miRNA - mRNA INTERACTION MAP IN BREAST CANCER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE
IN
LIFE SCIENCE

SUBMITTED TO

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

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CERTIFICATE

This is to certify that the thesis entitled "miRNA - mRNA INTERACTION MAP IN BREAST CANCER" submitted to National Institute of Technology; Rourkela for the partial fulfillment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by MITALI RANA under my supervision and guidance.

(Dr. Bibekanand Mallick)

ACKNOWLEDGEMENTS

The satisfaction and euphorbia that accompanies the successful completion of any task should be incomplete without the mention of the people who made it possible with constant guidance, support and encouragement that crowns all the efforts with success. It gives me immense pleasure to express my utmost respect and deepest sense of gratitude to my supervisor Dr. Bibekanand Mallick, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela for his esteemed guidance, assistance, time to time inspiration and encouragement throughout my project.

I also convey my deep gratitude to Prof. S.K. Sarangi, Director, National Institute of Technology, Rourkela for providing excellent facilities in the Institute for carrying out research.

I would like to take the opportunity to acknowledge quite explicitly with gratitude my debt to all the Professors and Staff, Department of Life Science, National Institute of Technology, Rourkela for his encouragement and valuable suggestions during my project work.

My heartfelt thanks to Ms. Devyani Samantray and Ms. Debashree Das for giving me experimental ideas throughout my project, without whose guidance it would not have been a success. My special thanks to all the other PhD scholars and Bini, Bibhudutta and Subhrata for their overwhelming inspiration and support throughout my project work.

Finally my deepest gratitude to my parents for their moral support and best wishes.

And to the almighty, who made all things possible……..

(MITALI RANA)

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ABSTRACT

MicroRNAs are a class of small endogenous RNA molecules that is involved in the post-transcriptional inhibition of gene expression. They directly interact with target gene transcripts and influence cellular physiology. MicroRNAs have been reported to be involved in breast cancer tumorigenesis and metastasis thus playing a vital role in cancer progression. Our study aims at identification of novel miRNA-mRNA target pairs that are hypothesized to play a role in breast cancer through a miRNA- mRNA interaction map analysis of microarray data and experimental validation of selected set of mRNAs. The target interaction map analysis revealed three novel target pairs, *hsa-miR-27a*–MARCKS, *hsa-miR-27a*–SIK1 and *hsa-miR-21*–BTG2 which can be potential therapeutic targets in breast cancer. Therefore, with the better understanding of the regulation of miRNAs, the gene networks and cellular pathways regulated by miRNAs, it will be of immense significance to further comprehend breast cancer pathogenesis and target interaction as a therapeutic for breast cancer.

Keywords: microRNAs, transcriptional inhibition, gene expression, tumorigenesis, metastasis, microarray, target interaction map

INTRODUCTION

INTRODUCTION

Breast cancer is the most commonly diagnosed cancers in women which is the second leading cause of cancer deaths that accounts for approximately 22% of all new cancer cases worldwide. It is seen that more than one million new cases arise every year. Globally, 0.45 million patients die from breast cancer annually, which constitutes 13.7% of female cancer deaths (Parkin, 2001 and Jemal et al. 2011). Breast cancer is prone to metastasis involving secondary sites such as the lung, liver, bone, and brain. Metastasis is seen to occur even after many years of the removal of the primary tumor, minimizing the survival rate from 85% for early detection to 23% for patients with lung or bone metastasis, therefore being the main cause of death for breast cancer patients (Lorusso, 2012). Genetic mutations are said to be the contributing causes of tumorigenesis and metastasis in breast cancer. Presently, the mechanisms for controlling metastasis are poorly understood and thus the treatments for metastatic late-stage breast cancer are still inefficient (Lee, 2012). Hence, it is of a great clinical importance to understand the molecular mechanisms involved in primary tumor cell invasion and its spread to distant sites, and thus to identify new molecular targets for cancer therapies.

In the recent past, microRNAs (miRNAs) got revealed as the focal point in the molecular dissection of human cancer (Calin et al., 2002). An emerging list of information recommends that microRNAs critically participate in cancer initiation and progression. Evidences have shown that microRNAs play a vital role in breast cancer. Such MicroRNAs have been described as a family of small, noncoding, double-stranded RNA molecules of short 18 to 25 nucleotides noncoding genes excised from 60-110 nucleotide hairpin RNA precursors that is involved in the regulation of expression of protein-coding genes (PCGs). Consequently it is seen that the events that activate or inactivate miRNAs were said to cooperate with PCG abnormalities in human tumorigenesis (Porter et al., 2008).

Hundreds of miRNAs have been identified till date in mammals, some of which are expressed in a tissue-specific and developmental stage-specific way. Since the discovery of this regulatory RNA phenomenon, much progress has been made in recent times towards understanding the mechanisms by which this process occurs and in the identification of cellular machinery involved in RNA-mediated silencing (Novina et al., 2004 and Meister et al., 2004). The miRNAs generally interact with target mRNAs with only partial or imperfect complementarity by causing either mRNA degradation or translation inhibition and can

negatively regulate the expression of target genes with their complementary sequence in cells (Novina et al., 2004 and Meister et al., 2004). More recently, it is cited that miRNA down regulation was recommended to play a role in cancer progression (Johnson et al., 2005 and Cimmino, et al., 2005).

MicroRNAs play essential roles in normal cellular development but may functionally act as either oncogenes or tumor suppressors by targeting analogous oncogenes or tumor suppressor genes (Chen, 2005). MicroRNAs possess the capacity to directly target gene transcripts and influence cellular physiology that is involved in cancer etiology. As miRNAs can elucidate their function through regulation of specific mRNAs, there has been an immense interest in identifying their targets. Among the differentially expressed miRNAs in breast cancer, miR-10b, miR-125b, miR-145, miR-21, and miR-155 were revealed to be the most consistently deregulated. The down regulation of miR-10b, miR-125b, and miR-145 and up regulation of miR-21 and miR-155 suggested that these miRNAs could play a role as tumor suppressor genes or oncogenes (Enders et al, 2009).

The efficacy of miRNA-based breast cancer therapy has been explored by emerging studies emphasizing their importance in breast cancer. A better understanding of the gene networks and cellular pathways regulated by miRNAs will facilitate further elucidation of breast cancer pathogenesis and therapy. This can be accomplished by identifying the genome-wide targets of miRNAs that is vital.

In our present study, we sought to generate an miRNA- mRNA interaction map for identification of novel mRNA-miRNA target pairs that are hypothesized to play a role in breast cancer through an mRNA- miRNA interaction map analysis of microarray data and experimental validation of selected set of mRNAs which has not been reported yet and may be helpful in treatment of breast cancer through miRNA therapeutics.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Cancer is defined by the uncontrollable growth and proliferation of abnormal cells inside the body. Characteristic immortality of cancer cells lead to their growth and invasion into other adjacent tissues and form a tumor. Cancer cells have the potential to pass into the bloodstream or lymph vessels and travel to other parts of the body, where they can grow and form new tumors that replace normal tissue through a process called metastasis.

Based on such characteristics tumors are categorized into two types. They are:

- **Benign tumors**: Generally considered as non-cancerous, benign tumors form mass of cells that lacks the ability to invade adjacent tissue or spread to distant sites by metastasizing and generally have a slower growth rate. These tumors are typically surrounded by an outer surface or fibrous sheath of connective tissue or remain with the epithelium. They can usually be removed from the body and in most case they never come back.
- **Malignant tumors**: These are characterized by a mass of cells which divide and grow uncontrollably, are capable of invading into adjacent tissues and spreading to distant tissues to become progressively worse and potentially resulting into death. Malignancy in cancers is characterized by invasiveness, anaplasia and metastasis.

Characterized by a myriad of aspects, Cancer is considered not just as a disease rather as a group of diseases that causes uncontrolled growth of abnormal cells in the body. Cancers can be classified on basis of the tissue from which they originate or on basis of the location in the body where they first develop.

Therefore, the different types of cancer classification are illustrated below:

a) **On the basis of Tissue and Blood classifications of cancer:**
    - **Carcinoma-** It is a cancer found in body epithelial tissue that covers or lines surfaces of organs, glands, or body structures. It accounts for almost 80-90% of all cancer cases. For example: Stomach cancer and Breast cancer.
    - **Sarcoma-** It is a malignant tumor growing from connective tissues, such as cartilage, fat, muscles, tendons and bones. It usually occurs in young adults. For example: Osteosarcoma (bone) and Chondrosarcoma (cartilage).

- **Lymphoma-** It is a cancer that originates in the nodes or glands of the lymphatic system, in the function in production of white blood cells and cleaning body fluids, or in organs such as the brain and breast. It is of two types: Hodgkin's lymphoma and Non-Hodgkin's lymphoma.
- **Leukemia-** It is a cancer of the bone marrow that restricts the marrow from producing normal red and white blood cells and platelets. It is also known as Blood cancer. WBCs are needed to resist infection; RBCs are needed to prevent anemia and Platelets keep the body from easy bruising and bleeding. For example: Acute lymphocytic leukemia, chronic lymphocytic leukemia myelogenous leukemia and chronic myelogenous leukemia.
- **Myeloma-** It grows in the plasma cells of the bone marrow. It is of two types: Plasmacytoma where the myeloma cells accumulate in one bone and form a single tumor and Multiple myeloma where the myeloma cells form many bone tumors by accumulating in many bones.
- **Blastoma-** It is a cancers derived from immature "precursor" cells or embryonic tissue. It is more common in children than in older adults.

b) **On the basis of tissue origin**: **(Table 1)**

- **Epithelial tissue tumor-** Epithelial tissue consists of skin tissue that covers and lines the body as well as covering all the body organs such as the digestive system organs and lining the body cavity such as the abdominal cavity and chest cavity. The epithelial cells cancers are called carcinomas. There are different types of epithelial cells and these can develop into different types of cancer. For example: Squamous cell carcinoma (squamous cells of the skin lining of oesophagus), Adenocarcinoma (glandular cells of kidney cells or breast cells), Transitional cell carcinoma (transitional cells of lining of bladder).

- **Mesenchymal or Connective tissue tumor-** Cancers of connective tissues are called sarcomas. Mesenchymal or Connective tissues are the supporting tissues of the body such as the bones, cartilage, tendons and fibrous tissue that support the body organs. Sarcomas are much less common than carcinomas and can develop

from bone, cartilage and muscle. They are usually grouped into two main types - bone sarcomas (osteosarcoma) and soft tissue sarcomas. For example: Chondrosarcoma (cancer of cartilage) and Rhabdomyosarcoma (cancer of a muscle).

- **Blood and Lymph tissue tumor**- There are many different types of blood and lymph tissue cells. Haematopoetic tissue is the tissue present in the bone marrow that is responsible for the formation of blood cells. Blood tissue can develop into Leukemias (cancer of the blood cells) and lymph tissue can develop into Lymphomas (cancer of the lymphatic system).These are the most common type of cancer affecting children. Brain tumors are the biggest group of rare cancers which develop from special connective tissue cells called glial cells that support the nerve cells in the brain. The cancers of the glial cells are called Gliomas.

c) On basis of location in the body:

Cancers are also named by their origin of initiation apart from its spread to other areas. For example, breast cancer that has spread to the liver is still called breast cancer, not liver cancer. Similarly, prostate cancer that has spread to the bone is called metastatic prostate cancer, not bone cancer. Other examples are cervical cancer, oral cancer, etc.

Table 1. Tumor Nomenclature

ORIGIN	CELL TYPE	BENIGN TUMOR	MALIGNANT TUMOR
Epithelial			
Adeno	Gland	Adenoma	Adenocarcinoma
Basal cell	Basal cell	Basal cell adenoma	Basal cell carcinoma
Squamous cell	Squamous cell	Karatoacanthoma	Squamous cell carcinoma
Melano	Pigmented cell	Mole	Melanoma
Terato	Multipotential cell	Teratoma	Teratocarcinoma
Mesenchymal			
Chondro	Cartilage	Chondroma	Chondrosarcoma
Fibro	Fibroblast	Fibroma	Fibrosarcoma
Hamangio	Blood vessels	Hemangioma	Hemangiosarcoma
Leiomyo	Smooth muscle	Leiomyoma	Leiomyosarcoma
Lipo	Fat	Lipoma	Liposarcoma
Meningio	Meninges	Meningioma	Meningiosarcoma
Myo	Muscle	Myoma	Myosarcoma
Osteo	Bone	Osteoma	Osteosarcoma
Rhabdomyo	Striated muscle	Rhabdomyoma	Rhabdomyosarcoma
Blood and Lymph			
Lympho	Lymphocyte		Lymphoma
Erythro	Erythrocyte		Erythrocytic leukemia
Myelo	Bone marrow		Myeloma

Breast cancer is a malignant tumor originating from breast epithelial tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. It is found mostly in women, but men can get breast cancer rarely. It is estimated that approximately greater than 1,300,000 cases of breast cancer are reported each year worldwide out of which around 450,000 resulting in deaths.

Types of Breast cancer:

- **Pre-Invasive Breast Cancer**

It is an early stage of cancer, when it is still confined to the layer of cells where it began and is named for cancer cells that stay inside the milk ducts or milk sacs (lobules) of the breast. They do not spread into deeper tissues in the breast or to other organs in the body.

1. **Ductal carcinoma in situ (DCIS)**

When cancer cells are confined to the ducts it is called Ductal carcinoma in situ. It increases the risk of developing invasive breast cancer. It's important to treat DCIS, to lower the risk of developing invasive breast cancer. DCIS can be found in women at any age mostly between 50 and 59 years old.

2. **Lobular carcinoma in situ (LCIS)**

When cancer cells are confined to the lobules it is called lobular carcinoma in situ. It is not a true cancer or pre-cancer. The cells on the inside of the lobules become abnormal in shape and size, proliferate and stay inside the lobules in the breast this is called Atypical lobular hyperplasia (ALH). If the abnormal cells stay inside the ducts in the breast this is called Atypical ductal hyperplasia (ADH). LCIS, ALH and ADH cannot be felt as a breast lump or other breast change and these situations are usually found by chance when a woman has undergone a breast biopsy.

- **Invasive Breast Cancer**

It is a type of cancer which already grown beyond the layer of cells spreading to the lymph nodes in the breast or armpit area from where it started. These can be either invasive ductal carcinoma or invasive lobular carcinoma.

- **Locally Advanced Breast Cancer**

It is larger than 5cm and may have spread from the breast into the lymph nodes or other tissues adjacent to the breast.

- **Metastatic Breast Cancer**

It is the most malignant stage of breast cancer where the disease has spread to distant metastases. It primarily metastasizes to the bone, lungs, lymph nodes, liver and brain. Metastatic breast cancer cells frequently differ from the preceding primary breast cancer as it has a developed resistance to previous treatment. It has a poor prognosis and causes about 90% of deaths.

The accurate cause of breast cancer is unidentified and there are no permanent causes for breast cancer. Some of the causes that are associated with breast cancer are:

- **Age:** Older woman is at the higher risk of developing breast cancer including over 80% of all female breast cancers occurring among women aged 50+ years after menopause.
- **Inheritance:** Family history of close relative like sister, daughter and mother who has been diagnosed with breast cancer increases the risk factor.
- **Genetics:** It plays a more significant role by causing a hereditary syndrome for breast and ovarian cancer carrying the BRCA1 and BRCA2 gene mutation. Other mutated genes involve p53, PTEN and STK11, CHEK2, BRIP1, ATM and PALB2.
- **Early menopause:** Early start onset of menses and early menopause are also associated with breast cancer.
- **Radioactivity:** Radioactive rays' exposure is carcinogenic and increases the chances of breast cancer.
- **Hormone Replacement Therapy:** Prolonged exposure to the hormones estrogen and progesterone for uninterrupted periods can affect breast cancer risks.
- **Exposure to harmful chemicals:** Chemical factory workers use harmful chemicals like organochlorines.
- **Nullyparity or Late childbearing:** Late childbearing or nullyparity also appear to be a minor risk factor in the development of breast cancer.
- **Alcohol consumption** - More alcohol for a woman regularly creates higher risk of developing breast cancer. (DeSantis et al., 2012 and Breast cancer overview, American Cancer Society, 2012)

MicroRNAs

MicroRNAs have been recently reported to be actively participating in initiation and progression of breast cancer. Such microRNAs (miRNAs) are described as a class of genes that encodes for small non-coding double-stranded RNA molecules of short 18 to 25 nucleotides noncoding genes excised from 60-110 nucleotide hairpin RNA precursors that is involved in the regulation of expression of protein-coding genes (PCGs). It mainly binds the 3'UTR of the target mRNA depending on the respective miRNA directed sequence, thus promoting mRNA degradation at a post transcriptional level or inhibiting the initiation of translation by translational repression (Quesne and Caldas, 2010) (Figure 1).

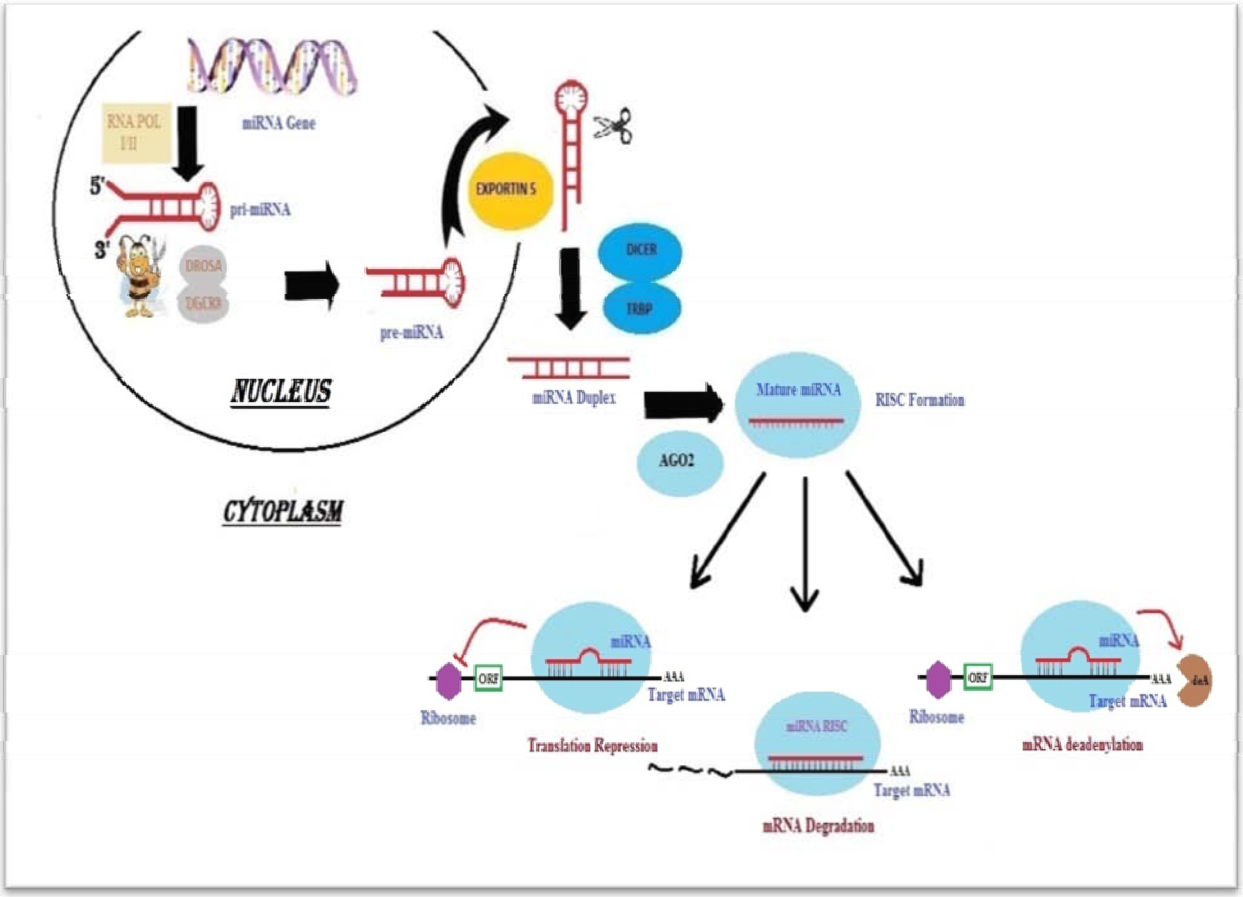

Figure 1: Biogenesis of MicroRNA (Samantarrai et al. 2013)

Rules of microRNA targeting

MicroRNA targeting follows certain rules. According to such rules pairing of mRNA and miRNA requires conserved Watson-Crick paring to the 5' region of the miRNA, centered on nucleotide 2-7 called as the miRNA "seed". Conserved paring to the seed region can also be sufficient on its own for predicting conserved targets above the noise of false positive prediction. The highly conserved target has many conserved targets. Nonconserved targeting is even more widespread than conserved targeting. The 3'- supplementary pairing are the numerous potential pairing possibilities involving the 3' portion of the miRNA and the UTR. The 3' pairing optimally centers on miRNA nucleotides 13-16 and the UTR region directly opposite this miRNA segment. Pairing to the 3' portion of the miRNA can not only supplement a 7-8mer match and can also compensate for a single nucleotide bulge or mismatch in the seed region. Presentation of 2-8 nucleotides prearranged in a geometry resembling an A-form helix enhances both the affinity and specificity for matched mRNA segment, enabling 7-8 nucleotide sites to suffice for most target functions.

Affinity to the seed is stronger than that to the other regions of the RNA. The positioning within the 3'UTR at least 15 nucleotide from the stop codon increases site efficacy whereas away from the center of long UTR also increases site efficacy. Site efficacy is also boosted by AU rich nucleotide composition near the site or other measures of site accessibility and by proximity to sites for co-expressed miRNA. Although most miRNA function has been for sites in 3' UTR, targeting can also occur in 5'UTR and open reading frames. The 3'UTRs with non-conserved sites are most often found in genes primarily expressed in tissues where the cognate miRNA is absent. Target recognition that relies heavily on 7mer nucleotide matches to the seed region creates possibility for a lot of non conserved targeting. ORF targeting is expected to be much more effective in messages that are already inefficiently translated. The hierarchy of site efficacy follows: 8mer>>7mer-m8>7mer-A1>>6mer>no site, with the 6mer differing only slightly from no site at all. For genes that should not be expressed in a particular cell type, the cell can come to depend on its miRNAs to act as binary off-switches to help to repress target protein output to inconsequential levels. Conserved targets have the tendency to be present at low levels in the same tissues as the miRNAs. It is seen that more than 90% of the conserved miRNA: target interactions involve only a single site to the miRNA, and therefore most of these

targets would be expected to be down regulated by less than 50%. miRNA targeting interaction can be disrupted by perturbing an endogenous site through homologous recombination. MicroRNA target sites tend to reside in an unstable region, and tend to lack stabilizing elements called as long stems (Bartel, 2009).

Role of microRNAs in cancer

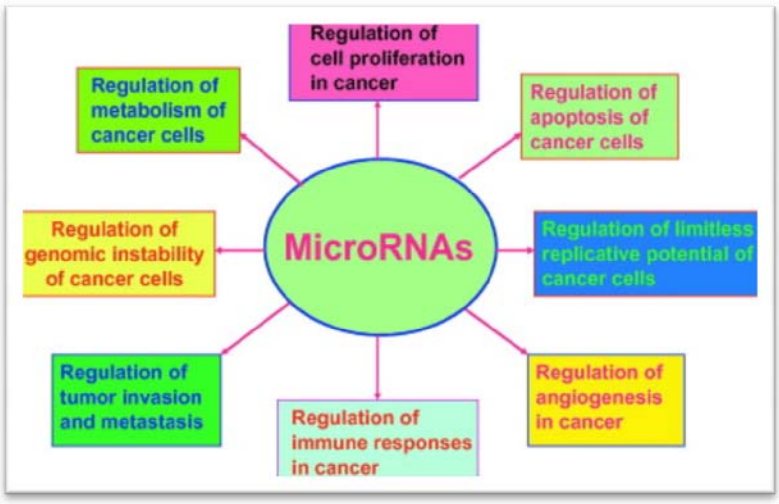

Figure 2. Role of microRNAs in hallmarks of cancer

Role of microRNAs in Breast cancer metastasis

MicroRNAs as balancers between suppression and activation:

The involvement of microRNAs in the development of metastases was initially discovered by Ma and coworkers from Robert Weinberg's group who revealed that up regulation of miR-10b promote breast cancer invasion and metastasis and are over-expressed in about 50% of metastatic breast cancers. They also proved that HOXD10, a homeobox transcription factor is the target of miR-10b. Later, Tavazzoie and colleagues of the Joan Massague group revealed that miR-335 suppresses metastasis and migration by targeting the transcription factor SOX4 and tenascin C which is an extracellular matrix component. At the same time, Huang and coworkers including the Reuven Agami group reported that miR 373 and miR-520c induce cancer cell migration and invasion by suppression of CD44. These landmark studies reveal a fine balance of microRNAs as activation and suppression of metastasis and identify several targets.

Balance between repression of miRNA targets and regulation of miRNA expression:

MicroRNA profiling studies have led to the identification of miRNAs that are aberrantly expressed in human breast cancer with miR-145, miR-10b and miR-125b being down regulated and miR-21 and miR-155 being up regulated. More recent studies have identified microRNA downstream targets and associated particular miRNA expression with prognostic information. Also microRNA has been shown either to be consistently down regulated or up regulated. Tumor formation may arise due to down regulation of a tumor suppressor miRNA and/or over expression of an oncogenic miRNA. Therefore, microRNA has been regarded as the key factors in breast cancer that possess both oncogenic and tumor-suppressing roles (Sidney et al. 2011; Negrini and Calin. 2008) (Figure 2).

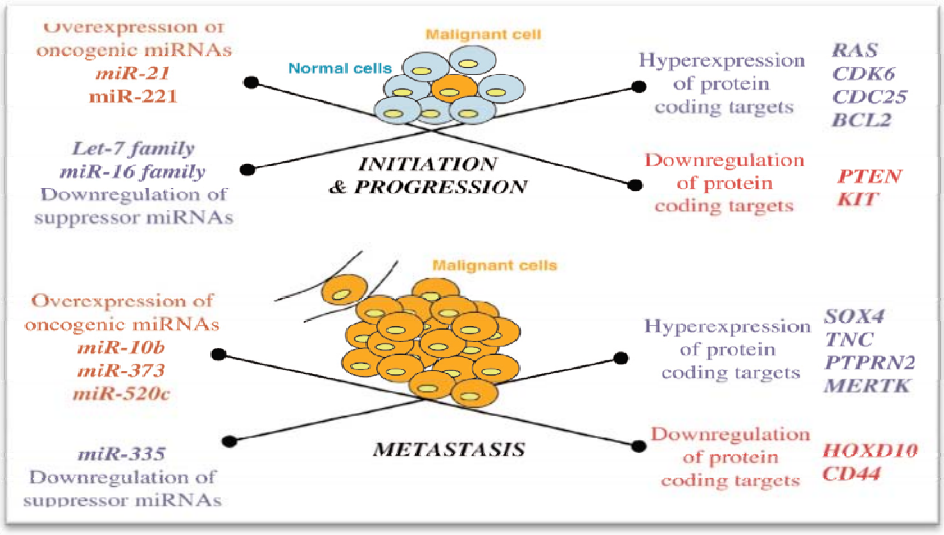

Figure 3: MicroRNAs and the significant confirmed targets (PCGs) are shown to be involved in initiation (early), progression (intermediate) and metastasis (late) stages in tumorigenesis. Initiation and progression stages involves the over expression of oncogenic miRNAs i.e. miR-21 and miR-221 results into the down regulation of PTEN & KIT whereas down regulation of tumor suppressor miRNAs i.e. miR-16 family and Let-7 family results into hyper expression of RAS, CDK6, CDC25 & BCL2. Simultaneously, over expression of oncogenic miRNAs i.e. miR-10b, miR-373 and miR-520c results into the down regulation of HOXD10 & CD44 whereas down regulation of tumor suppressor gene i.e. miR-335 results into hyper expression of SOX4, TNC, PTPRN2 & MERTK respectively in metastasis (Negrini and Calin. 2008).

The expression and function of various miRNAs in breast cancer is summarized (Table 2).

Table 2. miRNAs and their targets in breast cancer

MicroRNAs	TARGET	FUNCTIONAL PATHWAY
Tumor suppressor miRNAs		
miR-206	ESR1	ER signaling
miR-17-5p	AIB1, CCND1, E2F1	Proliferation
miR-125a, b	HER2, HER3	Anchorage-dependent growth
miR-200c	BMI1, ZEB1, ZEB2	TGF-β signaling
let-7	H-RAS, HMGA2, LIN28, PEBP1	Proliferation, differentiation
miR-34a	CCND1, CDK6, E2F3, MYC	DNA damage, proliferation
miR-31	FZD3, ITGA5, M-RIP, MMP16, RDX,	Metastasis
miR-355	RHOA	Metastasis
miR-27b	SOX4, PTPRN2, MERTK, TNC, CYP1B1	Modulation of the response of tumor to anti-cancer drugs
miR-126	IRS-1	Cell cycle progression from G1/G0 to S
miR-101	EZH2	oncogenic and metastatic activity
miR-145	p53-mediated repression of c-Myc	Suppresses Cell Invasion and Metastasis
miR-146a/b	NF-Kb	Negatively regulate factor-κB, and impaired invasion and migration capacity
miR-205	ErbB3 and VEGF-A expression	Inhibits tumor cell growth and cell invasion
Oncogenic miRNAs		
miR-21	BCL-2, TPM1, PDCD4, PTEN, MASPIN	Apoptosis
miR-155	RHOA	TGF-β signaling
miR-10b	HOXD10	Metastasis
miR-373/520c	CD44	Metastasis
miR-27a	Zinc finger ZBTB10, Myt-1	Cell cycle progression G2- M checkpoint regulation
miR221/222	p27Kip1	Tamoxifen resistance

Source: O'Day and Lal (2010) Sidney W. Fu et al. (2011)

Gene interaction network for breast cancer miRNA targets

The effectiveness of miRNA-based breast cancer therapy has been explained by a better understanding of the gene networks and cellular pathways regulated by miRNAs, by identifying the genome-wide targets of miRNAs. It is reported that miRNAs inhibit the expression of many genes which signifies that inclusive regulation can be achieved by over expressing a single miRNA. Simultaneously the deregulation of miRNAs would consequently change the expression of many genes resulting into induced tumorigenesis. The functions of the experimentally validated breast cancer miRNAs and their gene targets that might be incorporated within the pathogenesis of breast cancer can be better understood by a gene interaction network analysis.

In a study, a list of 34 genes was generated that is recognized to be altered by the 11 miRNAs. 19 genes formed a well-connected gene interaction network in which MYC act as the central node that is a target of miR-34a. Similarly, more highly interacting genes were *BCL-2*, *E2F1*, *CCND1* and *ESR1*, respectively. The results suggested that breast cancer is associated with the alteration in the expression of multiple miRNAs that disturb a network of genes which can either activate or inhibit each other's expression. The down regulation of miR-206 enhanced *ESR1 and MYC* expression. CCND1, E2F1 and E2F3 expressions are activated by MYC elevation. Additionally, miR-17-5p (regulates *CCND1* and *E2F1*) and miR-34a (regulates *E2F3*, *CCND1* and *CDK6*) also elevate the levels of these proteins. Thus suggested that down regulation of several tumor suppressor miRNAs may lead to up regulation of oncogenes in breast cancer via direct or indirect mechanisms. Increased levels of miR-21 regulate TPM1, miR-31 target MMP16 and miR-373/520c target CD44 that is seen to be repressed in breast cancer cells. The tumor suppressor or oncogenic miRNAs regulate the transcription of some of these genes by targeting transcription factors. The gene interaction network essential for breast cancer progression undergoes a change due to loss of miRNA regulation leading to a cascade of events at different stages of progression and metastasis possibly in most gene regulatory events. Other several miRNAs involved in breast cancer are miR-7, miR-128a, miR-210, miR-27b, miR-335, miR-126, miR-145 and miR-27a (Blenkiron et al., 2007) (Figure 3).

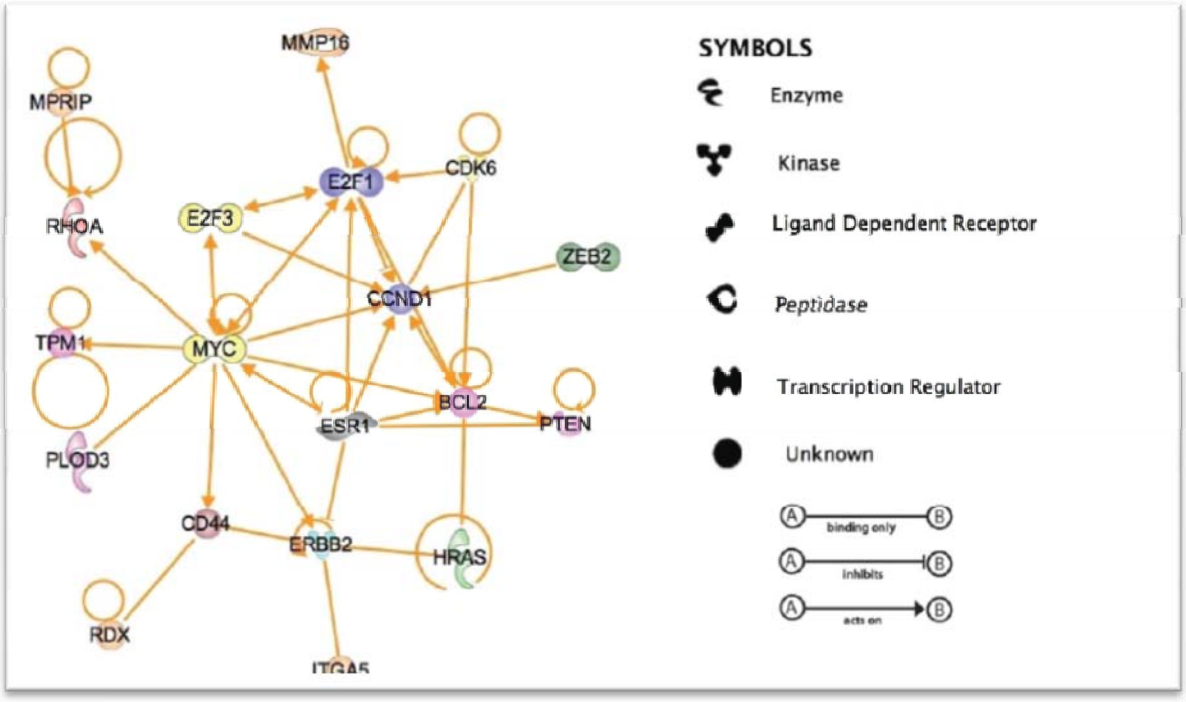

Figure 4. Gene interaction network analysis of miRNA targets in breast cancer. The direct interaction network of 34 published targets of 11 miRNAs implicated in breast cancer pathogenesis is determined using Ingenuity software and is shown: miR-206, miR-17-5p, miR-125a/b, miR-200, let-7, miR-34, miR-31, miR-21, miR-155, and miR-373/520c. Central highly connected network of targets are formed on MYC. Protein-protein or protein-DNA interactions are indicated by arrows suggesting these targets coordinate the expression and/or function of one another (Blenkiron et al., 2007).

MicroRNA profiling is a promising and powerful diagnostic tool that can be useful to differentiate the characteristics of different tumor types in breast cancer, such as the miRNA signatures that can clearly distinguish normal and malignant breast tissue and also categorize between breast cancer subtypes. Thus, the detection of the genes regulated by miRNAs and the clarification about their integrating mechanisms in breast cancer gene interaction network can better support in understanding this malignant disease.

OBJECTIVES

OBJECTIVES

OBJECTIVE 1

Microarray expression analysis for identification of differentially expressed mRNA in breast cancer

OBJECTIVE 2

Microarray expression analysis for identification of differentially expressed miRNA in breast cancer

OBJECTIVE 3

Target prediction and generation of miRNA–mRNA interaction map for differentially expressed sets of miRNA and mRNA

OBJECTIVE 4

Identification of novel miRNA–mRNA target pairs involved in breast cancer & experimental validation of mRNA expression by RT-PCR in MDA-MB-231 cell lines

MATERIALS AND METHODS

MATERIALS & METHODS

1) Gene Expression data

The gene expression data is taken in order to conduct a genome wide analysis of mRNA and miRNA expression of normal and diseased sample in breast cancer, and also to study and distinguish between the various expressions patterns for diagnosis and therapeutic mechanisms.

The gene expression data were retrieved from **Gene Expression Omnibus (GEO)** database. GEO a public functional genomics data repository supporting MIAME-compliant data submissions that archives and freely distributes next-generation sequencing, microarray and other forms of high-throughput functional genomic data submitted by the scientific community. A collection of web-based interfaces and applications are available to provide help users query and download experiments and curated gene expression profiles stored in GEO. The GEO data contains raw microarray data that involves images that are to be converted into gene expression matrices, where rows represent genes, columns represent various samples such as tissues or experimental conditions, and numbers in each cell illustrate the expression level of the particular gene in the particular sample. Analysis of the matrices can be further done in order extract any biological process and its understanding. Platform describes the list of features on the array (e.g., cDNAs, oligonucleotides, etc.). There is an importance of using different platforms as because of the diversity of technical and analytical sources that can affect the results of an experiment. Therefore, a comparison among experiments, its standardization within a single platform may be inefficient. So, large-scale comparison studies involving microarrays can be done for optimum reproducibility measurements using various platforms.

The gene expression data of mRNA and miRNA were taken. The mRNA breast cancer data taken were on breast epithelium from reduction mammoplasty of reduction mammoplasty patient and breast epithelium adjacent to tumor of breast cancer patient. The miRNA breast cancer data taken were on mastectomy samples of Normal breast tissue and Invasive breast cancer. Respective mRNA data were chosen in order to find out the mRNA differential expression in mammoplasty patient and breast cancer patients. The miRNA data

were selected in order to investigate the miRNA differential expression in normal breast tissue and invasive breast cancer tissues.

- The PLATFORM taken were:

mRNA	[HG-U133A] Affymetrix Human Genome U133A Array (GPL96)
miRNA	Agilent-031181 Unrestricted_Human_miRNA_V16.0_Microarray 030840 (GPL15018)

- The SAMPLE taken were:

mRNA	GSE9574 (Gene expression abnormalities in histologically normal breast epithelium of breast cancer patients)
miRNA	GSE38867 (MicroRNAs expression profiling during breast cancer progression)

2) Microarray analysis of gene expression data-

a) Retrieval of gene expression data –

- The SERIES taken for mRNA were: GSM242005
  GSM242006
  GSM242007
  GSM242020
  GSM242021
  GSM242022
- The data were taken in triplicates. Samples in an experiment have associated experiment parameters and corresponding parameter values, so triplicates were taken.
- The raw files were downloaded in .CEL format.

- Then the files were unzipped, extracted and renamed as control and test.

	Control	Test
mRNA	GSM242005 GSM242006 GSM242007	GSM242020 GSM242021 GSM242022

- The SERIES taken for miRNA were: GSM951044
  GSM951048
  GSM951052
  GSM951046
  GSM951050
  GSM951054

- The data were taken in triplicates. Samples in an experiment have associated experiment parameters and corresponding parameter values, so triplicates were taken.
- The raw files were downloaded in .CEL format.
- Then the files were unzipped, extracted and renamed as control and test.

	Control	Test
miRNA	GSM951044 GSM951048 GSM951052	GSM951046 GSM951050 GSM951054

b) Analysis of gene expression data-

The software used for gene expression analysis was Genespring GX software. It is a powerful microarray expression data analysis tool sold by Agilent, and something of a standard for such task which consists of a wide range of analytical algorithms for gene expression microarray experiments. It is used for identifying classes of transcripts that show expression patterns that are correlated with the experiment variables, displaying these transcripts against a backdrop of biochemical pathways and querying for transcripts using e.g., gene symbol, gene name or GO ontology terms.

Working in GeneSpring GX is organized into projects. A project comprises one or more related experiments. An experiment comprises samples (i.e. data sources), interpretations (i.e. groupings of samples based on experimental parameters), and analyses (i.e. statistical steps and associated results, typically entity lists). Statistical steps and methods of analysis are driven by a workflow which finds prominent mention on the right side of GeneSpring GX.

A **project** is the key organizational element in GeneSpring GX. It is a container for a collection of experiments. For example, one experiment measures gene expression profiles of individuals with and without breast cancer. A new project can be created from Project −New Project by just specifying a name for the project and optionally any user notes.

An **experiment** in GeneSpring GX represents a collection of samples for which arrays have been run in order to answer a specific scientific question. A new experiment is created from Project −New Experiment by loading samples of a particular technology and performing a set of customary pre-processing steps like, normalization, etc., that will convert the raw data from the samples to a state where it is ready for analysis. An experiment consists of multiple samples, with which it was created and multiple interpretations by grouping these samples by user-defined experimental parameters, and all other objects created as a result of various analysis steps in the experiment.

An experiment comprises a collection of **samples**. These samples are the actual hybridization results. Each sample is associated with a chip type or its technology and will be imported and used along with a technology.

GeneSpring GX technology contains information on the array design as well as biological information about all the entities on a specific array type. A technology initially must be installed for each new array type to be analyzed. For standard arrays from Affymetrix, Agilent and Illumina, technologies have been created. An experiment comprises samples which all belong to the same technology.

Steps followed for gene expression analysis in GeneSpring:-

- **For mRNA expression analysis:**

Normalization of the data was done to minimize systematic non-biological differences to reveal true biological differences, which may include systematic variations from sources like unequal quantities of starting RNA, differences in hybridization between chips and differences between manufactured chips in microarray experiments. Profile plot of Normalized intensity map values is obtained after Normalization of data. Data is normalized to 75th percentile of signal intensity to standardize each chip for cross-array comparison. The main objective is for eliminating redundancy and ensuring that the data make sense with minimum number of entities.

An option of **Create new experiment** was chosen that allows creating a new experiment. The **Experiment type** should then be specified as **Affymetrix Gene Chip-HG-U113A**. Once the experiment type is selected, the workflow type needs to be selected as **Guided workflow**.

An experiment can be created using **choose sample** option. **Experimental setup** was done by adding average parameter to help define the **experimental grouping** as test and control and replicate structure of the experiment.

Quality control of samples was done by **Filter Probesets by Errors.** This operation is performed on the raw signal values. The cutoff for filtering is set at 20 percentile of all the intensity values and generates a profile plot of filtered entities. The plot is generated using the normalized (not raw) signal values and samples grouped by the active interpretation.

Depending upon the experimental grouping, the **Significance Analysis** was done by performing **T-test unpaired** analysis as there are 2 groups, i.e. the Control and the Test, with replicates.

Statistical analysis was done by **T-test unpaired** as a test choice. The test has been used for computing p-values, type of correction used and P-value computation type by **Asymptotic** method. It assumes expression values for a gene within each population which is normally distributed and variances are equal between populations. The **p-value** cut-off taken was **≤ 0.05**.

Multiple testing correction was done by using **Benjamini-Hochberg FDR** algorithm. This algorithm is used to reduce the number of false positives or the false discovery rate. This correction is the least stringent and tolerates more false positives. There are chances of less false negative genes. If the p-value is **≤ 0.05**, it is significant.

Fold change analysis is used to identify genes with expression ratios or differences between a test and a control that are outside of a given cutoff or threshold. Fold change gives the absolute ratio of normalized intensities between the average intensities of the samples grouped. The entities satisfying the significance analysis are passed on for the fold change analysis. The fold change cut-off taken is **≥2.0**.

The analyzed data was exported by **export entity list** with normalized signal values consisting of Normalization values, Gene symbol, Entrez gene IDs etc. with interpretation of all samples. The entity list was then saved as .txt file.

The software used for clustering and generation of Heat map was done by using **CLUSTER 3.0**. It is a program that provides a computational and graphical environment for analyzing data from DNA microarray experiments by organizing and analyzing the data in a

number of different ways. The Cluster program provides several clustering algorithms. **Hierarchical clustering** methods organize genes in a tree structure, based on their similarity and assemble a set of items (genes or arrays) into a tree. Items are joined by very short branches if they are very similar to each other and longer branches if their similarity decreases.

The software used for visualization of Heat map was done by **Java TreeView** which allows the organized data to be visualized and browsed by a *.cdt* file generated through CLUSTER 3.0 and was exported as image.

- **For miRNA expression analysis:**

Normalization of the data was done to minimize systematic non-biological differences to reveal true biological differences, which may include systematic variations from sources like unequal quantities of starting RNA, differences in hybridization between chips and differences between manufactured chips in microarray experiments. Profile plot of Normalized intensity map values is obtained after Normalization of data. Data is normalized to 75th percentile of signal intensity to standardize each chip for cross-array comparison. The main objective is for eliminating redundancy and ensuring that the data make sense with minimum number of entities.

An option of **Create new experiment** was chosen that allows creating a new experiment. The **Experiment type** should then be specified as **Agilent_031181.** Once the experiment type is selected, the workflow type needs to be selected as **Guided workflow**.

An experiment can be created using **choose sample** option. **Experimental setup** was done by adding average parameter to help define the **experimental grouping** as test and control and replicate structure of the experiment.

Quality control of samples was done by **Filter Probesets by Flags.** This operation is performed on the raw signal values. The cutoff for filtering is set at 20 percentile of all the intensity values and generates a profile plot of filtered entities. The plot is generated using the normalized (not raw) signal values and samples grouped by the active interpretation.

Depending upon the experimental grouping, the **Significance Analysis** was done by performing **T-test unpaired** analysis as there are 2 groups, i.e. the Control and the Test, with replicates.

Statistical analysis was done by **T-test unpaired** as a test choice. The test has been used for computing p-values, type of correction used and P-value computation type by **Asymptotic** method. It assumes expression values for a gene within each population which is normally distributed and variances are equal between populations. The **p-value** cut-off taken was **≤ 0.05**.

Multiple testing correction was done by using **Benjamini-Hochberg FDR** algorithm. This algorithm is used to reduce the number of false positives or the false discovery rate. This correction is the least stringent and tolerates more false positives. There are chances of less false negative genes. If the p-value is **≤ 0.05**, it is significant.

Fold change analysis is used to identify genes with expression ratios or differences between a treatment and a control that are outside of a given cutoff or threshold. Fold change gives the absolute ratio of normalized intensities between the average intensities of the samples grouped. The entities satisfying the significance analysis are passed on for the fold change analysis. The fold change cut-off taken is **≥2.0**.

The analyzed data was exported by **export entity list** with normalized signal values consisting of Normalization values, Gene symbol, etc. with interpretation of all samples. The entity list was then saved as .txt file.

c) Analysis of the gene list-

The common set of genes was analyzed using GO database (Gene Ontology database), various technologies like Genomatrix and web based tools like Web based gene set analysis tool kit and their involvement in various pathways was studied. From the common set of genes two genes were selected based upon their regulation and association with cancer for further validation by qRT-PCR.

A set of genes involved in breast cancer with its regulation and fold change value was created through export list in an excel file. Similarly, a list of miRNAs involved in breast cancer with its regulation and fold change value with cut off ≥2.0 was created.

- For **mRNA** gene list analysis:
  The analysis was done by comparing the fold change value and regulation of the control and test samples. The list of down regulated genes in breast cancer was sorted out.
- For **miRNA** gene list analysis:
  The analysis was done by comparing the fold change value and regulation of the control and test samples. The list of up regulated miRNAs in breast cancer was sorted out too.

The genes and miRNAs are chosen on basis of their regulation i.e. highly down regulated and highly up regulated.

3) Target Interaction Map analysis through Magia^2 software:

The integrated analysis of *in silico* target prediction, miRNA and gene expression data for the reconstruction of post-transcriptional regulatory networks is performed by using software called Magia$^{2.}$ The gene expression profile can be resulted because of different levels of regulation and a highly connected network of regulatory elements and their interactors. Magia^2 software is a web based tool designed to cope with low sensitivity of target prediction algorithms by exploiting the integration of target predictions with miRNA and gene expression profiles to

improve the detection of functional miRNA–mRNA, for in silico target prediction through miRNA-target expression where regulatory elements and their integrators generate a highly interconnected network of mRNA, miRNA and Transcription Factor (TF). Functional enrichment of the gene network component can be performed directly using DAVID platform. The miRNA-target interactions experimentally validated (as reported in miRecords and TarBase databases) are specifically marked.

The respective data i.e. the gene expression data of mRNA and miRNA expression data of miRNA were uploaded and submitted. The analysis was done using **Pearson correlation** method that aims to display the target interaction map for matched miRNAs and gene expression data.

An interaction map was generated where the interconnected network of mRNA, miRNA and Transcription Factor were seen. From the interaction map, two genes and two miRNAs were selected based upon their regulation and association with breast cancer for further validation by qRT-PCR.

These selected genes (mRNAs) and miRNAs or the target pairs are not yet been reported to be involved in breast cancer. Therefore, web based tool and technology like Genomatrix is also used to study their involvement in breast cancer and various pathways.

4) Experimental Validation-

Cell culture-

Human breast carcinoma cell line, MDA MB 231 was obtained from National Centre For Cell Science (NCCS), Pune, India. The medium used for culturing the cell is MEM (Invitrogen; MEM with NEAA (non essential amino acids) and L-Glutamine) with 10% FBS (Fetal bovine serum from HIMEDIA) and 1% antibiotic solution (Penstrep solution from HIMEDIA). The culture flask containing the cell line is kept in the CO_2 incubator with the level of CO_2 maintained at 5%. With the utilization of medium the color of the medium changes from red to orange and then pale yellow because of change in pH of the medium.

The steps for cell culture was as followed:

1. The cells were harvested first.

- Cells were grown in suspension i.e. 1 x 107 cells. The number of cells was determined. The appropriate number of cells was pelleted by centrifuging for 5 min at 300 x g in a centrifuge tube. Carefully removed all supernatant by aspiration completely from the cell culture medium.
- To trypsinize and collect cells: The number of cells was determined. The medium was aspirated, and the cells were washed with PBS. Then the PBS was aspirated, and 0.1–0.25% trypsin in PBS was added. After the cells detach from the flask, medium (containing serum to inactivate the trypsin)was added , the cells were transferred to an RNase-free glass or polypropylene centrifuge tube and centrifuged at 300 x g for 5 min. The supernatant was aspirated completely, and proceeded to step 2.

2. The cells was disrupted by adding Buffer RLT:

- For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. 350 µl Buffer RLT was added. Vortexed or pipetted to mix, and ensured that no cell clumps were visible and proceeded to step 3.

3. The lysate was homogenize for 30 s using a rotor–stator homogenizer and proceeded to step 4.
4. 1 volume of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting. Did not centrifuge.
5. 700 µl of each sample was transferred from step 4, including any precipitate to each RNeasy spin column on the vacuum manifold.
6. The vacuum was switched on and was applied until transfer was complete. Then switched off the vacuum and ventilated the vacuum manifold.
7. 700 µl Buffer RW1was added to each RNeasy spin column.
8. The vacuum was switched on and was applied until transfer was complete. Then switched off the vacuum and ventilated the vacuum manifold.
9. 500 µl Buffer RPE was added to each RNeasy spin column.
10. The vacuum was switched on and was applied until transfer was complete. Then switched off the vacuum and ventilated the vacuum manifold.
11. 500 µl Buffer RPE was added to each RNeasy spin column.

12. The vacuum was switched on and was applied until transfer was complete. Then switched off the vacuum and ventilated the vacuum manifold.
13. The RNeasy spin columns was removed from the vacuum manifold, and was placed each in a 2 ml collection tube. The lids were closed gently, and centrifuged at full speed for 1 min.
14. Each RNeasy spin column was placed in a new 1.5 ml collection tube. 30–50 µl RNase free water was added directly to each spin column membrane. The lids were closed gently, and centrifuged for 1 min at 8000 x g (10,000 rpm) to elute the RNA.
15. If the expected RNA yield is >30 µg, then step 15 was repeated using another 30–50 µl RNase free water or using the eluate from step 14 (if high RNA concentration is required). The collection tubes were reused from step 14.

Note: If using the eluate from step 14, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

RNA Isolation-

The kit used for RNA isolation was from QIAGEN.

1. A maximum of 1×10^7 cells was harvested, as a cell pellet or by direct lysis of/in vessel. The appropriate volume of Buffer RLT was added.
2. 1 volume of 70% ethanol was added to the lysates and mixed well by pipetting. Did not centrifuge. Proceeded immediately to step 3.
3. Up to 700 µl of the sample was transferred, including any precipitation, to an RNeasy Mini spin column placed in a 2ml collection tube (supplied). The lid was closed and centrifuged for 15s at ≥8000×g. The flow –through was discarded.
4. 700 µl Buffer RW1 was added to the RNeasy spin column. The lid was closed and centrifuged for 15s at 8000×g. The flow –through was discarded.
5. 500 µl Buffer RPE was added to the RNeasy spin column. The lid was closed and centrifuged for 15s at ≥8000×g. The flow –through was discarded.
6. 500 µl Buffer RPE was added to the RNeasy spin column. The lid was closed and centrifuge for 2 min at ≥8000×g.

7. The RNeasy spin column was placed in the new 1.5 ml collection tube. 30-50 µl RNase-free water was added directly to the spin column membrane. The lid was closed and centrifuged for 1min at ≥8000×g to elute the RNA.
8. If the expected RNA yield is >30 µg, then step 7 was repeated using another 30-50µl of RNase- free water, or using the eluate from step-7. The collection tubes were reused from step-7.
9. The purity and yield of RNA yield was measured by **Eppendorf NanoDrop**. It is a cuvette free spectrophotometer which eliminates the need for other sample containment devices and allows for clean up in seconds. It measures 1 µl samples with high accuracy and reproducibility. The full spectrum (220nm-750nm) spectrophotometer utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place. A 1 µl sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The instrument is controlled by PC based software, and the data is logged in an archive file on the PC.

cDNA synthesis-

cDNA synthesis was carried out using SuperScript First-Strand Synthesis System for RT-PCR by Invitrogen using oligo dT primers.

The steps in cDNA synthesis:

1. Each of the components were mixed and briefly centrifuged before use.
2. For each reaction, the following in a sterile 0.2 or 0.5ml tube was combined.

Components	Amount
RNA	4 µl
10 mM dNTP mix	1 µl
Primer (0.5µg/µl oligo $(dT)_{12-18}$ or 2µM gene specific primer)	1µl
DEPC treated water	4 µl

3. The RNA/primer mixture at 65·c for 5 minutes was incubated, and then placed on ice for at least 1 minute.
4. In a separation tube, the following 2X reaction was prepared by adding each component in the indicated order.

Components	1RXn	10 RXns
10X RT buffer	2 µl	20 µl
25mM $Mgcl_2$	4 µl	40 µl
0.1M DTT	2 µl	20 µl
RNase out ^{TM} (400/ µl)	1 µl	10µl

5. 9µl of the 2X reaction mixture was added to each RNA/primer mixture from step3, mixed gently and collected by briefly centrifuge.
6. It was incubate at 42°c for 2 minutes.
7. 1µl of super script TM II RT was added to each tube.
8. It was incubate at 42°c for 50 minutes.
9. The reaction was terminated at 70·c for 15 minutes. Chilled on ice.
10. The reaction was collected by brief centrifugation. 1µl of RNase H was added to each tube and incubated for 20minutes at 37·c. The reaction was used for PCR immediately.

Quantitative Real Time RT-PCR Analysis-

Real-time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles. Quantitative RT-PCR involves the conversion of the fluorescent signals from each reaction into a numerical value for each sample. Fluorescent marker is used which binds to the DNA. Therefore, as the number of gene copies increases during the reaction so the fluorescence intensity increases. This is advantageous because the rate of the reaction and efficiency can be seen. Intercalating fluorescent dyes (e.g. SYBR green) are the simplest and cheapest way to monitor a PCR in real-time. The SYBR green dye fluoresces only when bound to double-stranded DNA. The major disadvantage of using a dye such as this is the lack of specificity.

The Gene specific **primer sequence** were obtained from Primer Bank database (Harvard) and ordered from the SIGMA GENOSYS. All the primers were desalted and UV absorbance was used to assess the quality of primer synthesis.

Procedure:

To perform PCR using RNA as a starting template which must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction, where the cDNA is used as template for real-time PCR with gene specific primers.

Table 3: Primer name and sequence with length and its amplicon size

Primer name	5'< ----sequence---- >3'	Length	Amplicon Size (nts)
beta actin F	CATGTACGTTGCTATCCAGGC	21	250
beta actin R	CTCCTTAATGTCACGCACGAT	21	
MARCKS F	AGCCCGGTAGAGAAGGAGG	19	110
MARCKS R	TTGGGCGAAGAAGTCGAGGA	20	
SIK1 F	CTCCGGGTGGGTTTTTACGAC	21	93
SIK1 R	CTGCGTTTTGGTGACTCGATG	21	

Real-time PCR was carried out in Eppendorf Masterplex Real Time PCR.

1. The primer concentrations were normalized and gene-specific forward and reverse primer pair was mixed. Each primer (forward or reverse) concentration in the mixture was 3.5 µl.
2. The experiment was set up and the following PCR program was made on. A copy of the setup file was saved and all PCR cycles were deleted. The threshold frequency taken was 33%. The cycle temperatures taken were as follows:

Table 4: Cycle temperature and time for qRT-PCR

STAGE	TEMPERATURE (°C)	TIME	CYCLE
Stage 1	95	20 sec	1
Stage 2	95 55 68	15 sec 15 sec 20 sec	40
Stage 3	95 60 95	15 sec 15 sec 15 sec	1

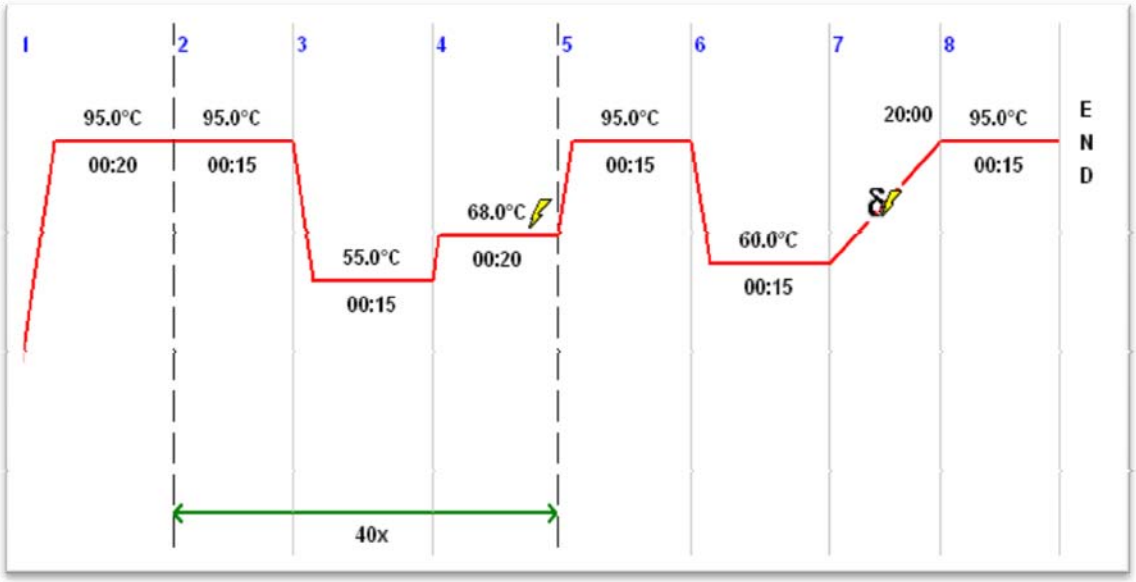

Figure 5. Cycle temperature and time for qRT-PCR

3. cDNA was diluted 1:20 ratio concentration and the primer was added.
4. 10 µl of a real-time PCR reaction volume was made.
5. The following mixture was made in each optical tube as follows:

SYBR Green Mix (2x)	35 µl
cDNA stock (cDNA: dH_2O [1:20])	40 µl
primer pair mix (3.5 µl each primer)	7 µl

6. The dissociation curve analysis was performed with the saved copy of the setup file.
7. The real-time PCR result was analyzed with the in-built software. It was also checked to see if there was any bimodal dissociation curve or abnormal amplification plot.
8. After PCR is finished, the tubes were removed from the machine.

RESULTS AND DISCUSSIONS

RESULTS & DISCUSSIONS

Microarray Analysis

mRNA Expression analysis result:

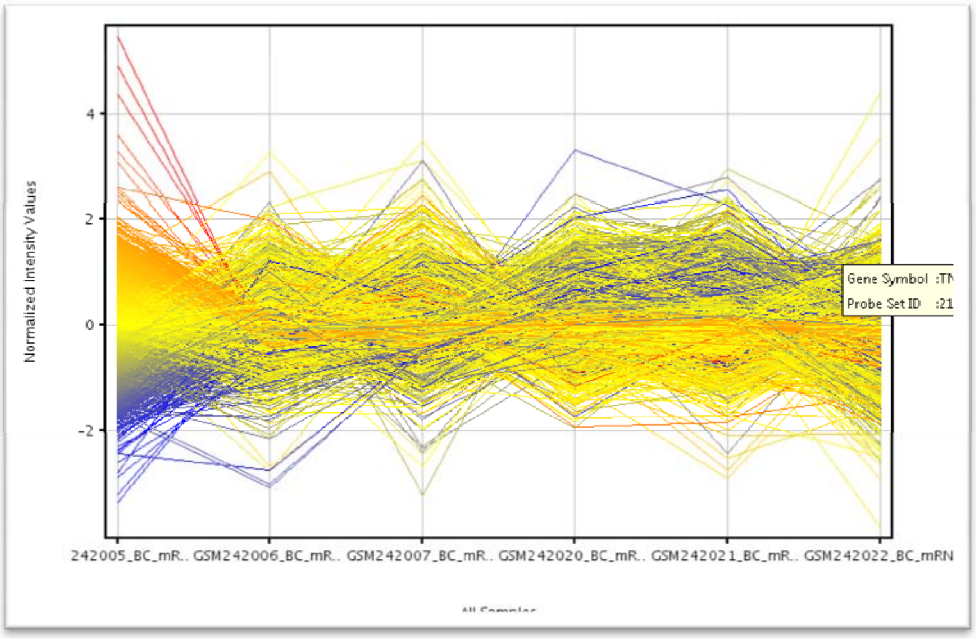

Figure 6. Analysis of all entities in the sample

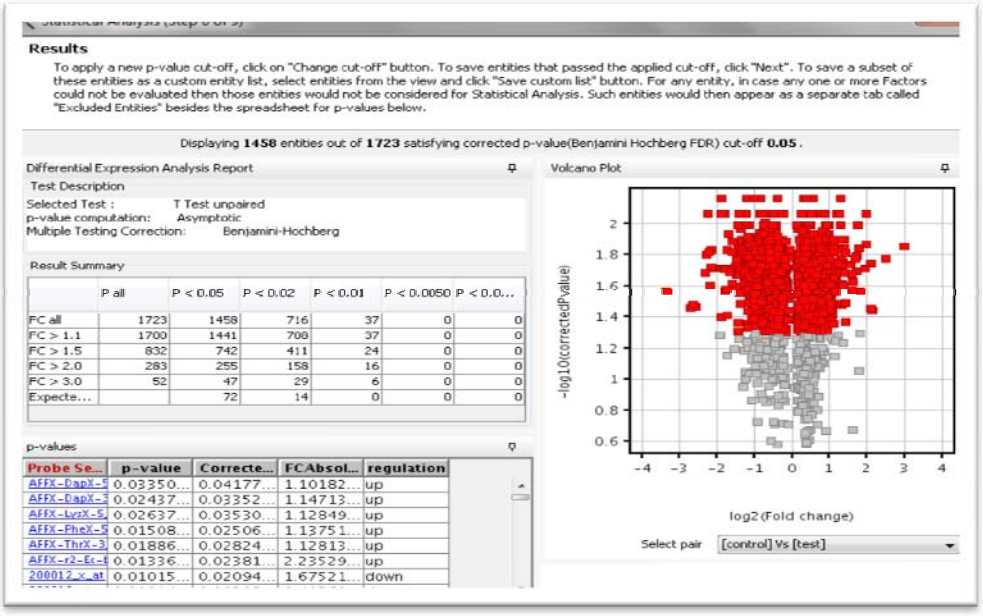

Figure 7. Statistical analysis by taking the p-value cut-off ≤ 0.05

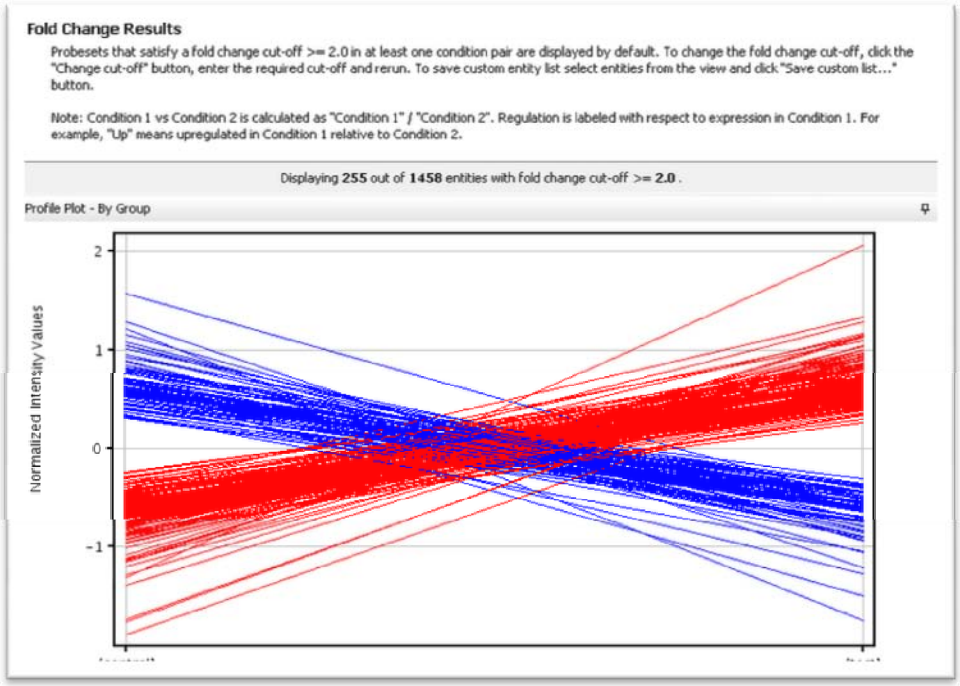

Figure 8. 225 entities (genes) are identified when Fold change cut-off was taken ≥2.0

The total entities of differentially expressed genes in breast cancer are 225, out of which 73 are down regulated and 152 are up-regulated.

miRNA expression analysis result:

From the miRNA expression analysis we found out that the total number of differentially expressed miRNAs in breast cancer is 94 by taking the p-value cut-off ≤ 0.05 and fold change cut off ≥ 2.0, out of which 82 are down-regulated and 12 are up-regulated.

Table 5: List of microRNAs with fold change and its regulation

microRNA	Fold change (cut off ≥2.0)	Regulation
hsa-let-7f	2.114769	Upregulated
hsa-let-7i	2.9767618	Upregulated
hsa-miR-1274a	5.035936	Upregulated
hsa-miR-1274b	2.7655098	Upregulated
hsa-miR-16	2.2772193	Upregulated
hsa-miR-193b	4.4158297	Upregulated
hsa-miR-200b	2.5386198	Upregulated
hsa-miR-21	8.594158	Upregulated
hsa-miR-331-3p	3.0384824	Upregulated
hsa-miR-24	2.3400607	Upregulated
hsa-miR-27a	2.127638	Upregulated
hsa-miR-22	2.2989354	Upregulated

Target interaction map analysis result:

The interconnected network of mRNA, miRNA and Transcription Factor was generated as an interaction map (Figure 4).

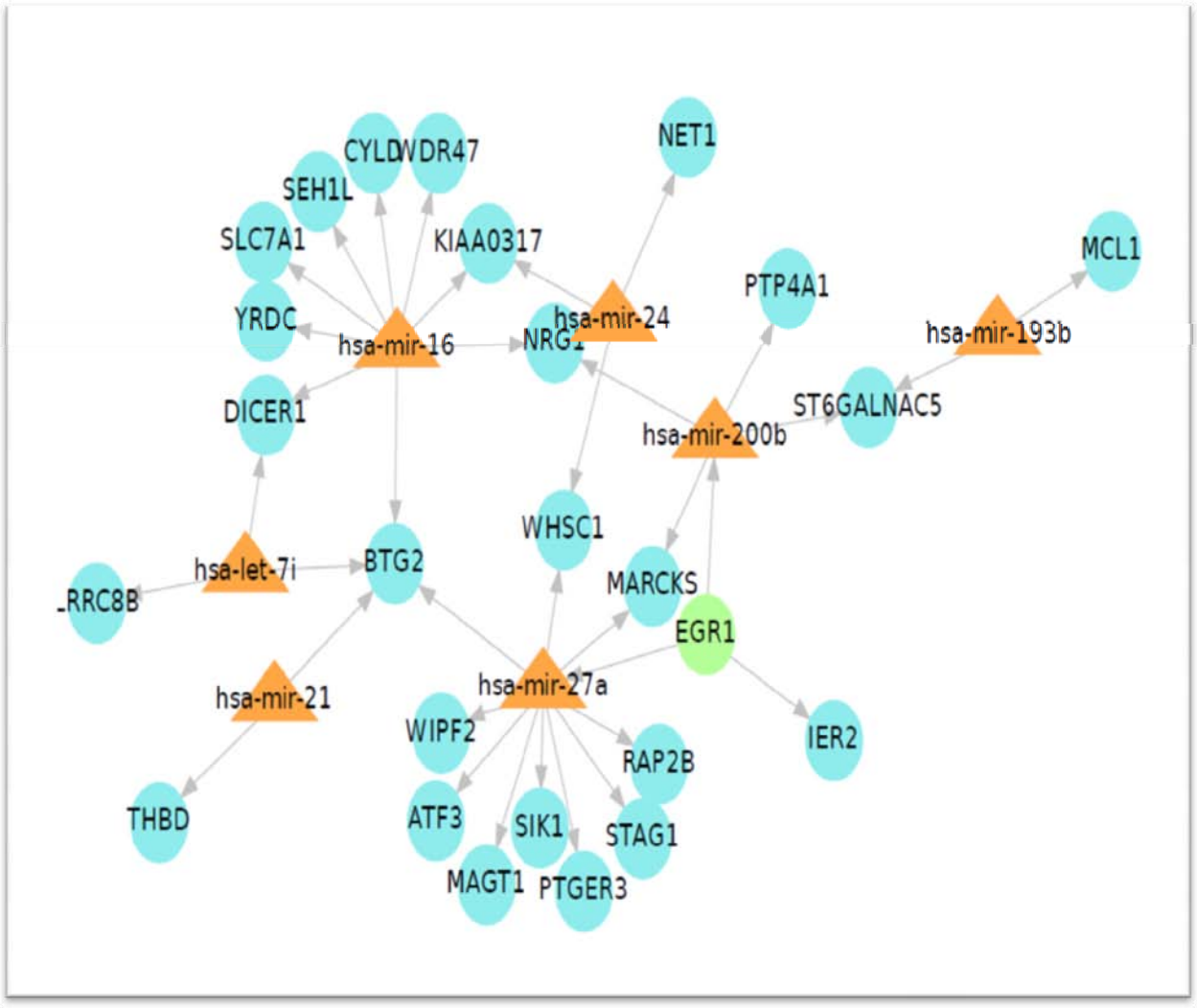

Figure 9. Interaction Map of mRNAs and miRNAs involved in breast cancer

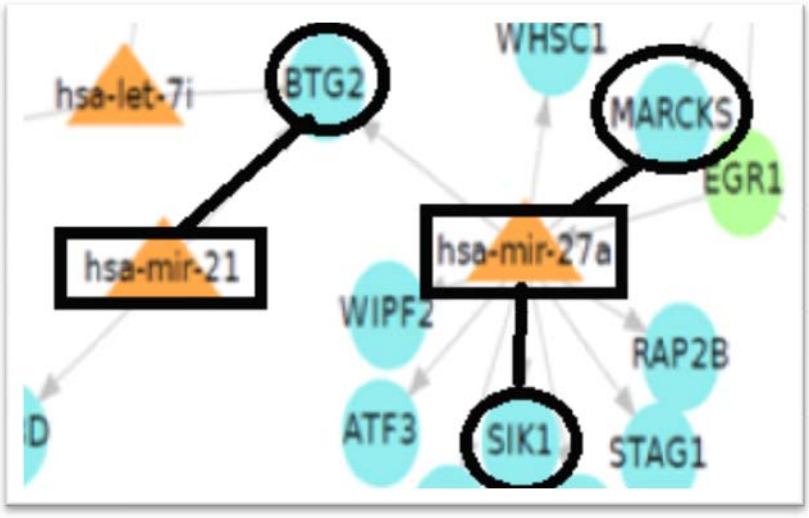

Figure 10. The interaction pairs where it is seen that *hsa-miR-21* target BTG2 and *hsa-miR-27a* target both SIK1 and MARCKS

From the interaction analysis it was seen that the two miRNAs i.e. *hsa-miR-21* target BTG2 whereas *hsa-miR-27a* target SIK1 and MARCKS. The genes MARCKS, SIK1and BTG2 are reported to be highly down regulated in breast cancer whereas the miRNAs *hsa-miR-21* and *hsa-miR-27a*, are reported to be highly up regulated in breast cancer.

- Using **GENOMATRIX** the respective pathway, regulation and function of the genes are known.

MARCKS

It is abbreviated as Myristoylated Alanine-rich C kinase Substrate gene which is the most prominent cellular substrate for protein kinase C (80 kDa protein, light chain). It is also known as MACS, PRKCSL (*Homo sapiens*). MARCKS gene is the most prominent cellular substrate for protein kinase C (Spizz and Blackshear, 1996, 1993). It is localised in the plasma membrane and is an actin filament cross-linking protein. Subcellular location of MARCKS is more prominent in cytoplasm than the plasma membrane (Seykora et al., 1996). MARCKS binds to plasma membranes via the dual actions of a hydrophobic myristoylated N-terminus and a polybasic stretch within the so called effector domain (ED) that mediates electrostatic interactions with acidic membrane Phospholipids (Brooks et al., 1996). Phosphorylation of

protein kinase C or binding to Calcium calmodulin displaces MARCKS from the plasma membrane inhibiting its association with filamentous actin linking activity with the plasma membrane and also its presence in the cytoplasm (Allen and Aderem, 1995). Myristic acid targets MARCKS at the N-terminal of glycine to bind the plasma membrane and phosphorylate by PKC. This leads to the down-regulation of MARCKS (Jonsdottir et al., 2005 & Arbuzova et al, 2002).

The protein is involved in cell motility, membrane trafficking, phagocytosis and mitogenesis. These are acidic proteins with high proportions of Alanine, Glycine, Proline and Glutamic acid. (Ramsden, 2000). It plays an important role in cell shape, secretion, transmembrane transport and cell-cycle regulation. (Finlayson et al.,2009). Recently, MARCKS has been implicated in the exocytosis of a number of vessicles and granules such as mucin and chromaffin. MARCKS are also seen to interact with TOB1. Also, MARCKS is seen to be reported in breast cancer (Finlayson and Freeman, 2009).

Function:

- Actin filament binding (in unphosphorylated form)
- Calmodulin binding
- Protein kinase C binding

Pathway:

- Fc gamma R-mediated phagocytosis
- BDNF signaling pathway
- Integration of energy metabolism
- Regulation of insulin secretion by Acetylcholine
- Regulation of insulin secretion
- Effects of Calcineurin in keratinocyte differentiation (Figure 6)

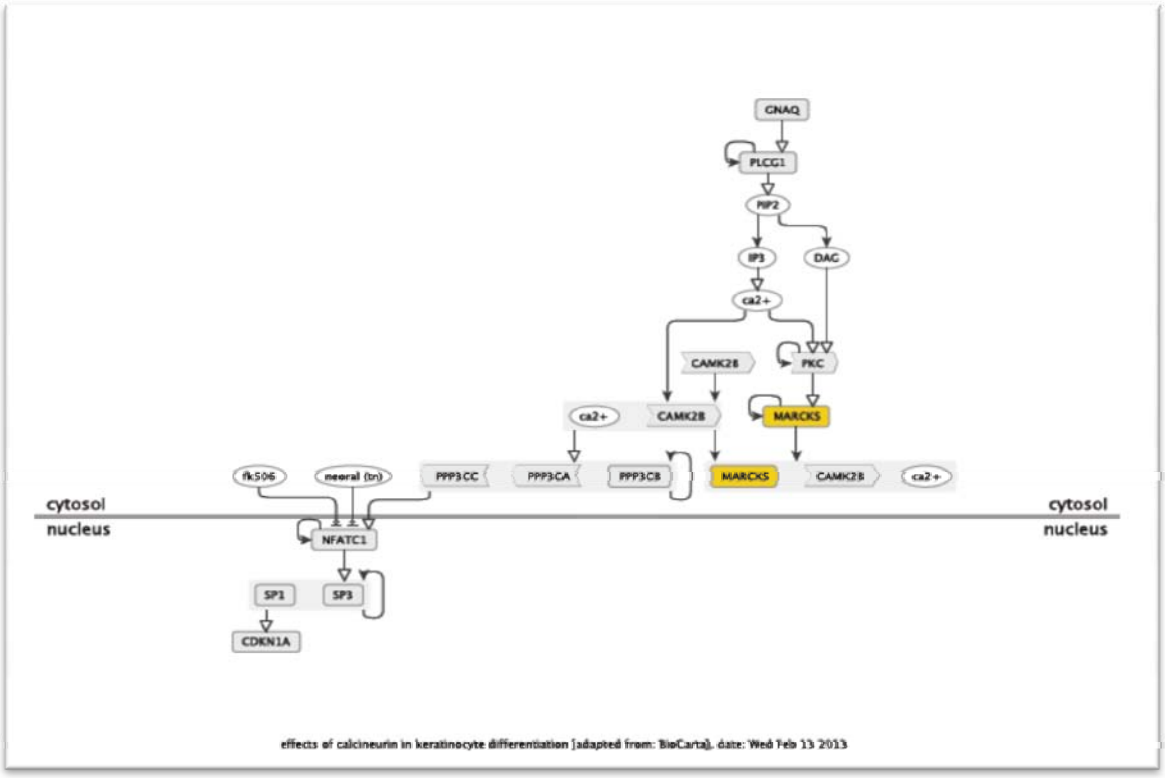

Figure 11. Involvement of MARCKS in keratinocyte differentiation pathway

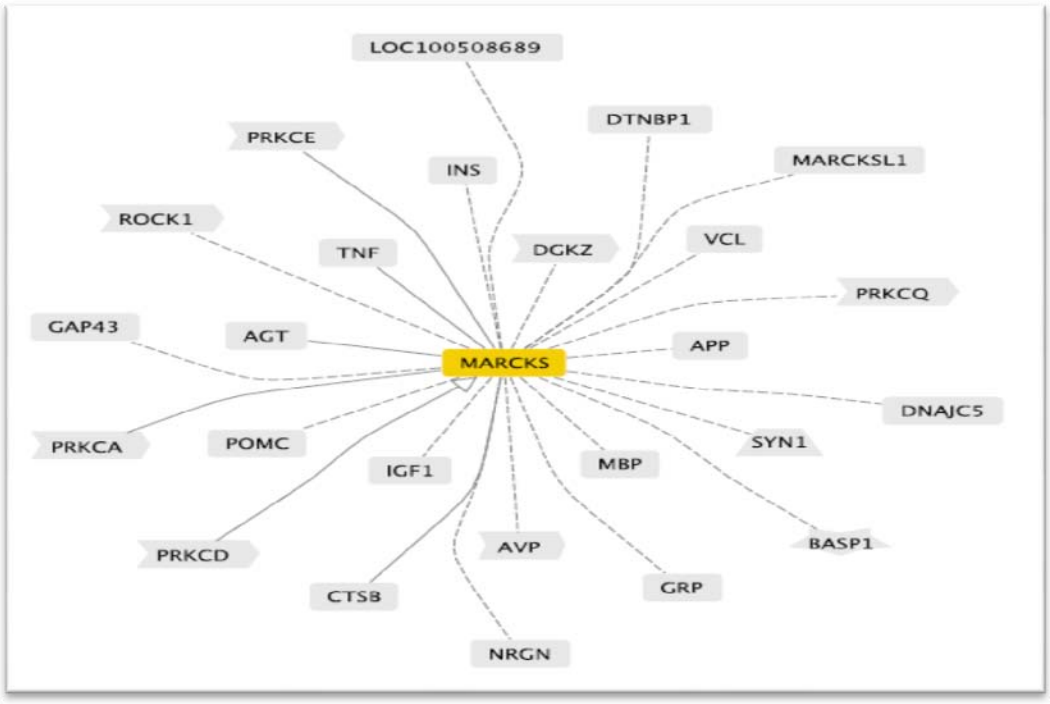

Figure 12. Gene network of MARCKS

SIK1

It is abbreviated as Salt Inducible Kinase 1 which is a tumor suppressor gene that plays a key role in p53 dependent anoikis. It belongs to protein kinase superfamily i.e. CAMK Ser/Thr protein kinase family and AMPK subfamily. It contains one protein kinase domain and one LBA domain (Katoh et al., 2004). The arginine-lysine RK region determines the subcellular location and its catalytic activity by phosphorylation on Thr-182 taking magnesium as a co-factor (Doi et al., 2002).

SIK1 is a part of sodium sensing signaling network, mediating phosphorylation of PPME1, increase in intracellular sodium. SIK1 is activated by CaMK1 phosphorylating PPME1 subunit of PP2A leading to dephosphorylation of NA+/K+ ATPase by increasing its activity (Kowanetz et al., 2008 and Stewart et al, 2013).

Defects in SIK1is reported to be involved in ovarian cancer and breast cancer (Cheng et al., 2009). SIK1 expression is found to be significantly lower or down regulated in primary breast cancer than the normal breast tissues (Sjöström et al., 2007).

Function:

- SIK1 acts as the regulators of muscle cells by phosphorylating and inhibiting HDAC4 and HDAC5 , leading to expression of MEF2 target genes in myocytes.
- It also regulate cardiomyogenesis by removing cardiomyoblast from cell cycle via down regulation of CDNK1C.
- It is also known as the regulator of hepatic gluconeogenesis by phosphorylating and repressing TORC1/CRTC1 & TORC2/CRTC2 inhibiting CREB activity.
- It regulates hepatic lipogenesis by phosphorylating and inhibiting SREBF1.

Pathway:

- Liver kinase B1 (LKB) signaling events (Figure 8)

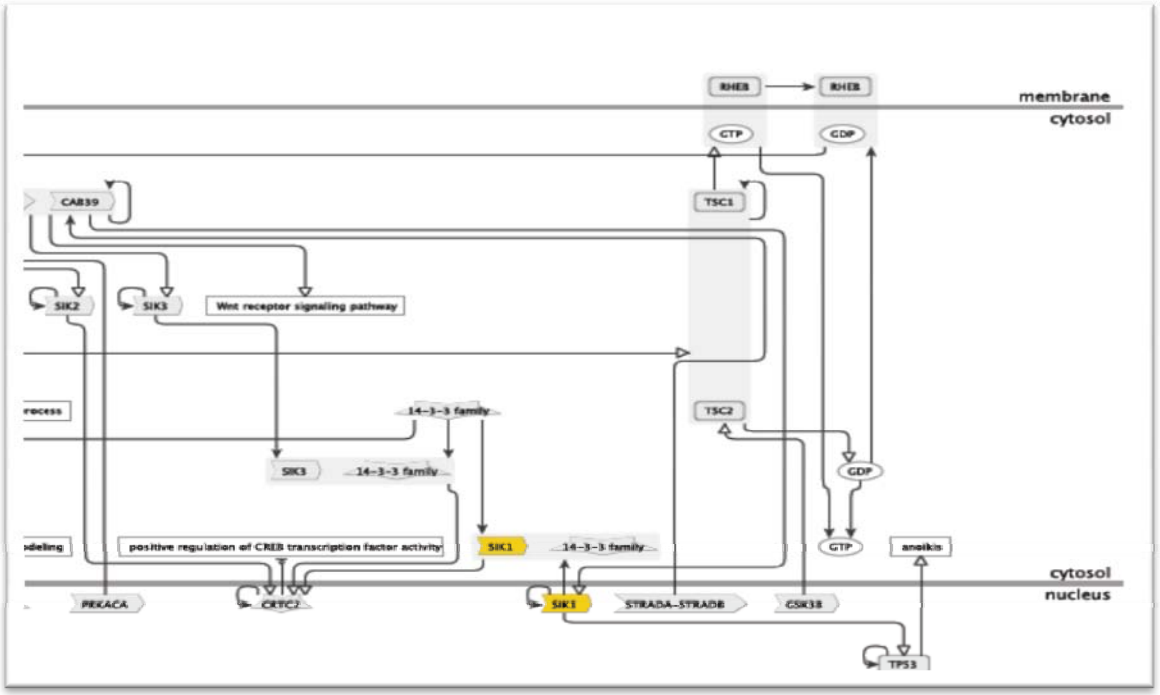

Figure 13. LKB1 signalling events in SIK1

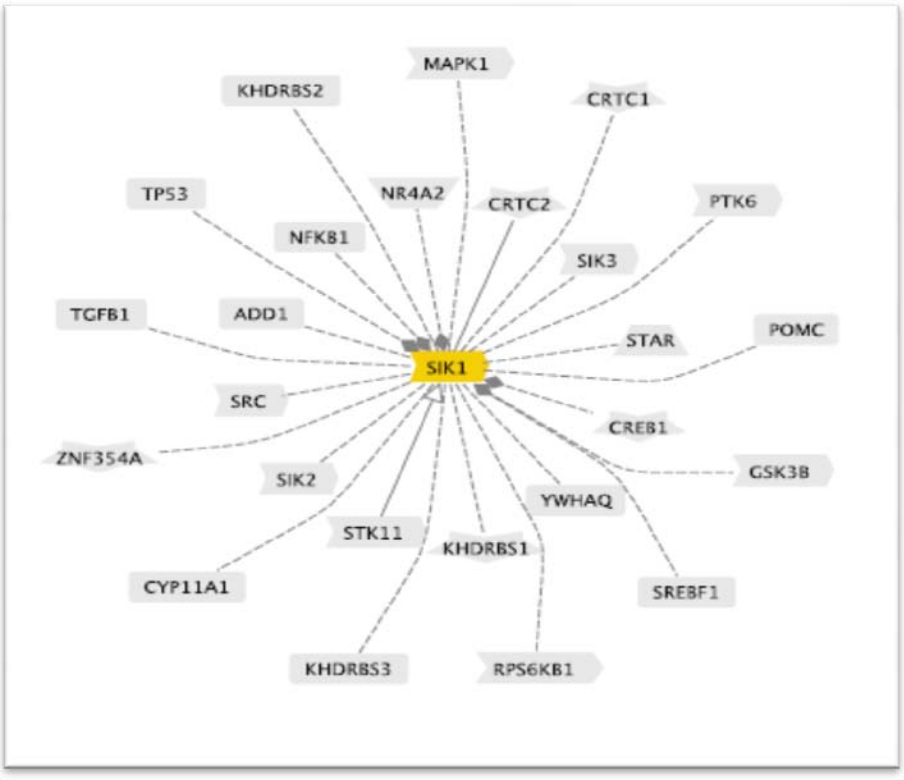

Figure 14. Gene network of SIK1

Table 6. Diseases reported to be involved with MARCKS & SIK1

MARCKS	SIK1
malignantsyringoma	medullary sponge kidney
syringoma	nephrocalcinosis
Bipolar disorder	nephrolithiasis
lymphocytic leukemia	breast cancer
neuroblastoma	gastric cancer
alzheimer's disease	hepatitis
Asthma prostatitis	malaria
melanoma leukemia	neuronitis
manic-depressive illness anoxia	Fibromyalgia
chronic lymphocytic leukemia	Arthritis
neuronitis	
liver cirrhosis hepatitis	
Colorectal cancer	
choroiditis cerebritis	
hepatocellular carcinoma	
retinoblastoma	
prostate cancer	
Breast cancer	
adenocarcinoma	

BTG2

It is abbreviated as B-Cell Translocation Gene. Also known as pheochromacytoma cell-3, NGF-inducible anti-proliferative protein PC3, B-cell translocation gene 2 and nerve growth factor-inducible anti-proliferative. The protein encoded by this gene is a member of the BTG/Tob family (Putnik et al., 2012). This family has structurally related proteins that appear to have anti-proliferative properties. This encoded protein is involved in the regulation of the G1/S transition of the cell cycle (Möllerström et al., 2010).

The post translational modification involves phosphorylation at Ser-149 by MAPK14 and at Ser-147 by MAPK1/ERK2 and MAPK3/ERK1, leading to PIN1-binding and mitochondrial depolarization (Takahashi et al., 2011). BTG2 is preferentially expressed in quiescent cells, anti-proliferative p53 dependent component of the DNA damage cellular response pathway, homolog to murine Pc3/Tis21 and human BTG1 (see symbol),at the onset of neurogenesis in single neuroepithelial cells that switch from proliferative to neuron-generating division, Tob/BTG1 family. BTG2 is a vital gene reported to be involved in breast cancer (Zhang et al., 2013).

Function:

- Involved in cell cycle regulation
- Involved in the growth arrest and differentiation of the neuronal precursors
- Modulates transcription regulation mediated by ESR1
- Anti-proliferative protein
- Involved in mitochondrial depolarization and neurite outgrowth

Pathway:

- Direct p53 effectors
- Deadenylation-dependent mRNA decay
- Cyclins and Cell Cycle Regulation

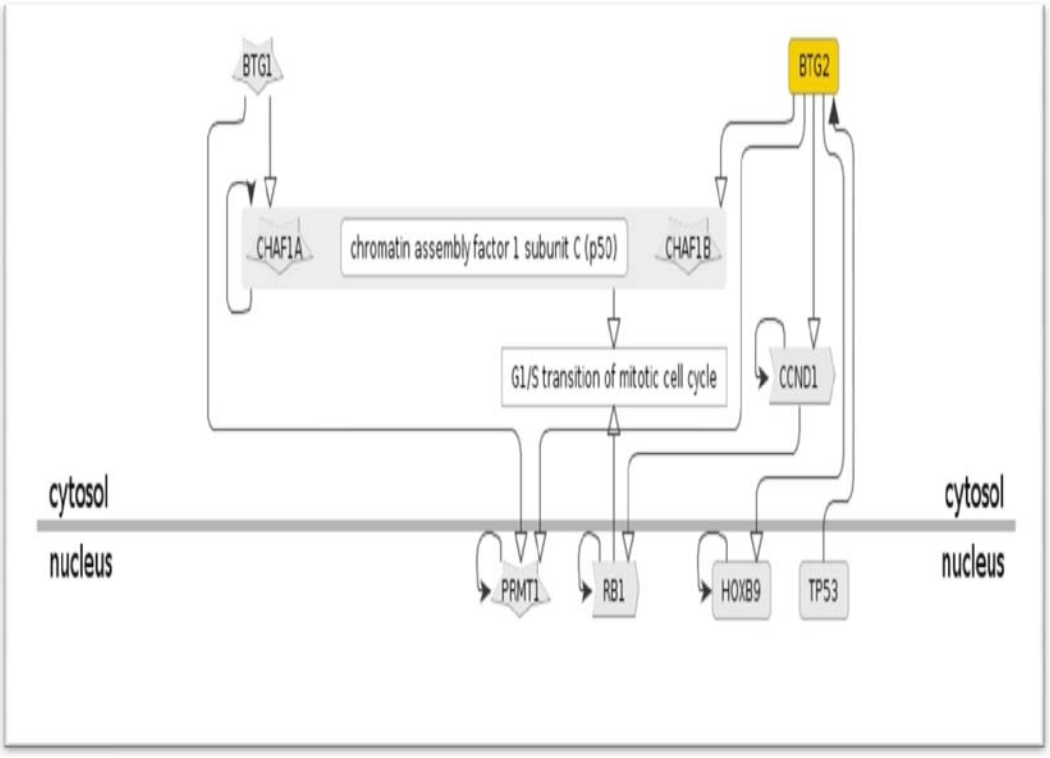

Figure 15. BTG family proteins and cell cycle regulation

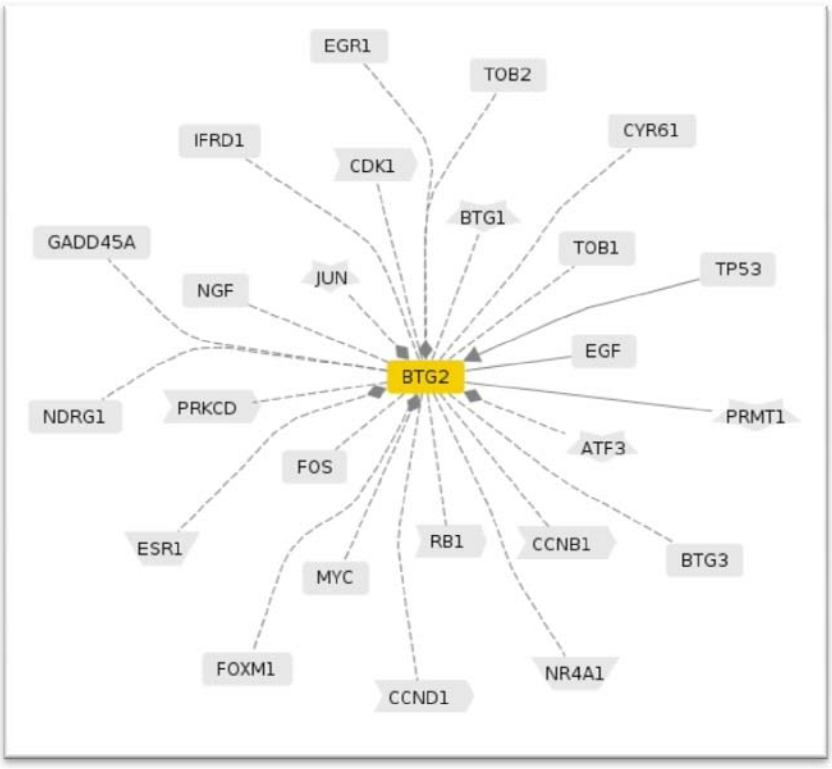

Figure 16. Gene network of BTG2

Experimental Validation

RNA isolation

- **260/280 Ratio:** This ratio indicates the absorbance of DNA and RNA at 260 nm and 280 nm, which is used to assess the purity of DNA and RNA. A ratio is expected approximately 1.8 and generally accepted as "pure" for DNA; a ratio of approximately 2.0 is generally accepted as "pure" for RNA. In either case, if the ratio is significantly lower it may indicate the presence of phenol, protein or other contaminants that absorb strongly at or near 280 nm.

- **260/230 Ratio**: This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are normally in the range of 2.0-2.2. If the ratio is significantly lower than expected, the presence of contaminants which absorb at 230 nm is indicated.

- Here, we got following results of two samples; Sample1 has perfect ratio absorbance in 260nm and 280 nm wavelengths where Sample2 has less value.

- **Sample1** = 500.6 µg/ml
  At (260/280) ratio = 1.99
  At (260/230) ratio = 2.01

- **Sample 2** = 125.2 µg/ml
  At (260/280) ratio = 1.54
  At (260/230) ratio = 1.10

qRT-PCR

- qRT- PCR melting curve analysis used to quantify nucleic acid, mutation detection and for genotype analysis.
- The melting temperature curve for the two gene MARCKS and SIK1 with respect to control , β-actin was observed.

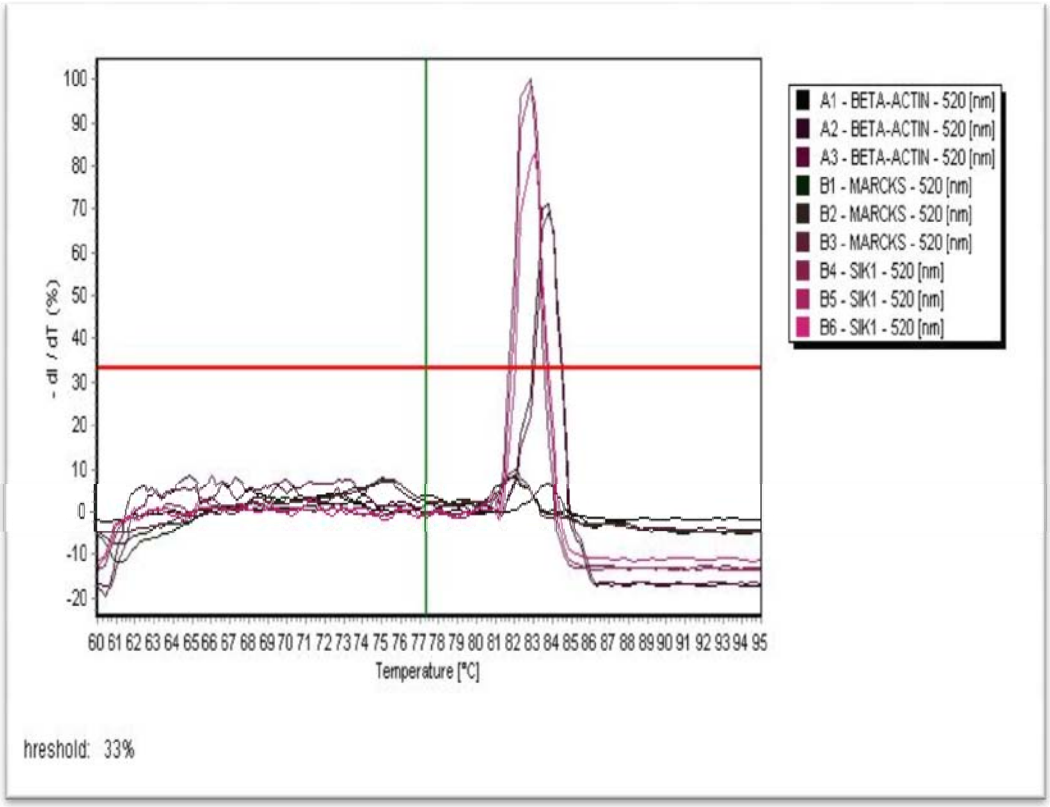

Figure 17. Melting temperature curve of MARCKS and SIK1 with respect to control

- From the qRT PCR analysis of SIK1, a fine melting temperature curve of SIK1 is observed in comparison to the control gene, β-actin. The melting temperature of SIK1 is 82°c. As the samples of gene were taken in triplicates. Three peaks of SIK1 positioned at one place were observed. The relative quantification of SIK1 with respect to β-actin shows high expression of SIK1 **(Figure 13).**

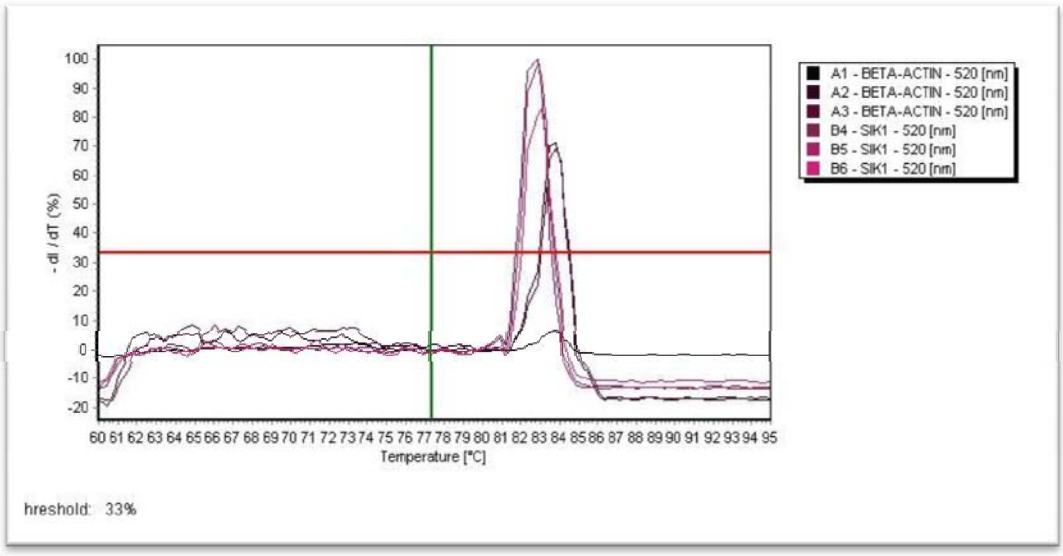

Figure 18. Melting temperature curve of SIK1 with respect to control

- But MARCKS has no result in qRT- PCR **(Figure 14).**

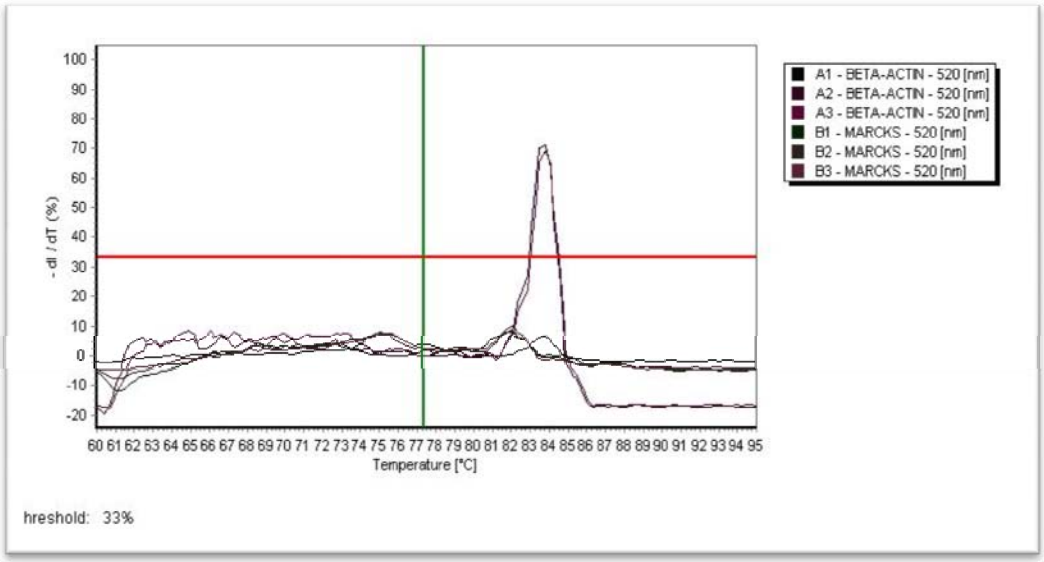

Figure 19. Melting temperature curve of MARCKS with respect to control

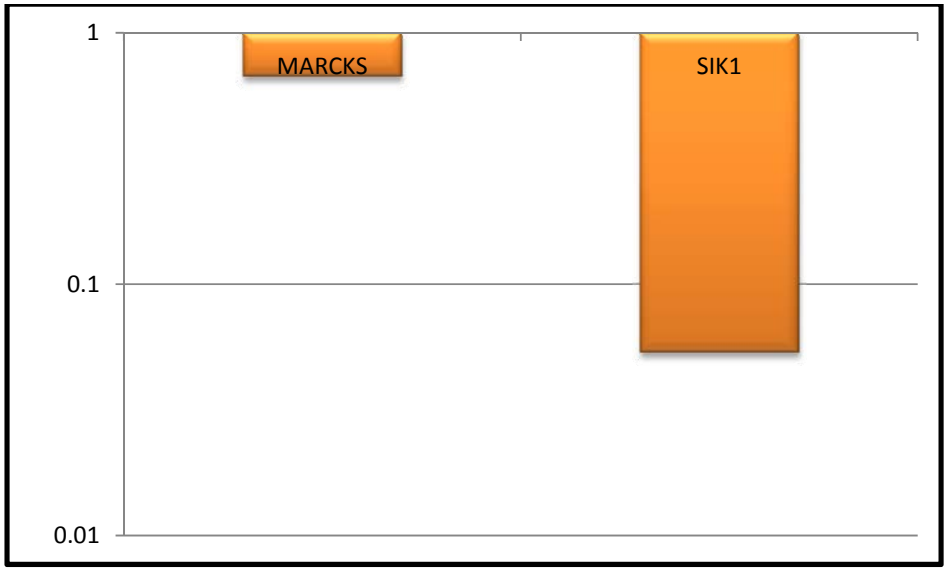

Figure 20. Relative expression of MARCKS and SIK1 with respect to control

The graph shows down-regulation of MARCKS and SIK1 with respect to control. According to our microarray data analysis these two genes were down regulated in BC. It was further validated by qRT PCR. This is in accordance to studies reported by Li et al., 2009 and Lönn P et al., 2012.

From the microarray data analysis we found out that MARCKS is down regulated with a fold change of 0.34107733 and SIK1 is down regulated with a fold change of 1.9210892.

As the genes are down regulated, therefore from the miRNA-mRNA interaction map we have seen that MARCKS and SIK1 are targeted by *miR-27a*. Also, BTG2 was found to be targeted by *miR-21*. Therefore, 3 target pairs, *hsa-miR-27a*–MARCKS, *hsa-miR-27a*–SIK1 and *hsa-miR-21*–BTG2 have been hypothesized to be good target pairs to be validated further by luminometer or Luciferase Reporter Assay.

CONCLUSION

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Our study aimed at identification of novel mRNA-miRNA target pairs that are hypothesized to play a role in breast cancer through an mRNA- miRNA interaction map analysis of microarray data and experimental validation of selected set of mRNAs. From mRNA microarray expression analysis, we found that the total number of differentially expressed genes in breast cancer is 225, out of which 73 are down regulated and 152 are up-regulated. Further, from miRNA expression analysis we found out that the total number of differentially expressed miRNAs in breast cancer is 94, out of which 82 are down-regulated and 12 are up-regulated. From the target interaction map analysis, we found that 7 miRNAs showed specific target binding for 25 genes, out of which these 3 pairs, *hsa-miR-27a*–MARCKS, *hsa-miR-27a*–SIK1 and *hsa-miR-21*–BTG2 are seen to be novel target pairs. MARCKS, SIK1 and BTG2 being tumor suppressor genes are seen to be significantly down regulated in breast cancer. *hsa-miR-21* and *hsa-miR-27a being* oncomiRs are seen to be highly up regulated in breast cancer because of their property to induce metastasis. The over expression of MARCKS and SIK1 can strongly repress the proliferation of cancer cells. The lower expression of *hsa-miR-21* and *hsa-miR-27a* can even contribute in suppressing metastasis in breast cancer. Therefore, *hsa-miR-27a*–MARCKS, *hsa-miR-27a*–SIK1 and *hsa-miR-21*–BTG2 have been hypothesized to be novel target pairs which can be further experimentally validated. Therefore, a better perceptive about the miRNA-gene networks and cellular pathways can be a promising concept in understanding and further elucidating the role of miRNAs involved in breast cancer pathogenesis and thereby may revolutionize the future of breast cancer therapeutics.

FUTURE PROSPECTIVES

FUTURE PROSPECTIVES

The effectiveness of miRNA-based breast cancer therapy can be a landmark in breast cancer studies. Identification of the genome-wide targets of miRNAs is a promising approach which can be experimentally validated to have a vital role in breast cancer. Henceforth, with a better perceptive about the gene networks and their cellular pathways regulated by miRNAs, the elucidation of breast cancer pathogenesis and therapeutics can be facilitated. Furthermore, experimental validation of *hsa-miR-27a*–MARCKS, *hsa-miR-27a*–SIK1 & *hsa-miR-21*–BTG2 through Luciferase Reporter Assay or any other molecular techniques will strengthen the foundation of miRNA-mediated regulation in breast cancer. Subsequent analysis of these novel miRNA-target pairs will enhance our understanding to manipulate pathways/networks for treatment of breast cancer through miRNA therapeutics.

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