Comprehensive Analysis of Differentially Expressed mRNAs and miRNAs in Breast Cancer

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "*Comprehensive Analysis of Differentially Expressed mRNAs and miRNAs in Breast Cancer*" submitted by **Tapaswini Singh** (Roll No: 412LS2061) in partial fulfilment of the requirements for the award of Master of Science in Life Science to the National Institute of Technology, Rourkela is an authentic and original record of research work carried out by her under my supervision and guidance.

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

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Place: Rourkela

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Date: 11.05.2014 Tapaswini Singh

List of symbols and abbreviation used

µg	Micro gram
Ml	Micro litre
°	Degree
C	Centigrade
Ml	Mili litre
mM	Mili molar
min	Minutes
%	Percentage
No.	Number
FC	Fold Change
Reg.	Regulation
miRNA	micoRNA

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ABSTRACT

Breast cancer is the second highest leading cause of mortality among women. There are many factors responsible for promoting breast cancer. One of the risk factor of this malignancy is molecular alterations. The molecular alterations include epigenetic regulations such as regulation by microRNAs (miRNAs). miRNAs are a group of small non-coding RNAs, about 18-25 nucleotides in length. These miRNAs have the potential to regulate mRNA expression by suppressing the translational activity. In this study, we aim to identify miRNAs which might have a role in breast cancer. For this, we analyzed expression data of miRNAs and mRNAs in breast cancer tissues and identified the sets of miRNAs and mRNAs that are differentially expressed in breast cancer. Considering sets of up-regulated miRNA and down-regulated mRNAs, we constructed miRNA-target interaction map and screened few miRNA-target pairs (hsa-miR-27a-3p:ADAMTS5 & hsa-miR-17-5p: CX3CL1) that are hypothesised to play a role in breast cancer malignancy. To confirm the expression of these miRNAs and mRNAs in breast cancer, we performed qRT-PCR of each of the mRNAs and miRNAs in MDA-MB-231 cell line and found that miRNAs- hsa-miR-27a-3p, hsa-miR-17-5p that target ADAMTS5 and CX3CL1 respectively are up-regulated and the corresponding targets are down-regulated. This inverse correlation in expression of these miRNAs and mRNAs supplemented with presence of target sites identified from target prediction indicate that these two miRNAs might be regulating the expression of these two mRNAs which in turn might be playing a crucial role in breast cancer. miRNA targeting can be further confirmed by luciferase reporter assays. We also performed methylation specific PCR (MSP) of ADAMTS5 to find the possibility whether its expression is modulated by methylation, rather than miRNAs before proceeding for reporter assays.

Key words: breast cancer, microRNA, epigenetic regulation, gene expression, MSP

INTRODUCTION

INTRODUCTION

Living bodies are generally made up of billions of living cells. These cells grow and divide into normal cells and die an orderly way. The normal cells replace the oldest and the apoptotic cells. When cells of the body grow in an uncontrolled way, cancer is caused. Cancer cells have properties of uncontrolled growth without dying and form new abnormal cells unlike normal cells. Cancer cells can invade the other tissues and spread to different locations. Cells become cancerous due to DNA damage. Normally DNA damages are repaired by various mechanisms involved in maintaining tissue homeostasis, however, in case of cancer cells, DNA damage is not repaired and the unwanted cells proliferate instead of being removed. Sometimes DNA damage is inherited, but mostly it is due to the change in normal cells and the environmental factors. So, cancer is a multifactor disease caused due to molecular alterations in the genome of somatic cells.

The process of formation of cancer called as oncogenesis or tumorogenesis is caused by interplay between genetic/epigenetic and environmental factors. There are specific traits which are called hallmarks that convert normal cells to cancerous cells. These hallmarks are- i) ability to stimulate their own growth, ii) ability to resist inhibitory signals iii) ability to resist their own programmed cell death (apoptosis), iv) ability to induce formation of blood vessels (angiogenesis) to supply nutrients to tumors, v) ability to multiply forever, vi) ability to invade local tissue and spread to distant sites known as metastasis. Apart from these six hallmarks, few hallmarks have been added recently to the list. These are- i) abnormal metabolic pathways, ii) ability to evade the immune system, iii) chromosome abnormalities and unstable DNA, and iv) inflammation (Weinberg and Hanana 2000).

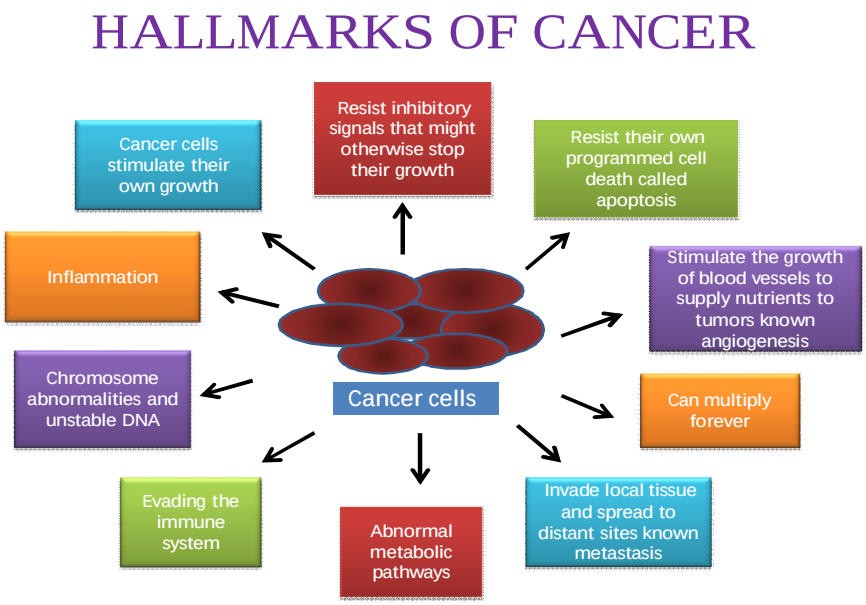


Figure 1. The hallmarks of Cancer

Most tissues of human body are not necessarily susceptible to cancer, but the breast tissue is unique in its composition with vulnerability and ability to change with the response of hormones. Breast cancer is a highly deadly disease and the second most susceptible among women, but not yet well understood. Globally, it is estimated that the incidence and mortality rate of breast cancer have increased over the past 30 years, at annual rates of 3.1% and 1.8% respectively (Luciani et al., 2009). Breast cancer patient have very good chances of a disease free survival if the cancer is diagnosed and treated in early stages.

A survey on breast cancer conducted in 2014 by the American Cancer Society, United States indicate below figures:

- New Diagnosed case of invasive breast cancer is about 232,670.
- Carcinoma in situ (CIS) breast cancer diagnosed cases are about 62,570.
- Mortality of women due to breast cancer is about 40,000.

Clinical data of breast cancer patients was recorded in different parts of India, particularly in Chennai, Bangalore, Mumbai, Barshi, Bhopal and Delhi from the year 1982/88 and 2007/08. By analysis of such data recorded at different places and different time intervals, it was found that the percentage of breast cancer cases increases when increasing time duration (Asthana et al., 2014).

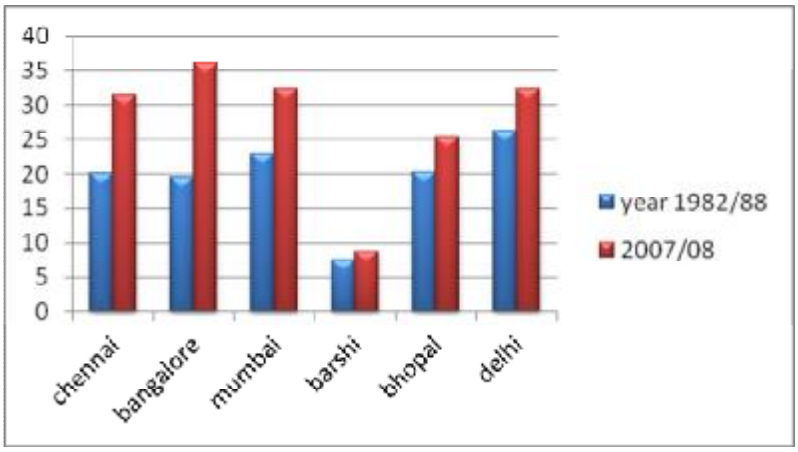


Fig 2: The percentage data of breast cancer patients reported in different cities of India in year 1982/82 and 2007/08.

There are several types of breast cancer depending upon the size of tumors-

- Ductal carcinomas in situ (DCIS) also known as intraductal carcinoma,
- Lobular carcinoma in situ,
- Invasive ductal carcinoma and
- Invasive lobular carcinoma.

The changes in molecular profile and morphology of breast tissue are observed because it has greater susceptibility to DNA damage through tissue damage. When a cell replicates more rapidly, its genetic codes are more vulnerable to damage by chemicals, viruses and radiations that later causes cancer. Various deregulated mechanisms in a cell can lead to cancer, such as genetic alternation, epigenetic mechanism and microRNA regulation etc. The germline mutations in genes like BRCA1(Breast cancer 1, early onset and BRCA2 (breast cancer 2, early onset) are reported to be associated with high risk of breast cancer in the early age. Besides genetic alterations, it has been reported recently that breast cancer is mainly regulated mainly by epigenetics mechanisms, either by DNA methylation or by the small non-coding RNA (ncRNA) mediated regulation.

DNA methylation, a biochemical process that involves addition of a methyl group to the cytosine residue is essential for normal mammalian development and is associated with a number of processes like genomic imprinting, inactivation of X-chromosomes, suppression of

repetitive elements, carcinogenesis and transcription of genes. The transcription of genes are affected by DNA methylation in two ways-

i) methylation of DNA itself may physically impede the binding of transcription factor to the gene,

ii) methylated DNA bound by protein known as methyl CpG binding domain proteins (MBDs) which impedes transcription.

It is known that methylation dependent mechanisms are associated with silencing of tumor suppressor genes which enhance the progression, initiation and development of breast cancer. The tumor suppressor gene, p16 has been reported to be silenced by hyper-methylation of promoter and promotes breast cancer (Stefansso et al., 2011). There can be many more genes that can be involved in breast cancer. Moreover, these genes may be regulated by another epigenetic mechanism, i.e., small ncRNAs.

MicroRNA (miRNA) is one such group of small ncRNAs that have potential role in cancer biology including breast cancer. miRNAs are mainly 20-24 nucleotides long. These recognise their target mRNA based on the sequence complementarity mainly to 3′UTR regions and inhibit protein translation. miRNAs are involved in cellular differentiation and proliferation in addition to numerous other functions and their de-regulation leads to cancer. Till date, many miRNAs have been identified that are associated with the breast cancer. In one recent study, it was found that 7 miRNAs (miR-200c, miR-205, miR-203, miR-141, miR-34a, miR-183, and miR-375) were down-regulated and 4 miRNAs) (miR-146a, miR-138, miR-125b1 and miR-100) were up-regulated in breast cancer as compared to normal cells (Luo et al., 2013). From another study, miR-21 was reported to be up-regulated and its target BCL2 which leads to breast cancer was found to be downregulated (Lindsey et al., 2012). The reason for high mortality rate of breast cancer is metastasis. When a solid tumor enters into adjacent tissues and spreads to the other parts of the body, the complete mechanism involved in the process is known as metastasis. Some miRNAs have been reported to be involved in metastatic process by targeting key genes like E-cadherin. E-cadherin plays an important role in cell-cell adhesion and maintaining the cell structure. It is a tumour suppressor gene and widely used as diagnostic biomarker in breast cancer. Loss of E-cadherin increases the tumour progression (Singhai et al., 2011). The mortality and invasion ability of breast cancer is increased by miR-9 which target E-cadherin (Ma et al., 2010). Similarly CD44 targeted by the miR-373/520 also helps in migration and invasion of breast cancer. miR-10b, miR-21, miR-29a, miR-155 also enhances the metastatic condition in breast cancer (Singh and Mo, 2012).

In this work, we planned to identify significant epigenetic regulations of few selected genes that might be responsible for breast cancer oncogenesis. To fulfil our proposed aim, we studied miRNAs and mRNAs that are significantly de-regulated in breast cancer and interactions among them followed by target analysis and quantification of both selected mRNAs and miRNAs by qPCR to establish their correlation of regulations. We also tried methylation-specific PCR to know whether these selected de-regulated mRNAs are modulated by methylation of their promoters in breast cancer cell lines (MDA-MB-231).

REVIEW OF LITERATURE

REVIEW AND LITERATURE

1. CANCER

Cancer refers to the uncontrolled growth and proliferation of abnormal cells which invades other tissues due to the DNA damage or mutation. Blood circulatory system and lymph nodes helps cancer cells to spread throughout the body. The unwanted extra cells aggregate to form mass of cell which is known as tumour. But all tumours are not cancerous. Based on the nature of tumor they can be classified into two types:

- **Benign tumors**

These are not cancerous. Benign tumour cells do not spread o other parts of the body. They can be removed and very rare to encounter again in the body.

- **Malignant tumors**

Malignant tumors are cancerous. These cells invade nearby tissues and spread to other parts of the body. This spreading of cancer from one part of body to other is called *metastasis*. Malignant tumors possess the nature of being converted into different cancer types. There are over 100 types of cancers discovered which affect different parts of body. All cancers are unique by its own effects, symptoms and methods of treatment.

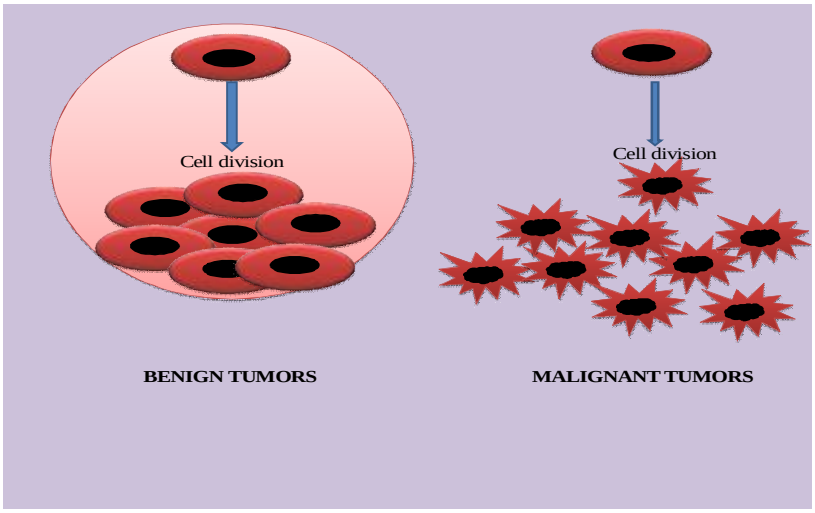


Figure 3: Benign tumors and Malignant tumors

A. Classification of cancer based on tissues of origin

Carcinoma: It is a type of cancer which originates in all epithelial tissues and covers the glands, surfaces and the body structure. 80-90% of cancers are carcinomas. Some examples of this type of cancer are cancer of lung, prostrate, breast, colon, pancreas, gastric and brain.

Sarcoma: It is a type of cancer that occurs in connective tissues like tendon, cartilage, ligaments, fat, bones and muscles. Sarcoma is developed from transformed mesenchymal origin cells. e.g. osteosarcoma.

Lymphoma: It is a cancer type that originates in the nodes or glands of the lymphatic systems.

Myeloma: Myeloma grows in the plasma cells and bone marrows.

Blastoma: Blastoma is caused by the malignancies in precursor cells and commonly seen in children. e.g. Chondroblastoma occurs due to the precursor of chondrocytes.

B. Organ specific cancer:

The cancer is classified according to their location in the body. Specific organs have specific type of cancer.

Table no: 1 common organ specific cancer type and their estimated new cases and mortality case reported by NCI (National Cancer Institute).

Cancer type	**new cases**	**Mortality case**
Bladder	74,690	15,580
Breast	232,670	40,000
Endometrial	52,630	8,590
Colon and rectal	136,830	50,310
Brain	23,380	14,320
Leukaemia	52,380	24,090
Lung	224,210	159,260
Melanoma	76,100	9,710
Non- Hodgkin lymphoma	70,800	18,990
Thyroid	62,980	1,890
Prostrate	233,000	29,480
Pancreatic	46,420	39,590

2. Breast cancer:

Breast cancer is a malignant tumour that starts from the cells of breast. It grows rapidly and may invades others areas of the body. Female mortality rate is greatly influenced by breast cancer.

GENERAL BREAST CANCER TYPES

Carcinoma

Carcinoma begins at inner layer (epithelial cell) of breast. Carcinoma exhibits abnormal malignant properties of tumour tissue derived from the putative epithelial cells.

Adenocarcinoma

Adenocarcinomas start from glandular tissue of breast that are present inside the duct and lobules of the breast.

Carcinoma in situ

The early stage of breast cancer is known as *carcinoma in situ* and is confined to the ducts. The cells do not grow deeper in these tissues and do not invade other organs in the body. *Ductal carcinoma in situ* of the breast is referred to as *non-invasive* or *pre-invasive* breast cancer but it sometimes develops into an invasive breast cancer if the cancer left untreated. *Lobular carcinoma in situ* is confined to the lobules of breast. It is not a true cancer also reffered to as the pre-cancer.

Invasive carcinoma

An invasive carcinoma develops beyond the starting point from where they have started growing. Invasive carcinoma is of two types, *ductal* invasive carcinoma and *invasive lobular* carcinoma.

Different risk factors influencing Breast cancer

Aging

Risk of breast cancer is increases with age. Most invasive breast cancer patients are found to be around the age of 55 or more.

Overweight

After menopause, generally there is a lot of weight gain which increases the risk of the breast cancer. The more production of the estrogen level in the body after menopause promotes breast cancer which is due to the high level of fat deposition. Being overweight also

triggers amount of insulin in the blood. The higher amount of insulin also acts as a risk factor for many cancers including the breast cancer.

Chest radiation:

Women, who are treated with radiation therapy for the treatment of other cancers like Hodgkin disease or non-hodgkin lymphoma in the chest area ever in their life, have risk of developing the breast cancer.

Drinking alcohol:

The alcohol consuming women have more risk towards breast cancer. It was reported that the women who are habituated of consuming one drink/day were less affected to those who used to consume 2-5 drinks/day (American Cancer Society 2012). Women who do not consume alcohol have less risk of breast cancer.

Tobacco Smoking:

Tobacco contains the chemicals that are carcinogenic in nature.

Molecular factors for breast cancer

Other than the above mentioned factors, some molecular alterations promote breast cancer. Breast cancer is caused by the mutation of genes and inherited from one generation to the next generation. About 5% to 10% of breast cancer is hereditary (American Society 2012). BRCA1 and BRCA2 genes are two well known tumour suppresser genes, which prevent cancer. If these genes were mutated and inherited from the parents, breast cancer risk will be high during the lifetime (Petrucelli et al., 2010). The TP53 gene helps in making the p53 proteins and stops the abnormal cell growth. Li-Fraumeni syndrome is caused due to the inherited mutations in this gene. People suffering from this syndrome were at high risk of developing breast cancer (Walsh et al., 2006). CHEK2 gene which have role in DNA damage and helps in the regulation of cell growth, also promotes breast cancer (Nevanlinna 2006 and Bartek 2006). The mutated PTEN causes Cowden syndrome. People suffering from Cowden syndrome have high risk of benign as well as malignant tumours (Liaw et al., 1997).

Signalling pathways involved in breast cancer

In breast cancer, many signalling pathways are interconnected. Signalling pathways are involved in cell cycle, cell division, migration, proliferation and all other biological processes. The tyrosine kinase signalling pathways help the normal cells to survive and proliferate (Lemmon and Schlessinger, 2010). The deregulation of the receptor tyrosine kinase by oncomirs causes cancer. HER2, a subtype of breast cancer is 30% more prevalent

and is caused mainly due to deregulation of EGFR signalling (Giancotti, 2003). Mutation in the subunit PIK3CA of PI(3)K in breast cancer alters the gene expression of PTEN and promotes various cancers including breast cancer (Brendan et al., 2006). In subsets of breast cancer, the PTK2, FAK is amplified by the deregulation of the integrin molecules. Integrin molecules are part of the supportive matrix which activate the receptor kinase activity (Giancotti, 2003) and reinforce the mitogenic signalling by regulating the Ras, PI(3)K and Rho family GTPase in the process of the cell adhesion and migration (Giancotti and Tarone, 2003). E-cadherin encoded by the CDH1 is inactivated in breast cancer. In breast cancer, the PI(3)K is mutated and activates the mTORC2, simultaneously activating the Akt, SGK kinase which inturn inactivates the TOR-PI(3)K which is involved in breast cancer. Notch receptors are more in the breast cancer.

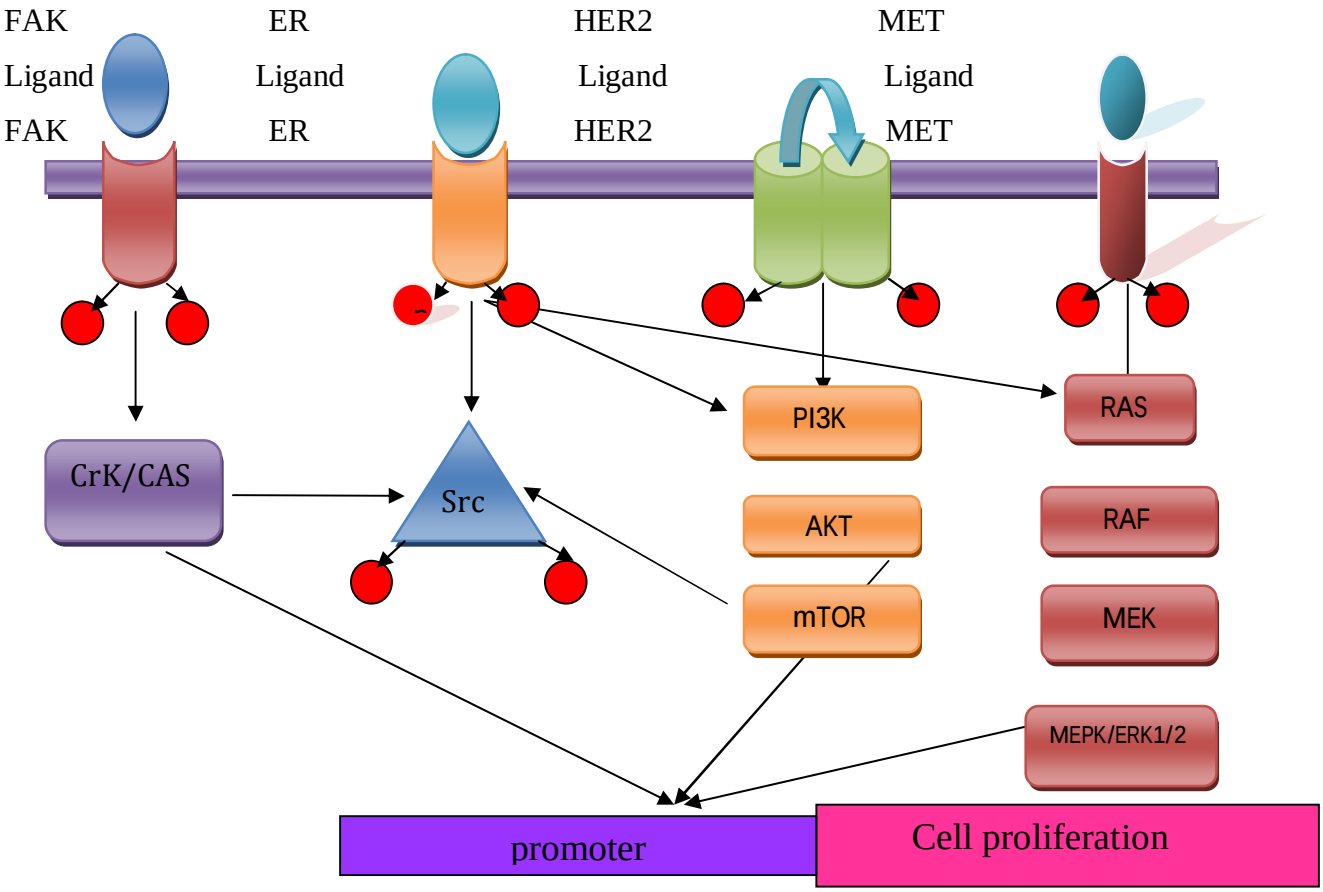


Figure 4: signalling pathways in breast cancer

Breast cancer is mainly progresses through tumour initiation and metastasis. In all the cases gene expression changes which alteres the normal cells. The change in gene expression is either due to activation of oncogenes or the repression of tumour suppressor genes.

Epigenetic mechanisms changes basic gene expressions without changing the composition of the sequences. Such mechanisms are active in breast cancer which causes a change in gene expression due to the methylation of the CpG islands in promoters of various genes and silencing the tumour suppressor genes by changing the chromatin structure. DNA methylation, post transcriptional histone modification and miRNA effect were vital component of epigenetic modification involved in silencing gene expression.

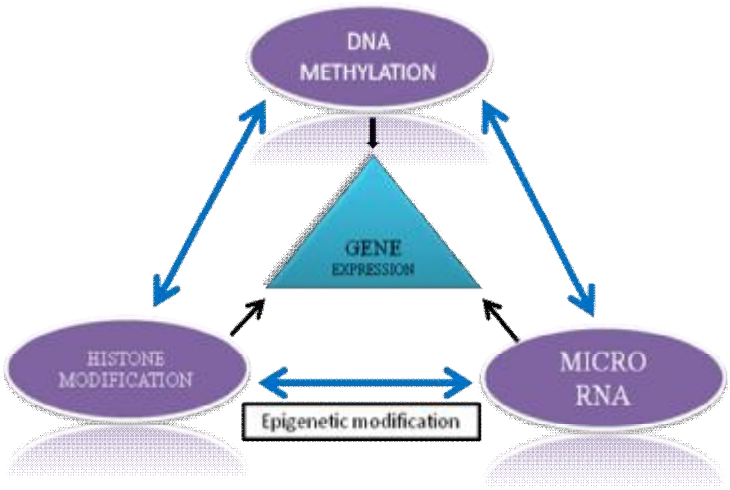


Figure 5: interaction of epigenetic modification

DNA METHYLATION:

DNA methylation is the process where the additional methyl groups are attached in the DNA. It mainly occurs at CpG dinuclueotides (cytosine located 5'guanine). They are present at promoter region of more than the 70% of all genes and form clusters known as CpG islands (Balch et al., 2004). Chromatin structure remodelling and the inactivation of transcription are due to the methylation in promoter sequence. The unmethylated CpG Island is open when the promoter region is surrounded by chromatin and activates the transcriptional factors and also the co-activators. The methyalated CpG Island have inactive promoter when the chromatin configuration is closed and this deactivates the transcription factors and co- activators. DNMTs (DNA methyl transferases) act like a catalyser in the DNA methylation process.

DNA methylations at the promoter site are regulated by two mechanism which helps inhibition of gene expression:

(i) Transcription factors recognise the methyl cytosine residues which blocks their binding site and inhibits the transcriptional activity (Comb and Goodman, 1990, Inamdar et al.,1991).

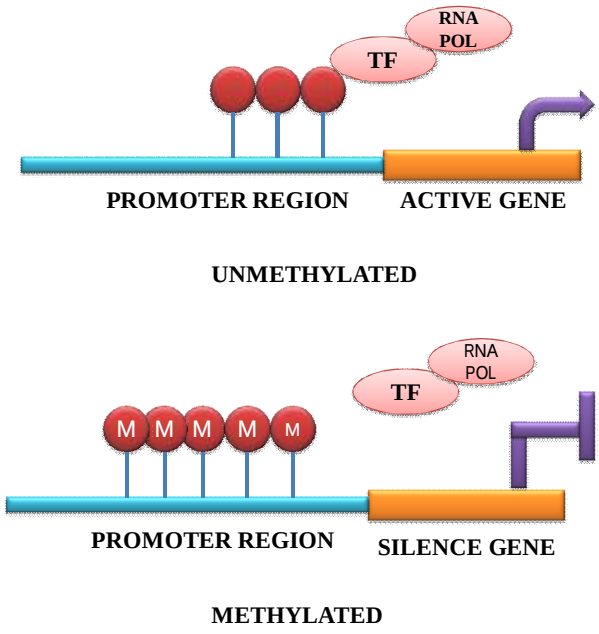


Figure 6: De-methylation and Methylation

ii) The second mechanism helps in recruitment of the MBD proteins (methyl DNA binding domains) to the methylated cytosine in the promoter site (Zhang et al., 2000). HDACs (histone deacetylases) are present in the histone modifying complexes and are recruited by the MBDs proteins like NurD complex, histone methyl transferase (HMTases) at the promoter which inactivates the chromatin configuration (Zhang et al., 2000).

DNA methylation role in Breast Cancer:

DNA methylation affects various aspects of cancer system by inhibiting the transcriptional activity. Hypermethylation as well as Hypomethylation processes are involved in triggering cancer. Not only are the promoters with high CGs have methylation sites but also promoters with low CGs site know as “shores” are methylated differentially in several cancers. BRCA1, a tumour suppressor gene when mutated causes breast cancer. In invasive breast cancer the promoter methylation is more in comparison to the in situ carcinoma (Xu et al., 1999). Mortality rate is also high in case of methylated BRCA1. It has been reported that

there is an increase in mortality rate by 45% in methylated BRCA1 breast cancer when compared with that of unmethylated BRCA1 (Xu et al., 1999). Tumour suppressor gene p16 is methylated and found as early biomarker in breast cancer (Berman et al., 2005).

MicroRNA

In all living system different biological events are mediated by biological machinery. Transfer of information via DNA to gene and generating the signal by protein synthesis are being regulated by RNAs which are also known as messenger. The non-coding or non translating RNAs are gaining important due to their diverse effects in biological process. One of such small non coding RNA is microRNA (miRNA), which have emerging role in breast cancer. miRNAs are endogenous and double stranded small RNAs. These are 18-25 nucleotides long and found that they down-regulated genes by suppress the activity of mRNA. It found that 30% of mRNAs are regulated by the miRNA and are involved in cell proliferation, haematopoiesis, apoptosis, virus offense and stress response in different organisms, including human (Fontana et al., 2007). A single miRNA can regulate multiple genes and also can several miRNAs can have single target gene.

miRNA BIOGENESIS

miRNA regulate the gene expression by post-transcriptional mechanism and degradation of mRNA. RNA polymerases actively participate in biogenesis process where pri-miRNA transcripts are generated by the RNA polymerase II (Bartel 2009). A DGCR8 (DiGeorge syndrome critical region 8) and Drosha protein trims the pri-miRNA (primary miRNA) to transcript pre-miRNA hairpin which is then exported from nucleus to cytoplasm by the exportin 5 (XPO5). A complex formed by the DICER and TRBP (trans-activator RNA (tar)-binding protein) proteins generates single stranded mature miRNA in the cytoplasm and incorporates it into a RNA induced silencing complex (RISC) by associating with AGO2 and GW182 (Hevens et al., 2012). This RISC complex delivers the miRNAs to the complementary site of mRNA. miRNAs are involved in gene silencing machinery. miRNAs binds at 3'-untranslated region (3'-UTR) of target mRNAs mainly by complementary base pairing at positions 2~8 nucleotide known as "seed region" of the miRNA. The efficiency of target suppression by miRNA depends upon the affinity at binding sites.

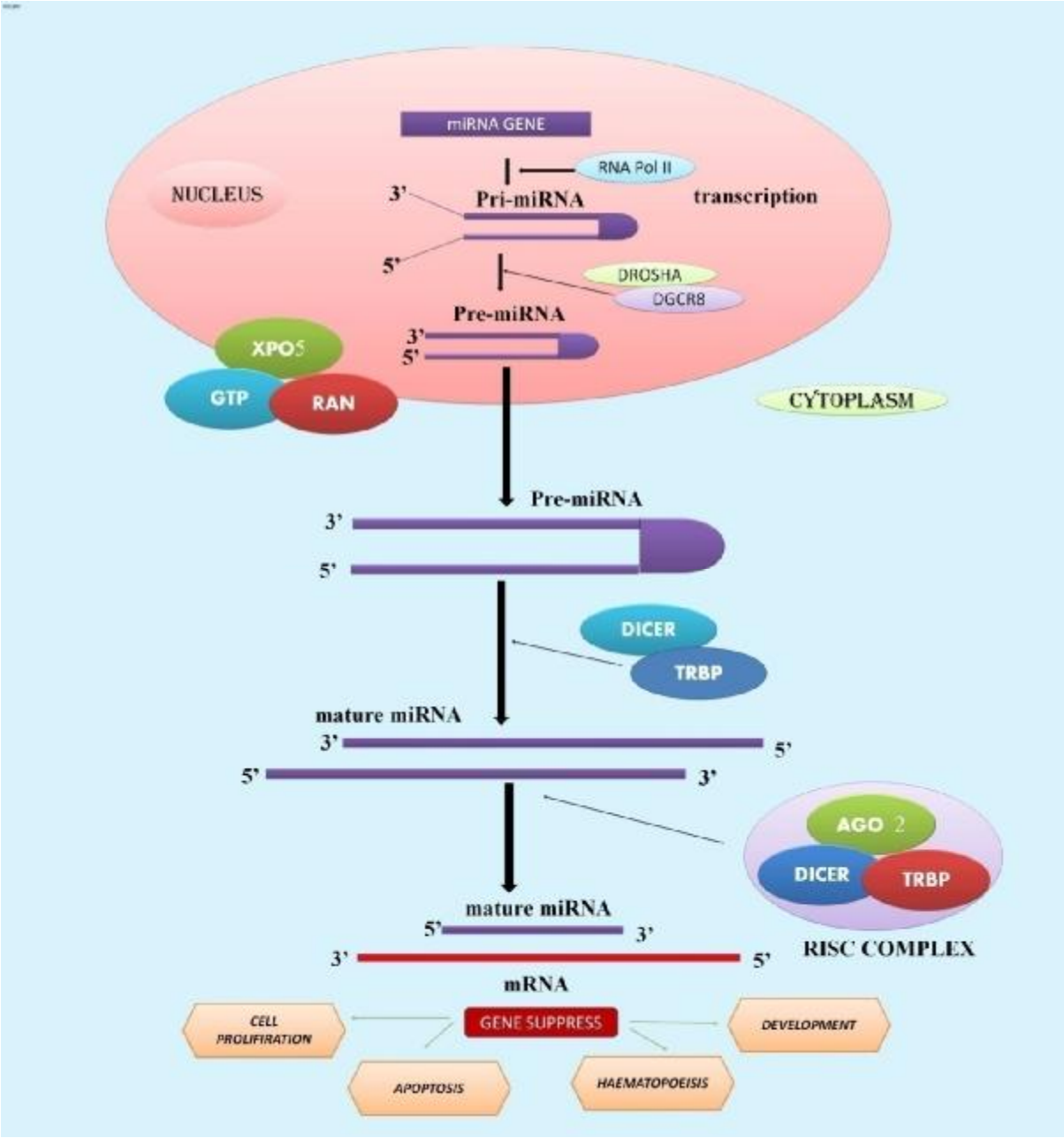
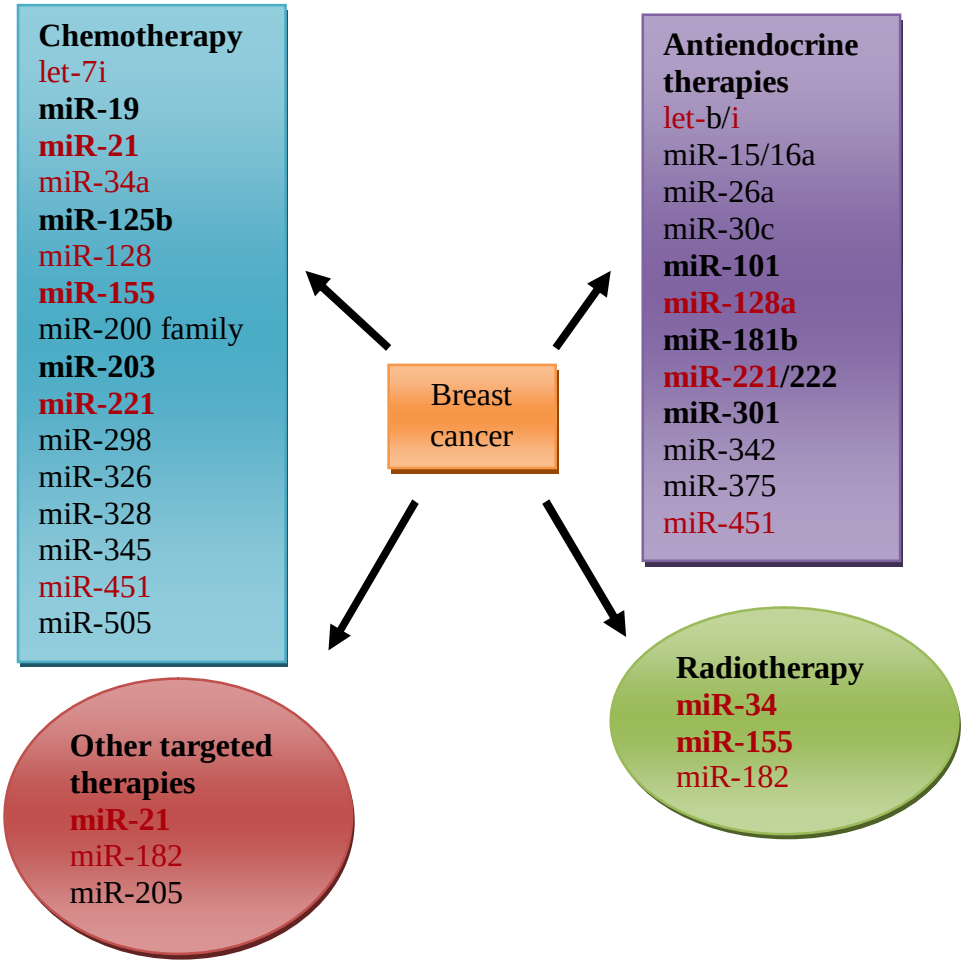


Figure 7: Biogenesis of miRNA

miRNA role in therapeutics

Some miRNAs are resistant to chemotherapy and deregulation of miRNAs play role in therapeutic interventions. The BRCP/ABCG2 breast cancer resistance proteins are negatively regulated by the miR-328 and act as chemotherapeutic agent (Pan et al., 2009). MDR1 is upregulated by the miR-21 and interact with its target PDCD4 (Bourguignon et al., 2009). Other miRNA that target MDR1 are miR-451(Kong et al., 2010), miR-7, miR-345. miR-34 targets the BCL2 and associated with the docetaxel treatment. miR-34 is highly

expressed and provides resistance for the radiotherapy in breast cancer (Kato et al., 2009). miR-221/222 is highly resistant to the anti-endocrine therapy which are prescribed for the ER-positive breast cancer (Miller et al., 2013).



Bold: indicates the expression of miRNA resistance to the therapy

Normal font: indicates the expression of the miRNA sensitive to the therapy

Red: indicates the miRNA alter resistance one and more therapy

miRNA role in Breast Cancer:

In different disease condition the genes are silenced by miRNA. miRNAs are either deregulated or over expressed with respect to their target genes. Breast cancer is caused by many miRNAs which repress various risk factor genes. Different miRNAs either behave as oncogenes or repress the tumor suppressor gene and promote cancer. In triple negative breast cancer miR-21, miR-210 and miR-221 are significantly over expressed whereas miR-10b, miR-145, miR-205 and miR-122a are under expressed in this cancer subtype. Some miRNA

like miR-21, miR-210, miR-221 are correlated with overall survival and play a significant role in primary triple negative breast cancer (Radojicic et al., 2011). In the ductal carcinoma in situ let-7d, miR-210 and miR-221 were down-regulated but were up-regulated in the invasive transition system (Volinia et al., 2006). miR-155 is highly expressed in many cancer type including the breast, lung, colon and hematopoietic cancer (Faraoni et al., 2009). In pre-leukaemia expansion the pre-B-cell are converted into full-blown B cell tumours which is due to the over expression of miR-155 in early B-cells.

Table 2: Role of miRNAs in breast cancer

MiR	Target genes	Role in breast cancer
Oncogenes		
miR-21	PDCD4, PTEN, TPM1, Maspin, BCL2, TIMP3, ANP32A, SMARCA4, MARCKS	Apoptosis, cell proliferation, invasion and metastasis, highly expressed
miR-10b	HOXD10, T1	Metastasis, up regulated in metastasis breast cancer
miR-155	RHOA, FOXO3a, SOCS1, SMAD5, SHIP1	Over expressed, involved in epithelial mesenchymal transition, in hormone receptor negative tumour highly expressed
miR-27a	FOXO1, ZBTB10, Myt1, SPRY2,	Regulated estrogen signaling, cell cycle
Tumour suppressor		
miR-31	FZD3, ITGA5, M-RIP, MMP16, RDX, RHOA, SATB2	Down regulates the metastasis related genes
miR-200c	BMI1, ZEB1, ZEB2, TUBB3, FAP1	Prevents epithelial mesenchymal transition, down regulated in BC stem cell, tumour suppressing properties, regulates apoptosis,
miR-18a	ERalpha	Higher expression in ER-negative tumours, regulates estrogen signalling
miR-205	HER3, ZEB1, ZEB2, VEGF-A	Down regulated, inhibits epithelial mesenchymal transition
miR-17-5p	AIB1, CCND1, E2F, HBP1	Regulates cell proliferation, invasion and migration

miR-17-5p and breast cancer:

miR-17-5p is located in the chromosome 13q31. The heterozygosity results in loss of corresponding genome region in breast cancer (Eiriksdottir et al., 1998). miR-17-5p directly targets the oncogene AIB1(Hossain et al.,2006), which encodes steroid receptor coactivators that enhance the transcriptional mechanism of ERα, E2F1. AIB1 expression is downregulated by miR-17-5p, which inhibits the function of ERα, E2F1 which suppresses the estrogen stimulated proliferation of the estrogen/ER-dependent and the estrogen/ER-independent cancers (Hossain et al., 2006).

miR-27a and breast cancer

miR-27a acts as an oncomir in breast cancer. In SKBR3 cell line, treated with pro-apoptotic dose of LAQ824 (a histone deacetylase), miR-27a is found to be down-regulated (Scott et al., 2006). miR-27a also potentially target the transcriptional cofactor ZBTB10/RINZF, which represses SP1, a transcriptional factor functioning in the cell cycle transition.

ADAM family role in breast cancer

In cancer the proteolytic enzymes have a crucial role. ADAMs (a disintegrin and metalloproteinase) and ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) are the matrix metallo-proteinases which help in cancer development and proliferation. Zinc dependent metalloproteinases also known as metzencin (Wolfsberg et al., 1995). The domains present in the ADAMs are independent but the complementary function.

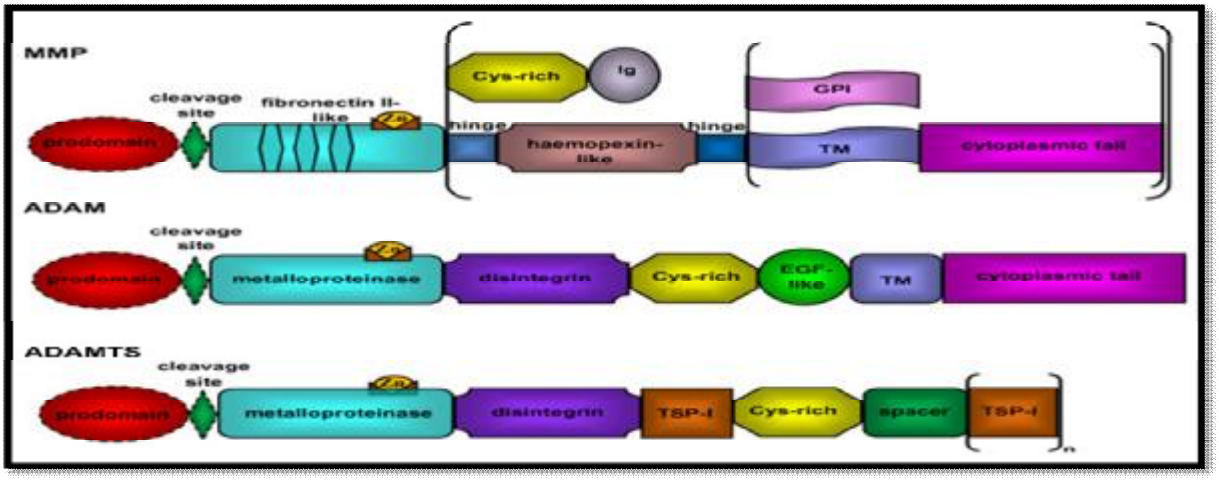


Figure 8: MMP, ADAM, ADAMTS domain structure (adopted from Paulissen et al., 2009)

The human genome encodes 19 ADAMTSs genes (Porter et al., 2005). In the C-terminal part additional thrombospondin type 1 motifs are present (TSP-I) and cytoplasmic domain and EGF like trans-membrane parts are missing. ADAMs are soluble proteins but some bind to the extracellular matrix by the thrombospondin motif or the spacer region (Porter et al., 2005). ADAM 17 is inhibited by TIMP-3(tissue inhibitor metalloproteinase). TIMP-1 and TIMP-3 both inhibit the ADAM 10 but TIMP-2 and TIMP-4 not have function on ADAM 10. TIMP does not have any effect on activities of ADAM-8, 9.

In breast cancer, the ADAM -9, 15, 17 are highly expressed. Involved in proliferation and apoptosis, ADAM 12 is an apoptosis modulating gene and it up-regulated in breast cancer but down-regulated in the non-malignant breast cancer. The over-expression of ADAM 17 causes invasion and proliferation (Kumar et al., 2012). Activation of ADAM 17 is correlated with the urokinase plasmogen activators. In breast carcinoma cell, ADAM 28 is over expressed and it helps regulation of the cell proliferation through IGFBP-3 cleavage. ADAM 9 and ADAM 15 are also detected in breast cancer.

ADAMTS5

ADAMTS5 also named as ADAMTS11 is located in chromosome 21q21.3. It secretes matrix metalloproteinase which is 100kDa. It has two TSR where one is the central TSR and the other is C-terminal TSR. N terminal signal peptides help in the secretion of ADAMTS5 (Abbaszade et al., 1999). At the C-terminal region the ADAMTS5 undergoes autocatalytic cleavage and forms two shorter N-terminal isoform fragments of 45 and 60kDa. the ancillary multi domain proteinase help in localisation and attaining substrate specificity. The aggrecanase activity is reduced with the loss of the c-terminal aggrecanase. The ADAMTS5 down regulated in the malignant breast cancer as compared with to non-neoplastic mammary tissues (Porter et al., 2004).

CX3CL1

CX3CL1 is known as the fractalkine or neurotactin. Chemokines secreted by the extracellular spacer chemikines (C-X3-C) ligand 1 behave as an anchored precursor. CX3CR1 is receptor for the CX3CL1 and function as adhesion molecules (González-Martin et al., 2012). Chemokines act as pro and anti tumour in various cancers. It regulates the survival and proliferation of malignant cells as well as it has role in angiogenesis. CX3CL1/CX3CR1 enhances the tumour progression by transducing the proliferation, pro-

survival signalling. CX3CL1/CX3CR1 also act as anti-tumour by enhancing the natural killer cells, CD8 T lymphocytes and tumour specific immune response. By targeting the epidermal growth factor receptor (EGFR) family the CX3CL1 promotes breast cancer (Tardáguila et al., 2013). CX3CL1 is down regulated in the breast cancer cell line as compared to the healthy tissues (Tardáguila and Mañes, 2013)

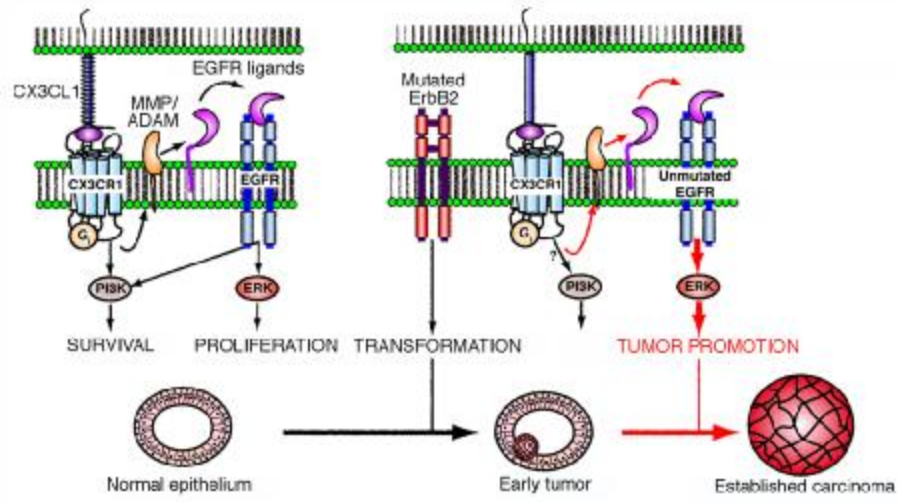


Figure 9: CX3CL1 regulation in breast cancer (adopted from Tardáguila and Mañes, 2013)

OBJECTIVES

OBJECTIVE 1

The Global expression Analysis of mRNAs in the breast cancer.

OBJECTIVE 2

Identification of differentially expressed microRNAs in the breast cancer.

OBJECTIVE 3

Identification of miRNA-mRNA interaction network and screening of important miRNA-mRNA duplexes operating in breast cancer by using different tools.

OBJECTIVE 4

Quantification and Validation of selected sets of miRNAs and mRNAs by different experimental validation in breast cancer cell lines (MDA-MB-231) and HaCaT cell lines.

OBJECTIVE 5

Investigating the mode of regulation of selected set of down-regulated mRNAs by Methylation-specific PCR (MSP).

MATERIALS AND METHODS

MATERIALS AND METHODS

GENE EXPRESSION

For the analysis of mRNA and miRNA gene expression of normal as well as disease state the gene expression data is convenient. Gene expression analysis is further useful in therapeutic process and comparison study of the various expression patterns. The gene expression microarray data of normal vs disease sample sets are retrieved from the GEO (Gene Expression Omnibus) database, available at NCBI (http://www.ncbi.nlm.nih.gov/geo/). GEO data base is an international public repository, which stores microarray, next generation sequencing, and functional genomics data submitted by different research communities which can be retrieved easily by public users. The GEO data bases has mainly 3 goals

i) provides durable, versatile database which storage high-thoughtful functional genomics data

ii) provides simple submission procedure and format to the research community also supports the annotated data

iii) user friendly mechanism that allows public query

For our present study expression profiles of mRNA and miRNA were taken from the GEO data base.

In microarray experiments platform describes the list of features on the array (e.g cDNA, oligonucleotides,etc).

Microarray analysis of gene expression data

The microarray data were retrieves from GEO database. The data was retrieved for normal (control) tissues and the breast cancer (test).

The GEO datasets Choosen

mRNA	**GSE10797**
miRNA	**GSE38867**

The PLATFORM used in the GEO datasets taken:

mRNA	**GPL571: [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array**
miRNA	**GPL15019:Agilent-031181 Unrestricted_Human_miRNA_V16.0_Microarray**

mRNA control samples:

GSM272727, GSM272728, GSM272729, GSM272730, GSM272731, GSM272732, GSM272733, GSM272734, GSM272735

mRNA test samples:

GSM272671, GSM272672, GSM272673, GSM272674, GSM272675, GSM272676, GSM272677, GSM272678, GSM272679, GSM272680, GSM272681, GSM272682, GSM272683, GSM272684, GSM272685, GSM272686, GSM272687, GSM272688, GSM272689, GSM272690, GSM272691, GSM272692, GSM272693, GSM272694, GSM272695, GSM272696, GSM272697, GSM272698, GSM272699, GSM272700, GSM272702, GSM272703, GSM272704, GSM272705, GSM272706, GSM272707, GSM272708, GSM272709, GSM272710, GSM272711, GSM272712, GSM272713, GSM272714, GSM272715, GSM272716, GSM272717, GSM272718, GSM272719, GSM272720, GSM272721, GSM272722, GSM272723, GSM272724, GSM272725.

miRNA control sample:

GSM951048, GSM951056, GSM951060, GSM951064, GSM951068

miRNA test sample:

GSM951054, GSM951062, GSM951066

The data sets were downloaded in .CEL format, unzipped and named as per convenience to differentiate the samples.

Microarray Expression Analysis:

The microarray data files were analysed using GeneSpring GX software. GeneSpring GX software provides powerful and accessible statistical tools for high speed visualization and

analysis of transcriptomics, genomics, proteomics and metabolomics data within a biological context. It includes all major microarray platform including Agilent, Affymetrix and Illumina. It helps to analyse all types of expressions like miRNA analysis, gene targets identification, correlative analysis on mRNA expression, miRNA expression, real time PCR data analysis etc.

For the analysis the breast cancer GEO data sets it crossed multistep process. First a project was created. This consists of the one or more related experiments. Experiment comprises samples, interpretations and analyses. Samples are mainly the data sets, interpretation was done with grouping of samples based on experimental parameters and analysis is statistic steps associates results, typically entity lists.

For mRNA expression analysis:

1. An option of *Create new experiment* is chosen that allows creating a new experiment. The *Experiment type* is specified as *Affymetrix Gene Chip-HGU113A.* Once the experiment type is selected, the workflow type needs to be selected as *Guided Workflow.*

2. An experiment is created using *choose sample* option. *Experimental setup* is done by adding average parameter which helps *grouping experiment* as test and control and replicate structure of the experiment.

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

4. 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.

5. *Statistical analysis* is 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.

6. The *p-value* cut-off is taken **≤ 0.05**. *Multiple testing corrections* were 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.

7. *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 cut off 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 is taken to be **≥2.0**.

8. The analyzed data is exported by choosing *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.

9. The software used for clustering and generation of Heat map is *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.

10. *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.

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

miRNA expression analysis:

1. An option of *Create new experiment* is selected that allows creating a new experiment. The *Experiment type* was specified as *Agilent_031181.* Once the experiment type is selected, the workflow type was selected as *Guided workflow.*

2. An experiment is created using *choose sample* option. *Experimental setup* was done by adding average parameter which helps *grouping experiment* as test and control and replicate structure of the experiment.

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

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

5. *Statistical analysis* is 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**.

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intensities of the samples grouped. The entities satisfying the significance analysis are passed on for the fold change analysis. The fold change cut-off is taken to be **≥2.0**.

8. The analyzed data is exported by choosing *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.

GENESPRING STEPs:

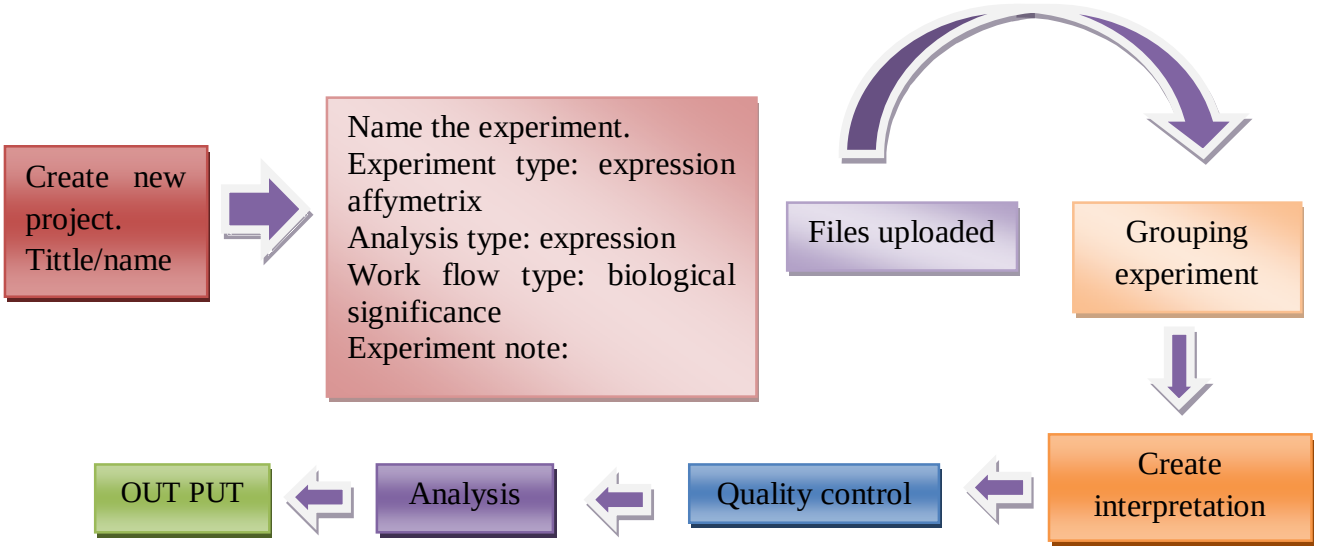


Figure 10: The multistep process of Genespring GX software

Analysis of differentially expressed gene list

The common set of genes were taken and analyzed by 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 choosen based upon their regulation and association with cancer for further validation done 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 file was created by selecting the list of miRNAs involved in breast cancer with its regulation and fold change value with cut off ≥2.0.

The highly up-regulated miRNA and down regulated mRNAs were taken for the further analysis.

TARGET INTERACTION ANALYSIS THROUGH MAGIA[2] SOFTWARE:

Magia[2] software is a web based tool which has low sensitivity of target prediction algorithms created by exploiting the integration of target predictions with miRNA and mRNA gene expression profiles for in silico target prediction where regulatory elements and their integrators generate a highly interconnected network of mRNA, miRNA and Transcription Factor (TF). The integrated analysis of *in silico* target prediction, miRNA and mRNA data for the reconstruction of post-transcriptional regulatory networks is performed by using Magia[2]. A file containing the intensity values representing the expression of mRNAs and miRNAs were uploaded. The analysis was done using **Pearson correlation** method that aims to display the target interaction map for matched miRNAs and mRNA expression data. An interaction map was generated which showed the interconnected network of mRNAs, miRNAs and Transcription Factors. The miRNA-target interactions which have been experimentally validated (as are reported in miRecords, TarBase) are specifically marked within the output file. From the interaction map, two mRNAs and two miRNAs were selected based upon their regulation and association with breast cancer for further validation.

Interaction study by NCI pathway analysis tool

The National Cancer Institute (NCI) in collaboration with Nature Publishing Group has established the Pathway Interaction Database (PID) in order to provide a highly structured, curated collection of information about known biomolecular interactions and key cellular processes assembled into signaling pathways. The database focuses on the bimolecular interactions that are known or believed to take place in human cells. It can be browsed as an online encyclopedia, used to run computational analyses, or employed in ways that combine these two approaches. In addition to PID's predefined pathways, search results are displayed as dynamically constructed interaction networks.

We used the batch query option from NCI database for functional enrichment of downregulated mRNAs in order to choose genes involved in crucial cellular processes.

EXPERIMENTAL VALIDATIONS

CELL CULTURE

Different Cell lines were cultured for experimental validations. Human breast cancer cell lines, MDA-MB 231 and normal keratinocyte cell line HaCaT were procured from the National centre of cell science (NCCS), Pune, India. The media used for both the control and breast cancer cell lines is DMEM with 10% FBS and 1% antibiotics solution. Cell lines were cultured in culture flask in a CO_2 incubator which maintained 5% CO_2 level.

CELL REVIVE

1. Water bath was heated up to 37^0C. A vial of frozen cells was removed from liquid nitrogen and placed in the water bath until thawed.
2. The outside of the vial was washed with 70% ethanol.
3. The thawed cells were slowly pipeted out into sterile centrifuge tube which contained pre-warmed growth medium and centrifuged at 200Xg for 2min.
4. The supernatant containing media with DMSO was decanted of aseptically and the cell pellet was resuspended in fresh media.
5. The resupended cells were then cultured in 20% FBS containing media at 37^0C and 5% CO_2.

TRYPSINIZING CELLS:

1. Trypsinizing is a technique that helps to detach adherent cells from the surface of a cell culture vessel. This procedure is performed whenever the cells are harvested.
2. The used media was aspirated and discarded.
3. The cells were washed with PBS (phosphate buffer saline).
4. 1-2ml of trypsine was added to the surface of the culture flask.
5. The flask is then placed in the CO_2 incubator at 37^0c for 1-2 min.
6. The flask is then removed from incubator and lightly tapped to assist detachment of cells.
7. Detached cells were resuspended in serum containing growth medium (the serum inactivates trypsin activity).
8. Then the cells were pippeted up and down gently for mixing properly.

9. The cells were the transferred to RNAase free glass or polypropylene centrifuge tube and centrifuge at 200Xg for 5min. The supernant was aspirated completely and taken for RNA isolation.

RNA ISOLATION

RNA isolation was done with the ***RNAeasy kit of QIAGEN.***

1. Cells were centrifuged at 1000rpm for 5min.
2. The pallet was retained and the supernatant was discarded.
3. The cells were disrupted by adding 350µl of RLT buffer and the pallet was dissolved with 1 volume of (350 µl) ethanol was mixed properly by pipetting (do not centrifuge).
4. Then the mixture was transferred to a RNasy mini spin column placed in a 2ml collection tube. The lid was closed and centrifuged for 15s at 13000rpm.Then the flow trough was discarded.
5. RW1 (700 µl) buffer was added to the column. The lid was closed and centrifuged at 13000rpm for 15s. flow through was discarded.
6. 500 µl RPE buffer was added to the column and centrifuged for 2min at 13000rpm, again it was centrifuged.
7. Then the column was transferred to a new 1.5ml collection tube. To this 30-50 µl RNase free water was added to the spin column membrane. Closed the lid and centrifuged for 1 min at 13000rpm for elution the RNA.
8. If the expected RNA yield is >30µg repeated last step using another 30-50µl of RNase free water or using the elute from last step. The spin column was reuse for last step.
9. Then the eluted RNA placed in ice.

MICRORNA ISOLATION:

The miRNA isolation was done by using the *mirVana™ miRNA Isolation Kit*

1. After trypsinization, cells were collected and washed with PBS. Cells pellet were taken and kept in ice.

2. For removal of PBS 600µl lysis/binding solution was added.

[*if cells are 100s in number-300µl]

[*if cells are 1000s in number-600µl]

3. The solution was Vortexed vigorously for homogenous lysate.

4. The $1/10_{th}$ volume (60 µl) of miRNA homogenate additive added to the cell and mixed well by vortexing for several times. The cells placed on ice for 10min.

5. 1 volume (600µl) of acid phenol chloroform was added which equal of the lysate volume and vortexed for 30-60sec for mixing.

6. Centrifugation was done for 5min at 10,000g at RT for separation the aqueous and organic phases.

7. The aqueous phase was removed carefully without disturbing the lower phase and transfers it to the fresh tube.

[*the volume removed was noted.]

8. 1.25 volume of removed aqueous phase was measured and 100% ethanol of same volume was added to it.

9. Then the filter cartridge was placed into one of the collection tubes. The ethanol was mixed properly with pipette and transferred onto the filter cartridge. The cells were centrifuged for 15s sec at 10000rpm. Flow through was discarded and repeated until the lysate/ethanol was through then the collection tube was reused for wash.

10. 700µl miRNA wash solution 1 was added to the filter catridge and centrifuge for 5-10sec. The flow through was discarded.

11. 2/3 of wash solution (500µl) was added and centrifuged for 5-10sec. This step was repeated again.

12. After discarding the flow through from last wash and filtered catridge in the same collection tube. The pallet was spin assembly for 1min for removal of residual fluid from filter.

13. Filter catridge was transferred into a fresh collection tube. The 100 µl of preheated elution solution added to the centre of the filter and closed the cap. Spin was done for 20-30 sec at maximum spead to recovery the RNA.

14. The eluent was collected and stored at -20^0 c.

NANODROP READING

RNA was quantified by using the nanodrop spectrophotometer. The OD was measured at 260nm wave length. The highest peak was at 260nm. The purity of DNA was determined by calculating the OD at 260/280 value. DNA concentration was determined based on the following formula.

1 OD_{260}=40µg ssRNA/ml

cDNA SYNTHESIS OF mRNA

The steps in cDNA synthesis:

1. For each reaction, the following components mixed in a sterile 0.2 or 0.5ml tube.

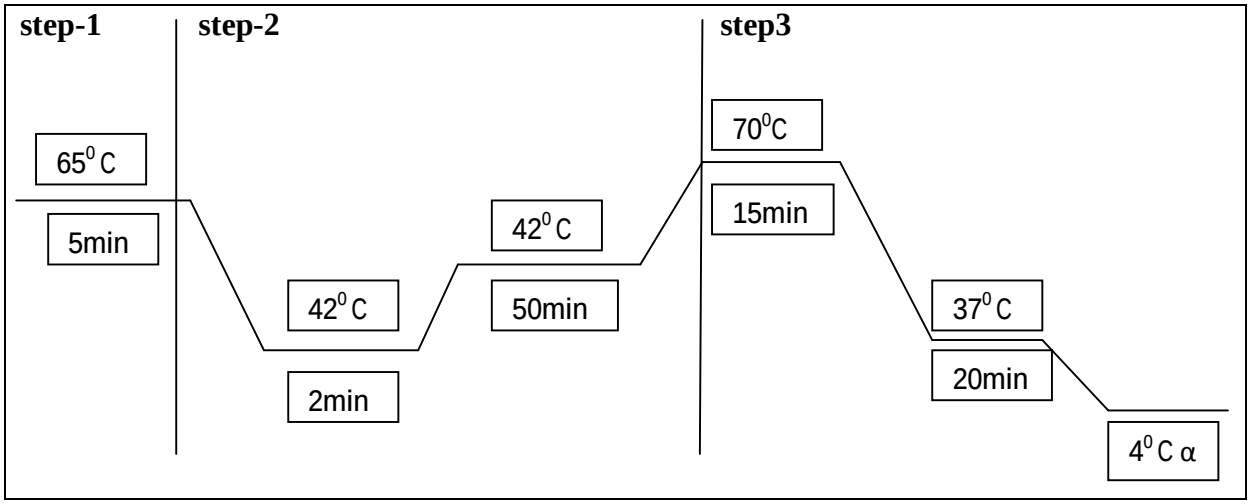
Components	Amounts
RNA	4 µl
10 mM dNTP mix	1µl
Primer (0.5µg/µl oligo (dT)12-18 or 2µM gene specific primer)	1 µl
DEPC treated water	4 µl

2. Each of the components were mixed and briefly centrifuged before use.
3. The RNA/primer mixture was incubated at 65·c for 5 minutes, 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	Amounts
10x RT buffer	2µl
25mM $Mgcl_2$	4 µl
0.1 M DTT	2 µl
RNase OUT	1µl

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 incubated at 42°c for 2 minutes.
7. 1µl of super script TM II RT was added to each tube.
8. It was incubated at 42°c for 50 minutes.
9. The reaction was terminated at 70·c for 15 minutes and then 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.



cDNA SYNTHESIS OF miRNA:

The cDNA was prepared from the miRNA by using the Invitrogen's *NCode™ VILO™ miRNA cDNA Synthesis Kit*

1. For the cDNA synthesis of the miRNA the undiluted cDNA are used.
2. For a single reaction the following component were taken in a tube on ice.

Component	Amount
5x Reaction mix	4µl
10x superscript enzyme mix	2µl
Total RNA	1.5µl
DEPC water	12.5µl

3. Then it was incubated at 37^0 c for 60min.
4. The reaction was terminated at 95^0 c for 5min and kept at hold at 4^0 c until the use.

Quantitative Real Time PCR (qRT-PCR) Analysis

Real-time PCR is the process where the DNA sequences are amplified exponentially and the fluorescent signal emitted during each step is measured over a range of cycles. Quantitative RT-PCR converts the fluorescent signals from each reaction into a numerical value for each sample. Fluorescent dyes was 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. The SYBR green dye fluoresces only when bound to double-stranded DNA. The disadvantage of using a SYBR green dye is the lack of specificity. We performed the quantitative analysis of two genes CX3CL1 and ADAMTS5 in breast cancer cell line.

Procedure for mRNA

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

i) **SYBR ® Green master mix:-**

The concentration stock solution was 2X and a working solution of 1 X was prepared.

ii) **cDNA Dilution:**

A 1:10 dilution of cDNA was prepared

iv) Primer: -

Final Concentration - 500nM

Preparation

2cDNA X 2Gene X 3(triplicate)=12 X10 µl= 120µl +10 µl(one reaction extra)=130 µ

Sybergreen(Master Mix)=5 µl X 13= 65 µl

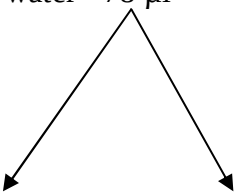
Forward Primer (F.P) = 0.5 X 13 = 6.5 µl

Reverse Primer (R.P)=0.5 X 13 =6.5 µl

cDNA = 3 X 13 =39 µl

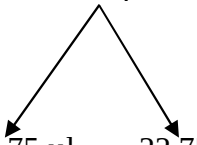
Total = 117 µl

Master Mix 65 µl +13 µl water =78 µl



	39 µl	39µl
F.P.(reference gene)	3.25 µl	3.25 µl(test gene)
R.P	3.25 µl	3.25 µl

Toatal =45 µl



	22.75 µl	22.75 µl
cDNA	9.75 µl	9.75 µl
(control sample)		(cDNA test sample)

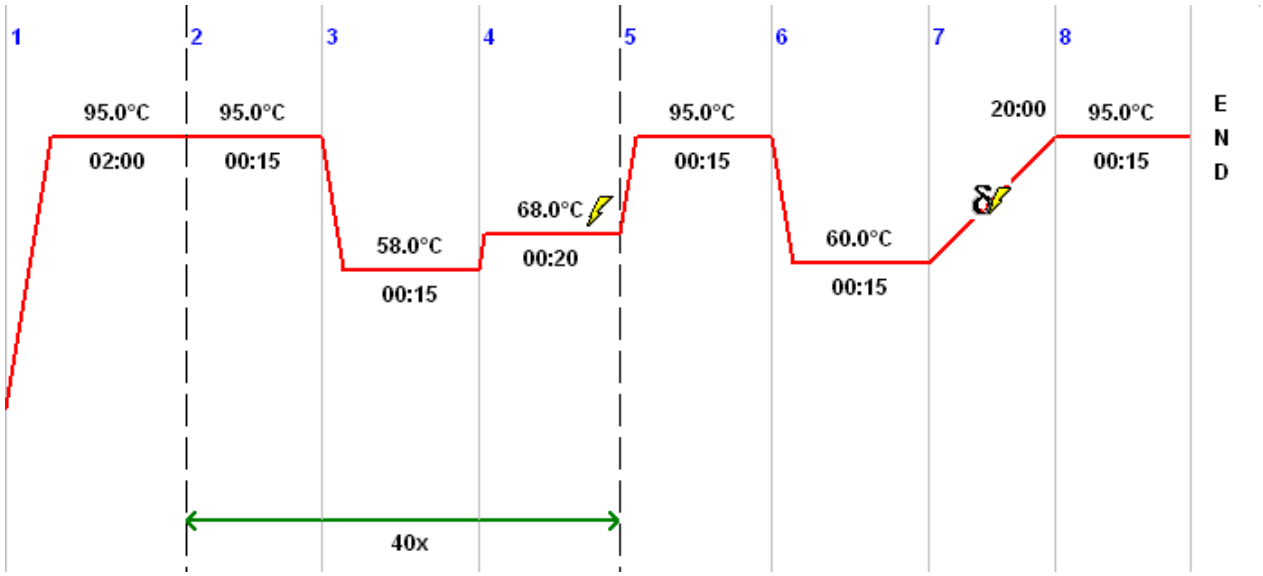
Total = 32.50 µl (then it is divided into 3 triplicate 10 µl reaction in each pcr tube)

Precaution: don't expose the reaction to the light

Pipeting error should be avoid.

mRNA PRIMER

PRIMER	START	TYPE	SEQUENCE
ADAMTS5	23	forward	**5'-GAACATCGACCAACTCTACTCCG-3'**
	20	reverse	**5'-CAATGCCCACCGAACCATCT-3'**
CX3CL1	19	forward	**5'-ACCACGGTGTGACGAAATG-3'**
	23	reverse	**5'-TGTTGATAGTGGATGAGCAAAGC-3'**
Beta Actin	21	forward	**5'- CATGTACGTTGCTATCCAGGC-3'**
	21	Reverse	**5'- CTCCTTAATGTCACGCACGAT-3'**

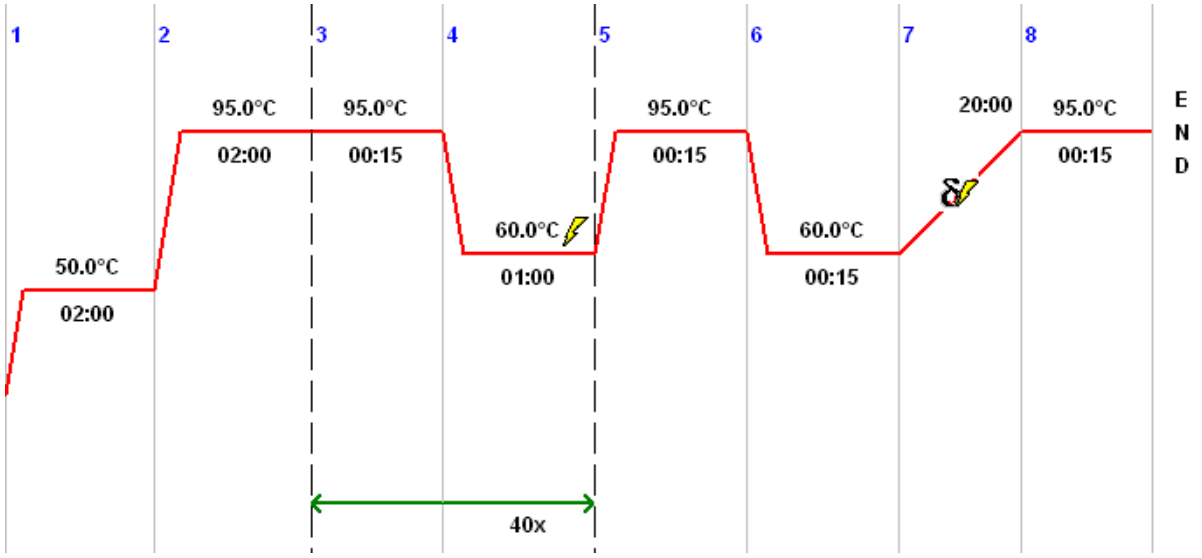


mRNA PCR Condition

miRNA PRIMER

PRIMER	LENGTH	TYPE	SEQUENCE
hsa-miR-17-5p	23	Forward	5'-**CAAAGUGCUUACAGUGCAGGUAG**-3'
hsa-miR-27-3p	21	Forward	5'-**UUCACAGUGGCUAAGUUCCGC**-3'

U6	25	Forward	**5'-GTGCTCGCTTCGGCAGCACATATAC-3'**
U6	27	Reverse	**5'-AAAAATATGGAACGCTTCACGAATTTG-3'**



miRNA PCR Condition:

DNA ISOLATION

1. The harvested cells were collected. 10^6 cells were pelleted down at 13000rpm for 6min.
2. Then it was washed with the PBS.
3. 60µl RIPA buffer and 5 µl proteinase K was added to it and incubated for the 5min at 4^0 C. Then tube was placed 25^0 C for 10min.
4. 600 µl phenol was added and kept for 10min.
5. Then it was taken for the centrifuged at 4^0 C at 6000rpm for 10min.
6. Supernatant was taken in a fresh tube.
7. Then phenol:chloroform + isoamylalcohol taken in 24:1 in 1:1 volume was added to the supernatant.
8. This process was repeated 2 times.
9. Then it was mixed for 10min and centriguge 6000rpm for 10min at 4^0 C.

10. The supernatant was taken and was added 600 µl chloroform and mixed it for 5min and centrifuge 6000rpm in 4^0 C for 10min.
11. Then 500 µl (same volume) isopropanol (chilled) was added and 1/10 volume of 3M sodium acetate was added and mixed properly.
12. After that it was kept under ice (4^0 C) for 2hr and then centrifuged at 13000rpm for 20min.
13. Then it was washed with 70% of ethanol for 2 times.
14. It was centrifuged at 13000rpm for 30min to remove excess ethanol. Then it was kept for air drying.

BISULFITE DNA CONVERSION

Bisulfite conversion is a method which provides detailed information on the methylation pattern of individual DNA molecules at single CG site. The method is based on the deamination of unmethylated cytosine residues to uracil in the presence of NaOH and sodium bisulfite where the methylated cytosines remains unchanged. The original methylation state of the DNA can be analyzed by sequencing of the converted DNA. After the conversion reaction, the DNA sequence under investigation is amplified by polymerase chain reaction (PCR) with primers specific for one strand of the bisulfite-converted DNA (Zhang et al., 2009).

PROCEDURE

1. in Bisulfite reactions the thaw DNA was used. Required number of aliquots of Bisulfite Mix was dissolved by adding 800 µl RNase-free water to each aliquot. Bisulfite Mix was completely dissolved by vortexing. This was taken up to 5 min.

Note: If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again.

Note: Do not place dissolved Bisulfite Mix on ice.

2. Bisulfite reaction was prepared in 200 µl PCR tubes according to Table 1.Each component was added orderly.

Note: The combined volume of DNA solution and RNase-free water must total 20 µl.

Bisulfite reaction components

Component	Volume per reaction
DNA solution	Variable (max 20µl)
RNase free water	Variable
Bisulfite mix	85
DNA protect Buffer	35
Total volume	140

3. PCR tubes were closed and bisulfite reactions mixed thoroughly. The tubes were stored at room temperature (15–25°C).

4. Bisulfite DNA conversion was performed using below thermal conditions.

Step	Time	Temperature
Denaturation	5min	95
Incubation	25min	60
Denaturation	5min	95
Incubation	85min	60
Denaturation	5min	95
Incubation	175min	60
Hold	Infinite	20

Cleanup of bisulfite converted DNA

6. When the bisulfite conversion was completed, PCR tubes containing the bisulfite reactions were centrifuged, and then transferred the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.

7. 560 µl freshly prepared Buffer BL was added which containing 10 µg/ml carrier RNA to each sample. The solution was mixed by vortexing and then centrifugation was done briefly.

8. Necessary number of EpiTect spin columns and collection tubes were placed in a suitable rack. Entire mixture was transferred from each tube in step 7 into corresponding EpiTect spin column.

9. Spin columns was centrifuged at maximum speed for 1 min. The flow-through was discarded, and placed the spin columns back into the collection tubes.

10. 500 µl Buffer BW was added to each spin column, and was centrifuged at maximum speed for 1 min. flow-through was discarded, and placed the spin columns back into the collection tubes.

11. 500 µl Buffer BD was added to each spin column, and incubated for 15 min at room temperature (15–25°C).spin columns was centrifuged at maximum speed for 1 min. flow-through was discarded, and spin columns was placed back into the collection tubes.

13. 500 µl Buffer BW was added to each spin column and centrifuged at maximum speed for 1 min. flow-through was discarded and spin columns were placed back into the collection tubes.

14. Step 13 was repeated again.

15. Spin columns were placed into new 2 ml collection tubes, and spin columns were centrifuged at maximum speed for 1 min.

16. Recommended: spin columns were placed with open lids into clean 1.5 ml micro-centrifuge tubes (not provided) and spin columns was incubated for 5 min at 56°C in a heating block. This step was enables evaporation of any remaining liquid.

17. Spin columns were placed into clean 1.5 ml micro-centrifuge tubes (not provided). 20 µl Buffer EB was dispensed onto the center of each membrane. Purified DNA was eluted by centrifugation for 1 min at approximately 15,000 x g (12,000 rpm).

MSP (Methylation specific PCR)

Methylation specific PCR is a quantitative technique used for detecting the presence of methylation in bisulfite converted DNA. Primers to detect methylated DNA are designed under the assumption that the region will be fully methylated and thus will contain cytosine in the bisulphite converted sequences at CG dinucleotides. Unmethylated DNA are designed assuming the region to be unmethylated and therefore containing thymine rather than cytosine at CG dinucleotides. The annealing temperature for methylated primer will be higher than that of unmethylated primers.

PROCEDURE

For experiment two types of primers are used, one for the methylated DNA and the other for unmethylated DNA.

PRIMER	TYPE	SEQUENCE
Cx3cl1 (methylated)	Forward	5'-**GGTGAGAAGTTTAGGGATATAAAGTC**-3'
	Reverse	5'-**AAACTCAATACTATCCCCTTACCG**-3'
Cx3cl1 (un-methylated)	Forward	5'-**GAGAAGTTTAGGGATATAAAGTTGG**-3'
	Reverse	5'-**AAACTCAATACTATCCCCTTACCAC**-3'

PRIMER	TYPE	SEQUENCE
ADAMTS5 (methylated)	Forward	**5'-TAAAATAAGAAAGGAAGTGGGTTTC-3'**
	Reverse	**5'-TCGAATAATAAAAATTCAACAAATACG-3'**
ADAMTS5 (un-methylated)	Forward	**5'-TCGAATAATAAAAATTCAACAAATACG-3'**
	Reverse	**5'-AAATAATAAAAATTCAACAAATACAAA-3'**

The following reaction mix was for prepared the pcr reaction.

Component	Final Concentration
PCR buffer	1x
MgCl2	1.5mM
Template	0.4
Forward primer	200nM
Reverse primer	200nM
dNTP	200nM
DNA polymerase	1.25 per 50µl

The above reaction mix was prepared properly and the template was amplified using Touch-Down PCR cycle conditions.

Phase1:

Step	Temperature	Time
Denature	95	3min
Denature	95	30sec
Anneal	Tm+10	45sec
Elongate	72	60sec

Phase 2

Step	Temperature	Time
Denature	95	30sec
Anneal	Tm/Tm-5c	45sec
Elongate	72	60sec

Termination:

Step	Temperature	Time
Elongate	72	5
Halt reaction	4	15
Hold	23	INFINITE

RESULTS AND DISCUSSIONS

RESULTS AND DISCUSSIONS

Microarray Analysis

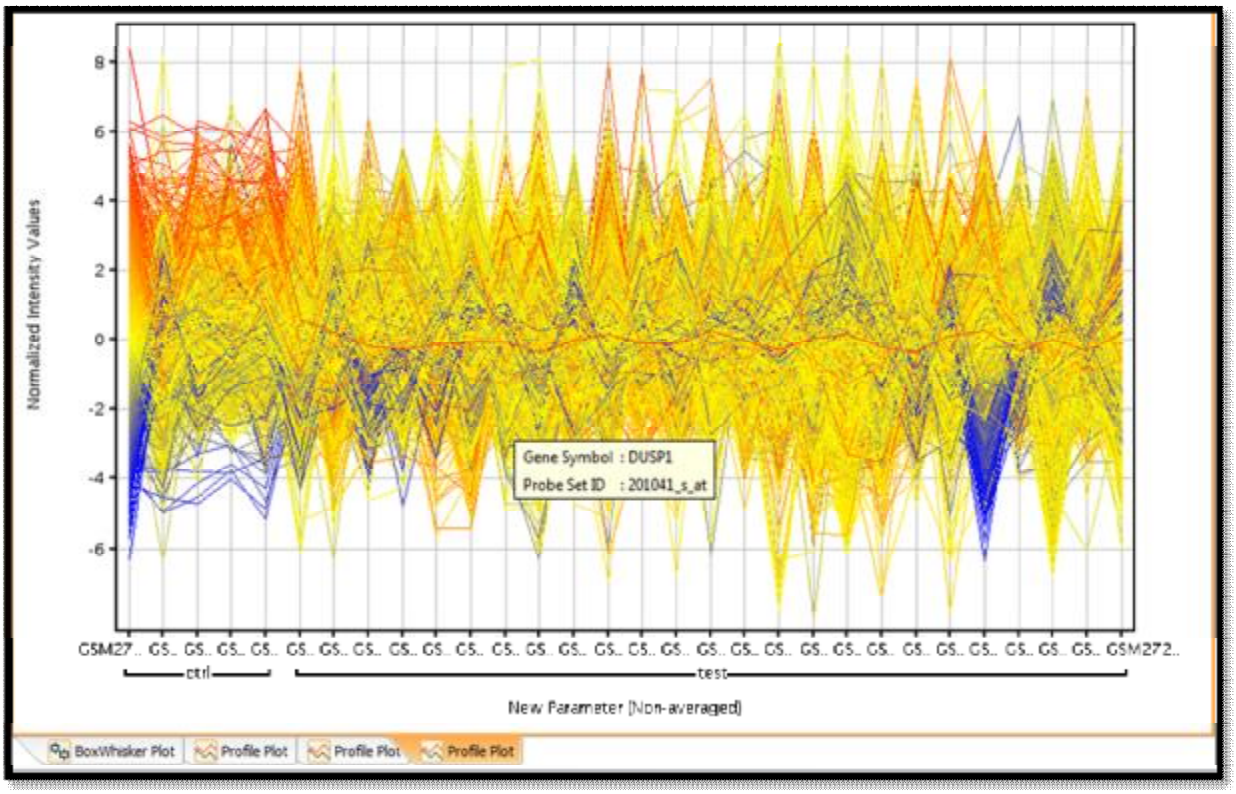
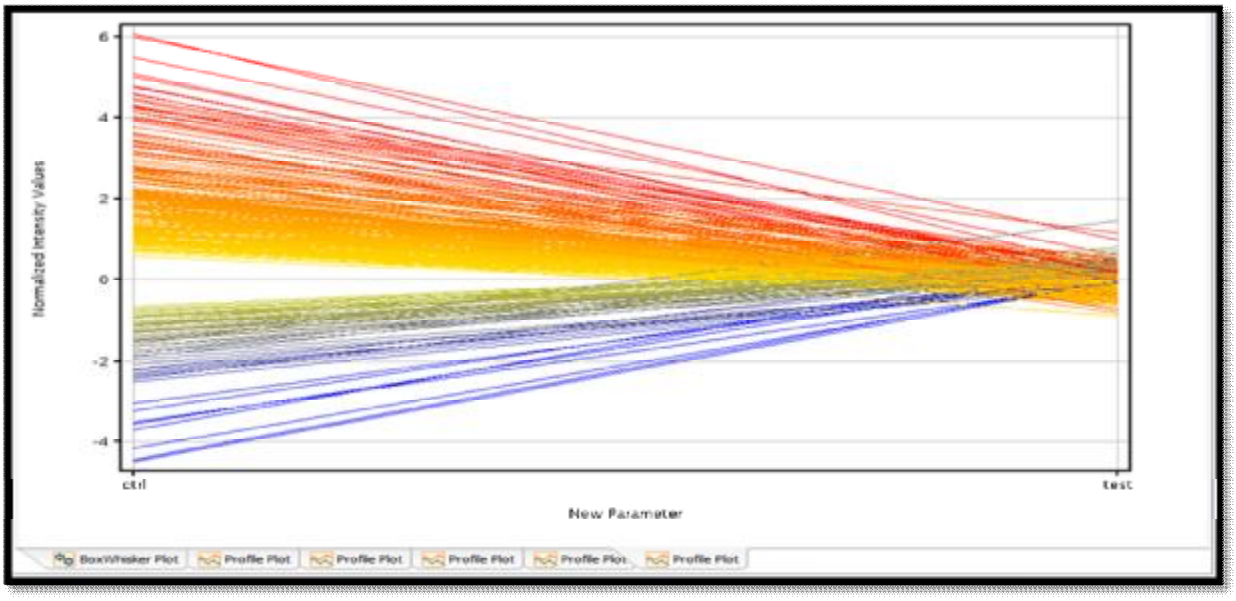


Figure 11: Analysis of all entities in the samples



HIERARCHIAL CLUSTERING OF THE mRNA and miRNA

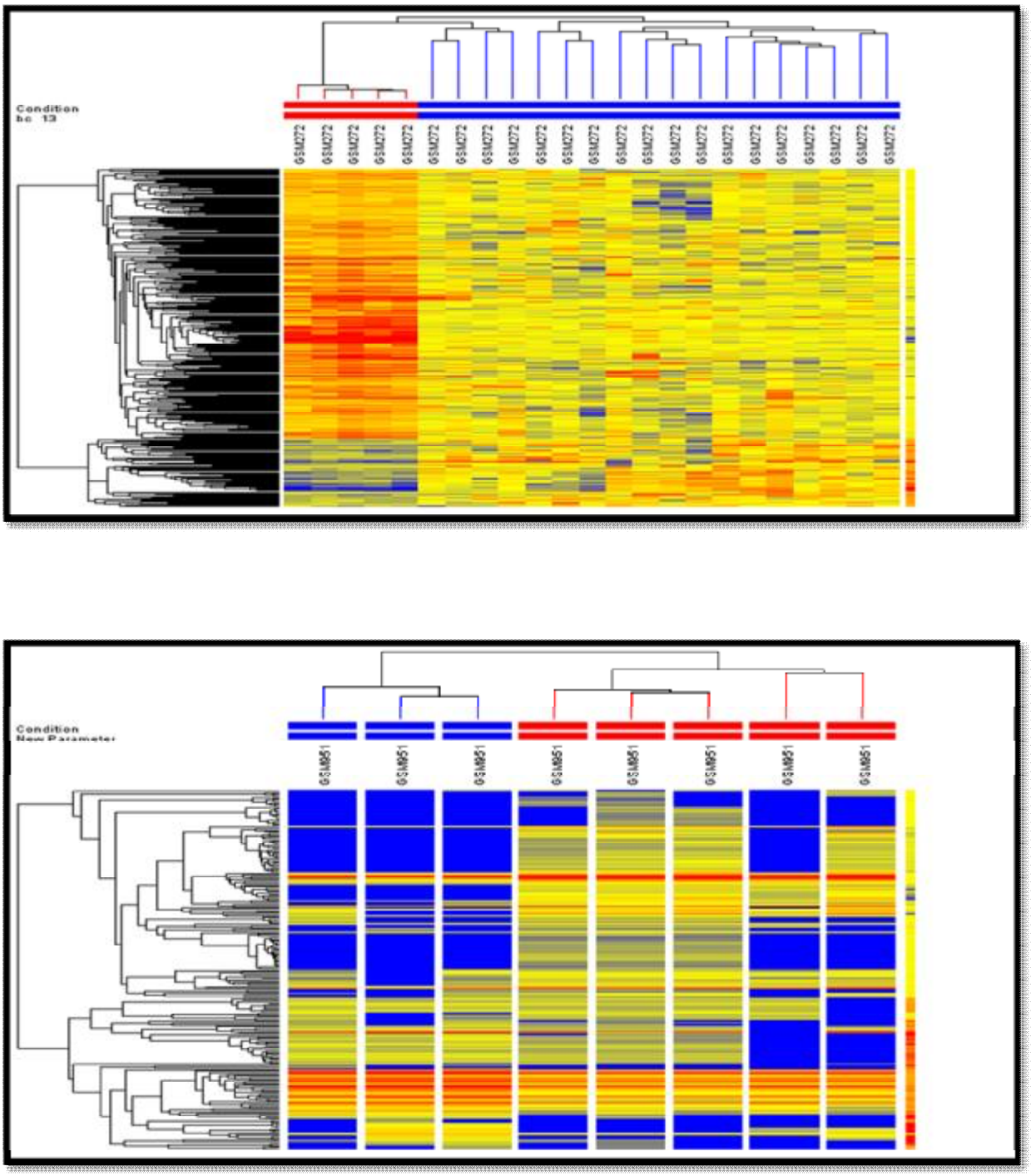


Figure 12: Hierarchical clustering of mRNAs and miRNAs

From above microarray data analysis, we found 195 mRNAs to be down-regulated and 42 to be up-regulated. Microarray analysis of miRNA datasets yielded 122 miRNAs that are down regulated and 75 up regulated.

TARGET ANALYSIS INTERACTION MAP:

The interconnected network of mRNAs-miRNAs-transcription factors was generated as an interaction map.

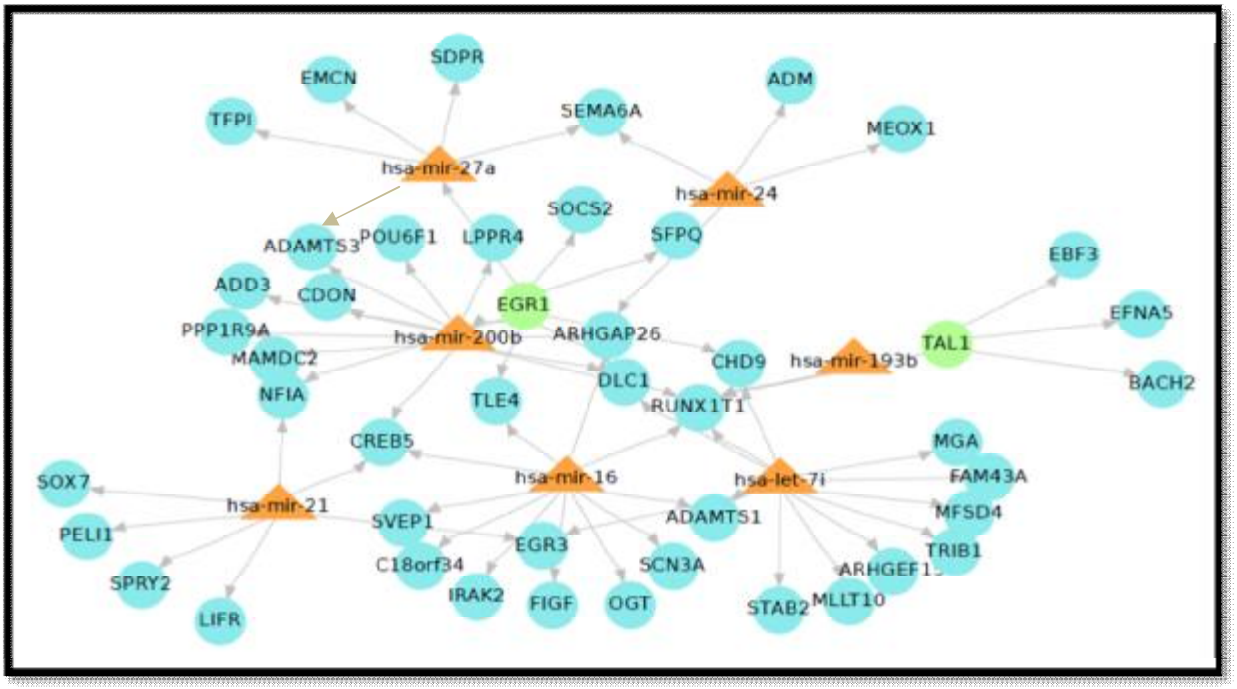


Figure13: Interaction Map of differentially expressed mRNAs and miRNAs

From the Interaction map, ADAMTS5 was selected which is targeted by the hsa-miR-27a. The ADAMTS5 is expected to be down-regulated in breast cancer and the hsa-miR-27a is expected to be up regulated is breast cancer.

Interaction study by NCI pathway analysis tool

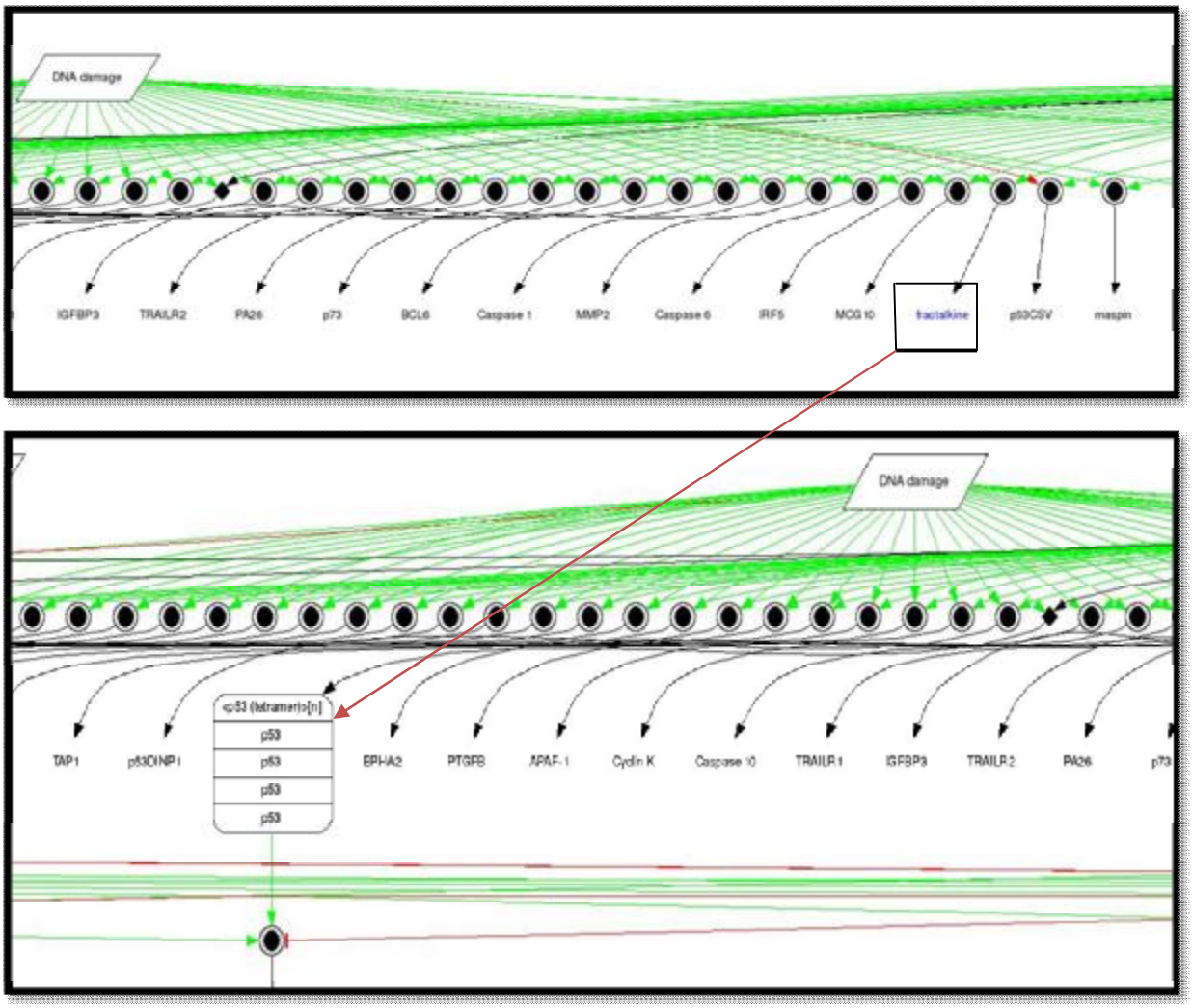


Figure 14: Interaction Map of CX3CL1 with p53

The NCI pathway analysis tool provided an enrichment analysis of the down-regulated genes in breast cancer. From this functional categorisation of the genes, CX3CL1 (fractalkine) a key gene involved in p53 mediated DNA damage response was chosen for further analysis.

EXPERIMENTAL VALIDATIONS

RNA ISOLATION

Cells were collected from test cell line (MDA-MB 231) as well as control cell line (HaCaT). RNAs was isolated by using the desired kit and was quantified using nanodrop spectrophotometer. The OD was measured at 260 nm wavelength and 280 nm wave length. The highest peak was at 260 nm. The purity of RNA was determined by calculating the OD at 260/280 value. The RNA concentration was determined based on the following formula

1 OD_{260} =40µg ssRNA/ml

Quantification of mRNA

RNAs	AMOUNT	260/280nm
Control	931.1 µg/ml	2.47
Infected	889.3 µg/ml	1.93

Quantification of miRNA:

RNAs	AMOUNT	260/280nm
Control	1611.5 µg/ml	2.04
Infected	1127.4 µg/ml	1.89

REAL TIME PCR

The qRT-PCR was performed for the quantitative analysis of the both the mRNAs as well as miRNA. The two gene CX3CL1 and ADAMTS5 were considered along with reference gene, beta Actin and their expression was quantified in both test and control samples.

ADAMTS5

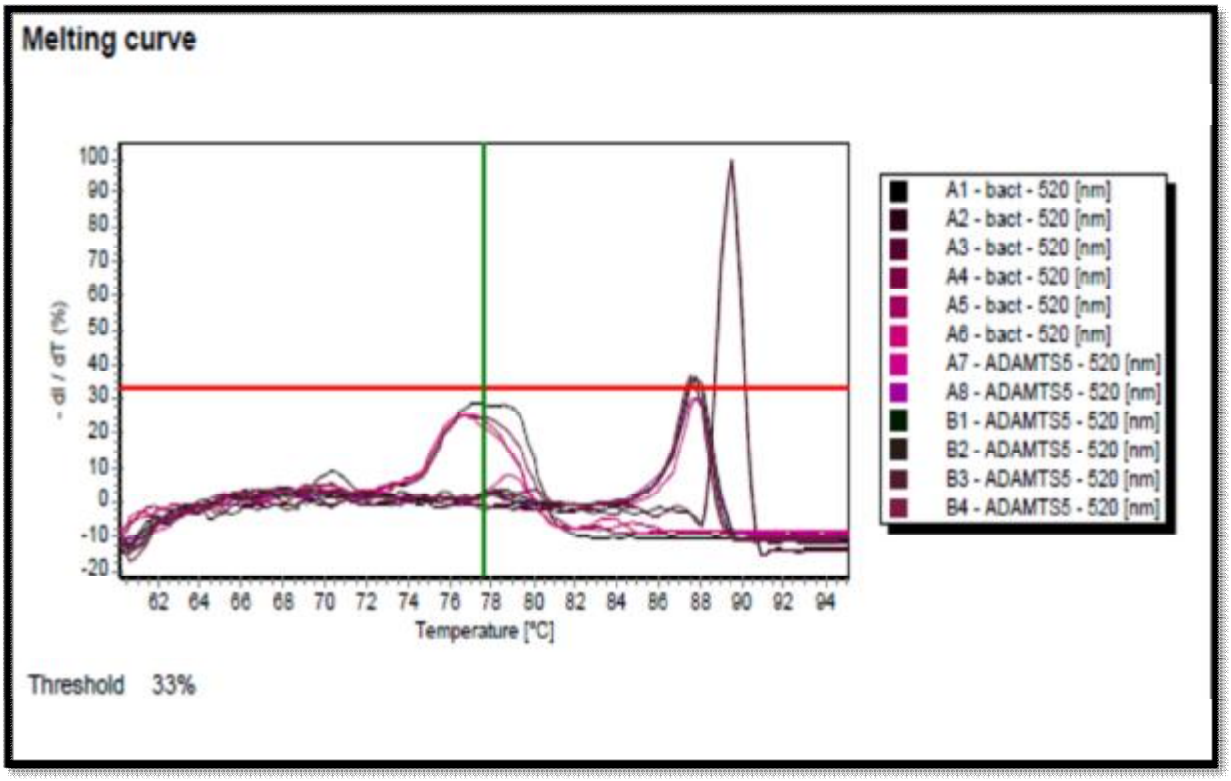


Figure15: Melting Curve of ADAMTS5 with respect to the control gene

miRNA qRT-PCR:

The two miRNA hsa-miR-27a-3p and hsa-miR-17-5p were taken with the control $U_{6.}$

hsa-miR-27a-3p:

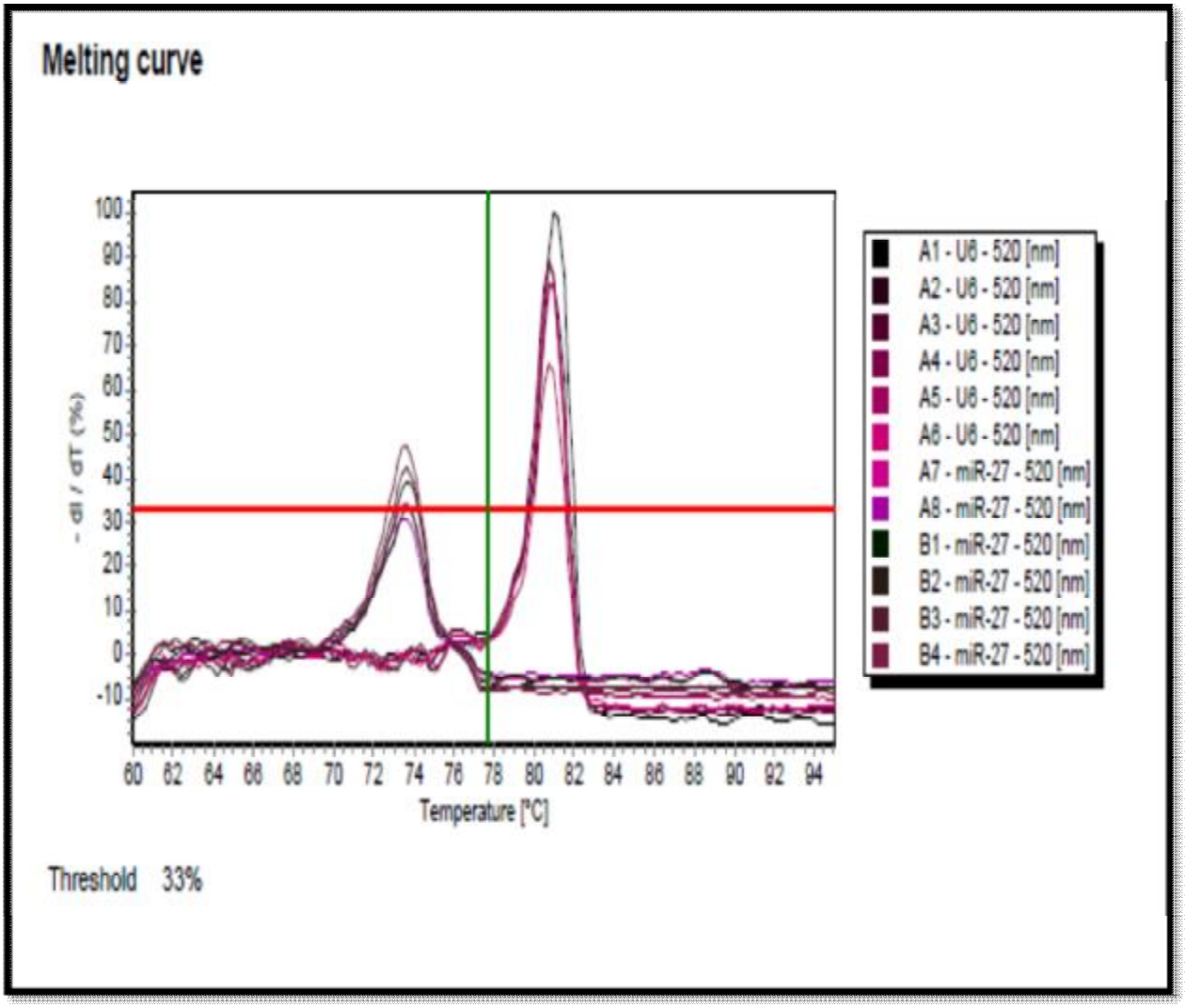


Figure16: Melting Curve of the hsa-miR-27a-3p with respected the U_6

MELTING CURVE OF hsa-miR-17-5p and hsa-miR-27a-3p

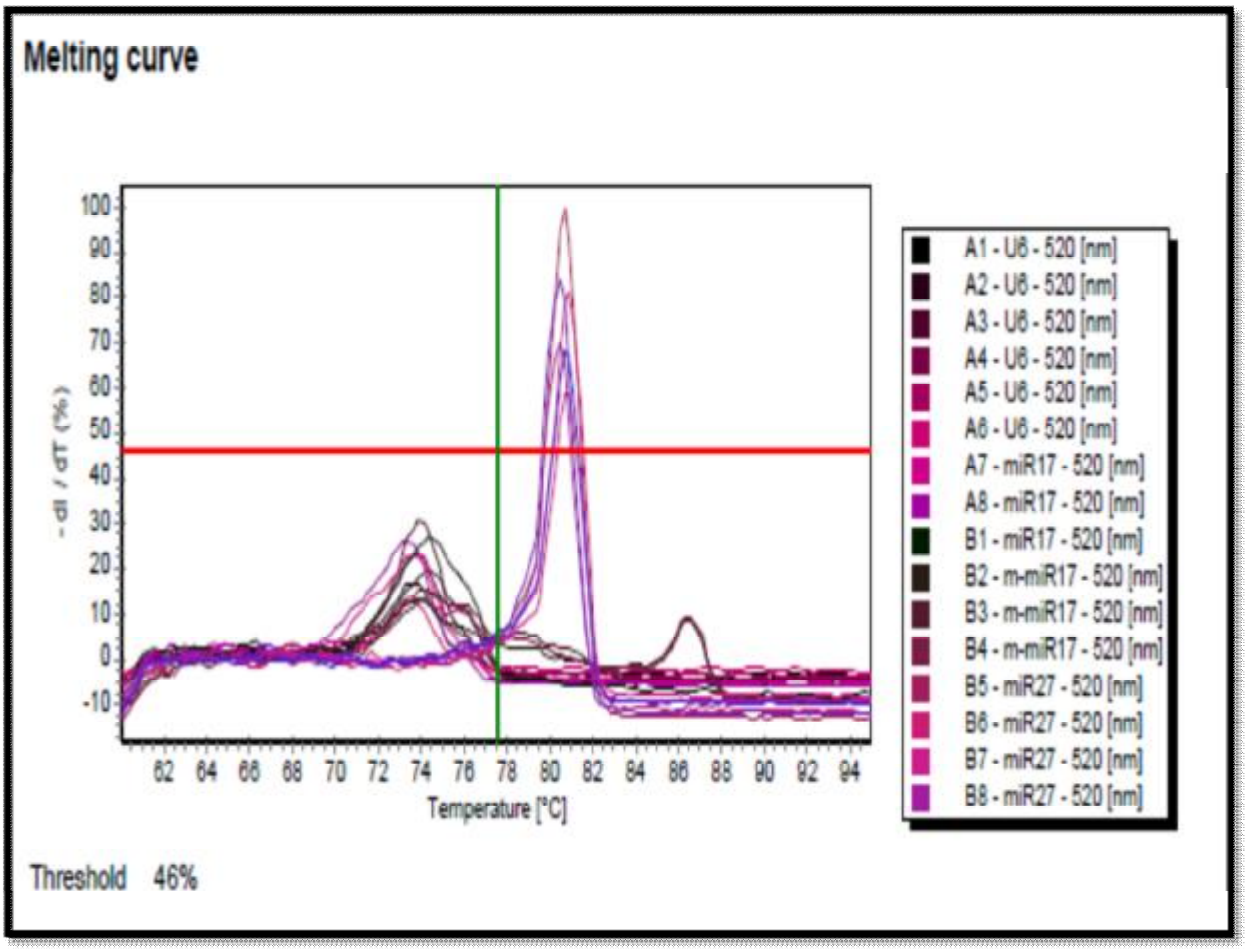
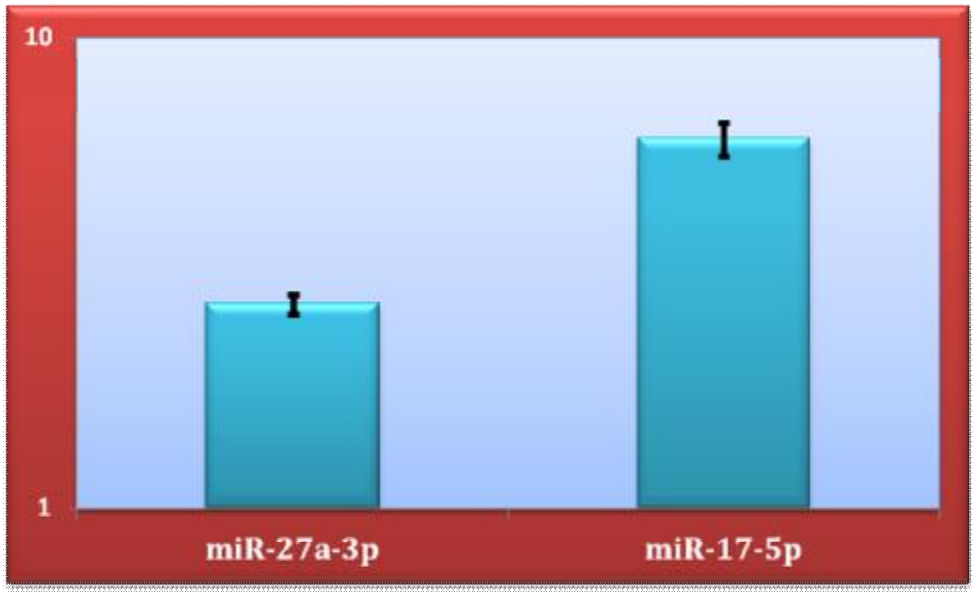
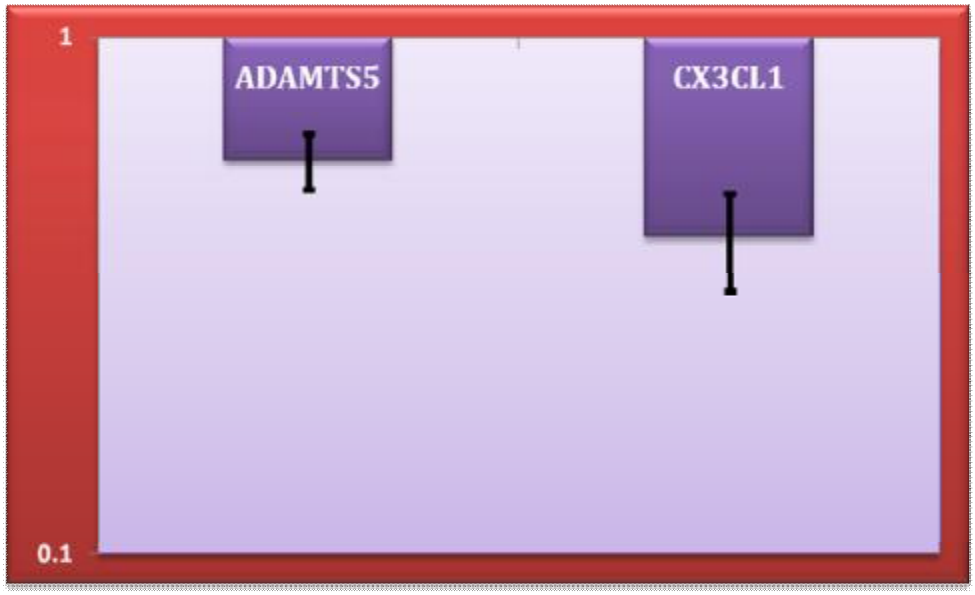


Figure17: Melting curve of hsa-miR-17-5p and hsa-miR-27a-3p

BAR DIAGRAM OF miRNA AND mRNA EXPRESSION:



(a)



(b)

Figure 18: Fold change of miR-27a-3p, miR-17-5p, ADAMTS5 and CX3CL1 in breast cancer (MDA-MB-231 cell lines) obtained from qRT-PCR

(a) Above bar graph represents miRNAs to be highly expressed in the test sample in comparison to normal sample. (b) Simultaneously the mRNA levels is found to be down regulated in the test sample with respect to their fold change ($FC=2^{-\Delta\Delta CT}$).

CONCLUSIONS

CONCLUSIONS

In our study, we mainly focused on the differentially expressed mRNAs and miRNAs which regulate the breast cancer. The targets are identified and studied to analyse the mechanism of miRNA targeting in breast cancer.

We analysed expression data of human breast cancer tissues obtained from Gene Omnibus database (GSE ID: 10797) to find out significantly expressed genes. 33 samples were used in this analysis, out of which 5 samples were from breast epithelial tissues (used as control) and 28 samples were from cancer epithelial tissues (used as test). We obtained a set of differentially expressed mRNAs that comprised of 195 down regulated genes and 42 up regulated genes. We also analysed the miRNA expression of breast cancer (GSE ID: 38867) and obtained 122 down regulated and 75 up regulated miRNAs.

By using the different web based tools, interaction studies were done. From that interaction map, we found that ADAMTS5 is targeted by hsa-miR-27a-3p. From the NCI pathway analysis, we found that CX3CL1 have more potential role by targeting p53 in breast cancer which has target site for hsa-miR-17-5p.

Experimental validation by was done for both of the above mentioned mRNAs and miRNA using quantitative RT-PCR and found that ADAMTS5 is down regulated and hsa-miR-27a-3p is up regulated in breast cancer. The down-regulation of this gene leaves scope for a hypothesis that it may be due to being targeted by has-miR-27a-3p which is highly expressed in breast cancer. From the qRT-PCR, we also found that CX3Cl1 is down-regulated and hsa-miR-17-5p is highly regulated.

For better understanding of the mode of regulation of such genes, we attempted Methylation analysis of respective promoter sequences through MSP, but were not successful. Further study is needed for the MSP validation.

FUTURE PROSPECTIVES

FUTURE PROSPECTIVES

Curable rate of breast cancer patient is less compared to increased incidence of breast cancer. Identifying the molecular mechanisms responsible for breast cancer malignancy will be helpful for improving therapeutic uses. The further study and validation of ADAMTS5 and CX3CL1 which are predicted to be targeted by hsa-miR-27a-3p and hsa-miR-17-5p respectively by luciferase assay will shed light on mode of regulation of these genes. Further functional experimental validations will disclose the significance of these de-regulations in breast cancer to understand the mechanisms underlying this malignancy which ultimately may help to discover new ways of incorporating miRNA in cancer therapeutics.

REFERENCES

1. Abbaszade, I.; Liu, R.; Yang, F.; Rosenfeld, S.; Ross, O.H.; Link, J.R.; Ellis, D.M.; Tortorella, M.D.; Pratta, MA.; Hollis, J.M.; et al. Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. J. Biol. Chem. 1999, 274, 23443–23450.

2. Asthana S, Chauhan S, Labani S. Breast and cervical cancer risk in India: an update. Indian J Public Health. 2014 Jan-Mar;58(1):5-10.

3. Balch C, et al. The epigenetics of ovarian cancer drug resistance and resensitization.Am. J. Obstet. Gynecol. 2004;191:1552–1572.

4. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell.2009;136(2):215–33.

5. Berman H, Zhang J, Crawford YG, Gauthier ML, Fordyce CA, McDermott KM, Sigaroudinia M, Kozakiewicz K, Tlsty TD: Genetic and epigenetic changes in mammary epithelial cells identify a subpopulation of cells involved in early carcinogenesis. *Cold Spring Harb Symp Quant Biol* 2005, 70:317-327.

6. Blaumueller, C. M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. (1997). Cell 90, 281-291.

7. Bourguignon LY, Earle C, Wong G, Spevak CC, Krueger K. Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. Oncogene. 2012 Jan 12;31(2):149-60.

8. Brendan D. Manning, Lewis C. Cantley. AKT/PKB Signaling: Navigating Downstream cancer cells. Oncogene Cancer Res Treat. 2006 Feb;95(3):211-8.Cancers (Basel). 2012 Nov 29;4(4):1252-99.

9. Cell. Author manuscript; available in PMC 2009 October 5.Published in final edited form as: Cell. 2007 June 29; 129(7): 1261–1274.

10. Comb M, Goodman HM. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. 1990;18:3975–3982.

11. Eiriksdottir, G., G. Johannesdottir, S. Ingvarsson, I. B. Bjornsdottir, J. G. Jonasson, B. A. Agnarsson, J. Hallgrimsson, J. Gudmundsson, V. Egilsson, H. Sigurdsson, and R. B. Barkardottir. 1998. Mapping loss of heterozygosity at chromosome 13q: loss at 13q12-q13 is associated with breast tumour progression and poor prognosis.Eur. J. Cancer 34:2076-2081.

12. Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. Biochim Biophys Acta. 2009 Jun;1792(6):497-505.

13. Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, Croce CM, Brunetti E, Grignani F, Peschle C. MicroRNAs 17–5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol.2007;9:775–787.

14. Giancotti F. G., Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. Annu. Rev. Cell Dev. Biol. 2003;19:173–206.

15. González-Martin A, Mira E, Mañes S. CCR5 as a potential target in cancer therapy: inhibition or stimulation? Anticancer Agents Med Chem 2012; 12:1045-57

16. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan7;100(1):57-70.

17. Havens MA, Reich AA, Hastings ML. Drosha Promotes Splicing of a Pre-microRNA-like Alternative Exon. PLoS Genet. 2014 May 1;10(5):e1004312.

18. Hossain A, Kuo MT, Saunders GF. Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Mol Cell Biol. 2006 Nov;26(21):8191-201

19. Inamdar NM, Ehrlich KC, Ehrlich M. CpG methylation inhibits binding of several sequence-specific DNA-binding proteins from pea, wheat, soybean and cauliflower.Plant Mol Biol. 1991;17:111–123.

20. Kong D, Li Y, Wang Z, Banerjee S, Ahmad A, Kim HR, Sarkar FH. miR-200 regulates PDGF-D-mediated epithelial-mesenchymal transition, adhesion, and invasion of prostate cancer cells. Stem Cells. 2009;27:1712–1721.

21. Kumar S, Rao N, Ge R. Emerging Roles of ADAMTSs in Angiogenesis and Cancer. Cancers (Basel). 2012 Nov 29;4(4):1252-99.

22. Li Ma, Jennifer Young, Harsha Prabhala, Elizabeth Pan, Pieter Mestdagh, Daniel Muth, Julie Teruya-Feldstein, Ferenc Reinhardt, Tamer T. Onder, Scott Valastyan, Frank Westermann, Frank Speleman, Jo Vandesompele, Robert A. Weinberg. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis Nat Cell Biol. Author manuscript; available in PMC 2010 September 1.Published in final edited form as: Nat Cell Biol. 2010 March; 12(3): 247–256.

23. Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet. 1997 May;16(1):647.

24. Lindsey E. Becker Buscaglia, Yong Li Chin J Cancer. Apoptosis and the target genes of miR-21 PMC 2012 April 4.Published in final edited form as: Chin J Cancer. 2011 June; 30(6): 371–380.

25. Longpré, J.-M.; McCulloch, D.; Koo, B.-H.; Alexander, J.; Apte, S.; Leduc, R. Characterization of proADAMTS5 processing by proprotein convertases. Int. J. Biochem. Cell Biol. 2009, 41, 1116–1142.

26. Luciani S, Cabanes A, Prieto-Lara E, Gawryszewski V. Cervical and female breast cancers in the Americas: current situation and opportunities for action.Bull World Health Organ. 2013 Sep 1;91(9):640-9.

27. Luo D, Wilson JM, Harvel N, Liu J, Pei L, Huang S, Hawthorn L, Shi H. A systematic evaluation of miRNA:mRNA interactions involved in the migration and invasion of breast cancer cells. J Transl Med. 2013 Mar 5;11:57.

28. Ma L , Reinhardt F , Pan E , Soutschek J , Bhat B , Marcusson EG , Teruya-Feldstein J , Bell GW , Weinberg RA . Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. Nat Biotechnol. 2010;28:341–347.

29. Mark A. Lemmon, Joseph Schlessinger Cell. Cell signaling by receptor-tyrosine kinases. Author manuscript; available in PMC 2011 June 25.Published in final edited form as: Cell. 2010 June 25; 141(7): 1117–1134.

30. Miller TE, Ghoshal K, Ramaswamy B, Roy S, Datta J, Shapiro CL, Jacob S, Majumder S. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. J Biol Chem. 2008;283:29897–29903.

31. Bogdanova N, Sonja Helbig, Thilo Dörk. Hereditary breast cancer: ever more pieces to the polygenic puzzle Hered Cancer Clin Pract. 2013; 11(1): 12. Published online 2013 September 11.

32. Nevanlinna H, Bartek J. The CHEK2 gene and inherited breast cancer susceptibility. Oncogene. 2006 Sep 25;25(43):5912-9.

33. Nielsen BS, Jorgensen S, Fog JU, Sokilde R, Christensen IJ, Hansen U, et al. High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. Clin Exp Metastasis. 2011;28:27–38.

34. Pan YZ, Gao W, Yu AM. MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. Drug Metab Dispos. 2009;37(10):2112–7.

35. Petrucelli N, Daly MB, Feldman GL. Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Genet Med. 2010 May;12(5):245-59.

36. Porter, S., Clark, I. M., Kevorkian, L. and Edwards, D. R. (2005). The ADAMTS metalloproteinases. Biochem. J. 386, 15-27.

37. Radojicic J , Zaravinos A , Vrekoussis T , Kafousi M , Spandidos DA , Stathopoulos EN . MicroRNA expression analysis in triple-negative (ER, PR and Her2/neu) breast cancer.Cell Cycle. 2011;10:507–517.

38. Rajeev Singhai, Vinayak W Patil, Sanjog R Jaiswal, Shital D Patil, Mukund B Tayade, Amit V Patil N Am J. E-Cadherin as a diagnostic biomarker in breast cancer. Med Sci. 2011 May; 3(5): 227–233.

39. Ramesh Singh, Yin-Yuan Mo Cancer Biol Ther. 2013 March 1; 14(3): 201–212.

40. Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem. 2007;282:1479–1486.

41. Shanchun Guo, Mingli Liu, Ruben R. Role of Notch and its oncogenic signaling crosstalk in breast cancer Gonzalez-Perez.Biochim Biophys Acta. Author manuscript; available in PMC 2012 April 1.Published in final edited form as: Biochim Biophys Acta. 2011 April; 1815(2): 197–213.

42. Stefansson OA, Jonasson JG, Olafsdottir K, Hilmarsdottir H, Olafsdottir G, Esteller M, Johannsson OT, Eyfjord JE. CpG island hypermethylation of BRCA1 and loss of pRb as co-occurring events in basal/triple-negative breast cancer. Epigenetics. 2011 May;6(5):638-49.

43. Tardáguila M, Mira E, García-Cabezas MA, Feijoo AM, Quintela-Fandino M, Azcoitia I, et al. CX3CL1 promotes breast cancer via transactivation of the EGF pathway. Cancer Res 2013; 73:4461-73

44. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257–2261.

45. Walsh T, Casadei S, Coats KH, Swisher E, Stray SM, Higgins J, Roach KC, Mandell J, Lee MK, Ciernikova S, Foretova L, Soucek P, King MC. Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. JAMA. 2006 Mar 22;295(12):1379-88.

46. Wolfsberg, T. G., Primakoff, P., Myles, D. G., and White, J. M. (1995).J. Cell Biol. 131, 275-278.

47. Xu, G.L., Bestor, T.H., Bourc'his, D., Hsieh, C.L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J.J., and Viegas-Pequignot, E. 1999. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* 402: 187–191.

48. Yu Z, Wang C, Wang M, Li Z, Casimiro MC, Liu M, Wu K, Whittle J, Ju X, Hyslop T, McCue P, Pestell RG. A cyclin D1/microRNA 17/20 regulatory feedback loop in control of breast cancer cell proliferation. J Cell Biol. 2008 Aug 11;182(3):509-17.

49. Zhang D, Holmes WF, Wu S, Soprano DR, Soprano KJ. Retinoids and ovarian cancer. J Cell Physiol. 2000;185:1–20.