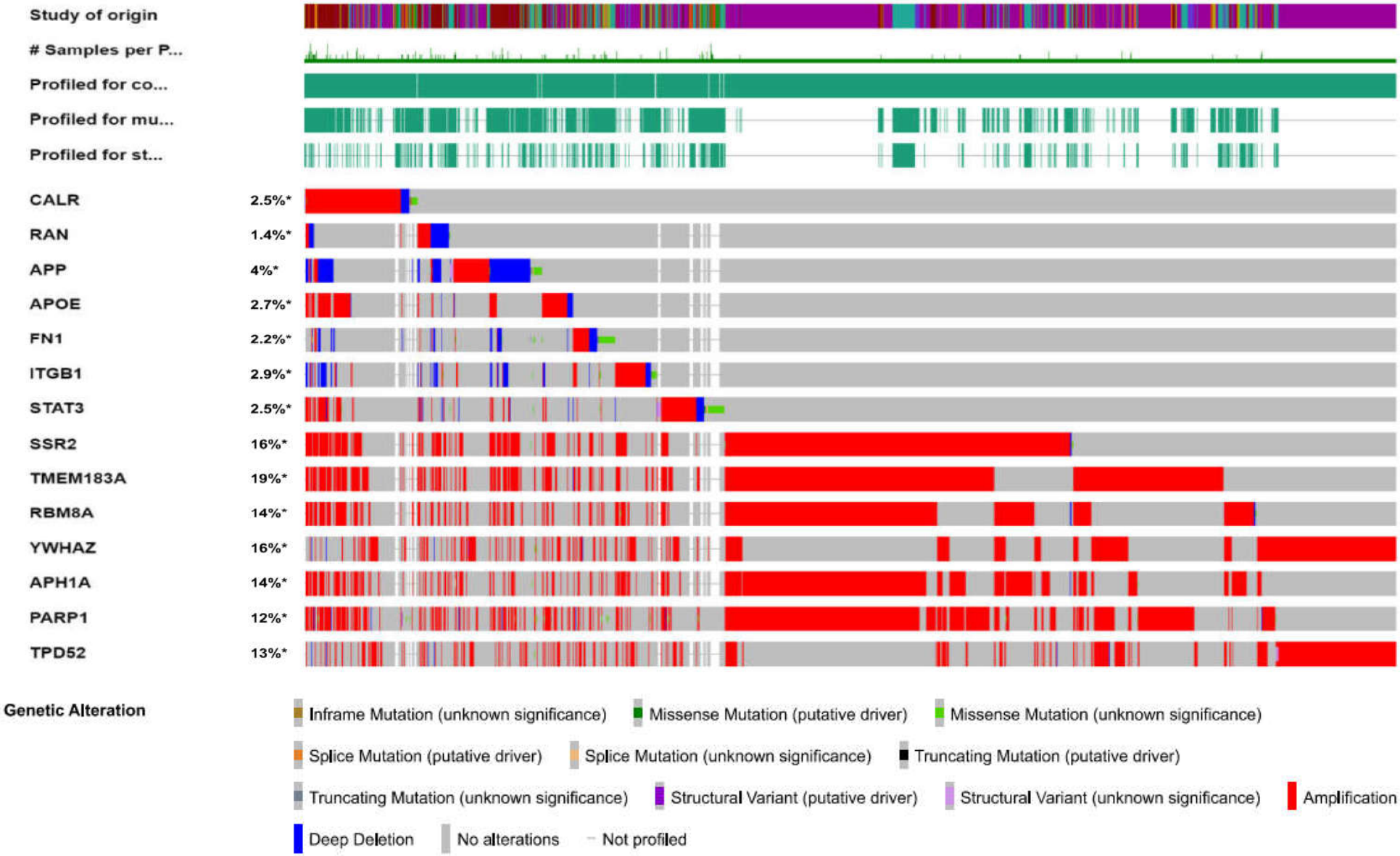
B


DE miR expression across stages in breast invasive carcinoma
Normal
Stage1
Stage2
Stage3
Stage4
RPM
hsa-miR-135b
hsa-mir-522
hsa-mir-4454
hsa-mir-514a-3
hsa-mir-519a-2
hsa-mir-451a
has-miR-144
hsa-mir-199a-1
hsa-mir-199b

A
Validated
hsa-miR-451a
hsa-miR-144-3p
hsa-miR-1910-5p
hsa-miR-514a-5p
hsa-miR-517c-3p
hsa-miR-519a-3p
MIF
ERH
YWHAZ
APP
SLC39A6
HNRNPAB
YWHAQ
CALR
ITGB1
HNRNPA1
ESR1
EEF2
P4HB
TIMP1
APH1A
RPS27A
HSPA8
PARP1
RAN
MFAP2
CSDE1
SYNM
PSMA7
PFN1
TPD52
NCL
ANXA5
TMEM59
APP
Normal
Tumor
B
Predicted
hsa-miR-451a
hsa-miR-144-3p
hsa-miR-135b-5p
hsa-miR-1299
hsa-miR-514a-5p
hsa-miR-519a-3p
PSMB8
ZFP36L2
HTRA3
FN1
VKORC1L1
CDV3
EFEMP1
PNRC1
MBNL1
YWHAG
SSR2
DDX3X
KLF4
STAT3
CAST
TM9SF3
MMD
ZFAND5
TGFBR2
FBXL5
APCDD1
NFIB
DPYSL2
JAK1
BLCAP
HBP1
Normal
Tumor
Log2(TPM+1)
0
5
10
15

Validated
Predicted
hsa-miR-199a-3p+hsa-miR-199b-3p
hsa-miR-199a-3p
hsa-miR-7975
ETS2
JUNB
SMARCA2
CAV2
APOE
PNRC1
QKI
IGF1
SOD2
HLA-B
TSPAN3
CCDC80
FGF1
STAT3
CD151
PNRC1
OCIAD2
CCDC80
SH3D19
RBM8A
MYADM
ITPR1
NCOA7
PAK4
GALNT7
TMEM183A
Normal
Tumor
Log2(TPM+1)
0
5
10
15


A
CAST
PNRC1
SLC39A6
APCDD1
TM9SF3
SYNM
MIF
TMEM59
JAK1
TIMP1
HTRA3
BLCAP
STAT3
KLF4
ITGB1
MMD
ESR1
MFAP2
PFN1
CSDE1
FN1
ANXA5
YWHAZ
EFEMP1
DDX3X
NFIB
TGFBR2
NCL
PARP1
CALR
APP
ZFP36L2
YWHAG
ZFAND5
P4HB
HSPA8
HNRNPA1
MBNL
EEF2
DPYSL2
APH1A
RPS27A
YWHAQ
RAN
ERH
SSR2
HBP1
HNRI
CDV3
TPD52
PSMA7
VKORC1L1
PSMB8
FBXL5
B
PAK4
FGF1
OCIAD2
QKI
ITPR1
PNRC1
IGF1
HLA-B
GALNT7
APOE
NCOA7
SOD2
SMARCA2
STAT3
CAV2
TMEM183A
CCDC80
RBM8A
SH3D19
JUNB
TSPAN3
CD151
MYADM
ETS2


Study of origin
Samples per P...
Profiled for co...
Profiled for mu...
Profiled for st...
CALR 2.5%*
RAN 1.4%*
APP 4%*
APOE 2.7%*
FN1 2.2%*
ITGB1 2.9%*
STAT3 2.5%*
SSR2 16%*
TMEM183A 19%*
RBM8A 14%*
YWHAZ 16%*
APH1A 14%*
PARP1 12%*
TPD52 13%*
Genetic Alteration
Inframe Mutation (unknown significance)
Missense Mutation (putative driver)
Missense Mutation (unknown significance)
Splice Mutation (putative driver)
Splice Mutation (unknown significance)
Truncating Mutation (putative driver)
Truncating Mutation (unknown significance)
Structural Variant (putative driver)
Structural Variant (unknown significance)
Amplification
Deep Deletion
No alterations
Not profiled