Molecular network analysis of microRNA and mRNA expression profiles in pancreatic cancer

to be submitted as Major Project in partial fulfilment of the requirement for the degree of

M. Tech

Submitted by

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Certificate

This is to certify that the M. Tech. dissertation entitled **"Molecular network analysis of microRNA and mRNA expression profiles in pancreatic cancer"**, submitted by **Priyanka Dimri** (**2K14/BIO/18**) in partial fulfilment of the requirement for the major project during M.Tech in Biomedical Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work which is to be carry out by him under my guidance.

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DECLARATION

I declare that my major project entitled **"Molecular network analysis of microRNA and mRNA expression profiles in pancreatic cancer"**, submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at "Genome Informatics Laboratory" Department of Biotechnology, as major project.

Date: 05/07/2016 Priyanka Dimri

Place: Delhi

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Molecular network analysis of microRNA and mRNA expression profiles in pancreatic cancer

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1. ABSTRACT

Micro RNAs (miRNAs) are important gene regulators. They control wide range of biological pathways and involved in several type of diseases including cancers. Generally, a single miRNA simultaneously regulates hundreds of target mRNAs called as “targetome”, hence playing a key role in fine tuning of the diverse cellular functions, such as proliferation, differentiation, metabolism and apoptosis.

The main aim of this study was to explore the underlying molecular mechanisms and potential target molecules of pancreatic adenocarcinoma. This analysis was done by determining differential gene expression in cancer using the open-source R programming environment in conjunction with the open-source Bioconductor software. The miRNA (GSE28955) expression profiles of patients with pancreatic ductal adenocarcinoma and healthy controls were downloaded from the Gene Expression Omnibus database. Gene expression profile of 4 tumor and 4 normal tissues by using aglient two colour GeneChip (microarray chip) was determined. Differentially expressed miRNA genes were identified by analyzing the microarray algorithm after data preprocessing. Functional analysis was conducted by the Database for Annotation, Visualization and Integrated Analysis. miRNA-mRNA regulation pairs were obtained in MirRecords database.

The expression of 15 miRNA are found to differ significantly of which 9 genes are down-regulated and 6 genes are up-regulated, in tumor arrays. This differential expression of genes was found using two independent statistical methods. Our study screened out some miRNAs and their target mRNAs for pancreatic ductal adenocarcinoma, which may be helpful in its diagnosis and treatment.

2. INTRODUCTION

Pancreatic cancer is one of the most fatal malignancies with increasing incidence and high mortality. Possibilities for early diagnosis are limited and there is currently no efficient therapy. Molecular markers that have been introduced into diagnosis and treatment of other solid tumors remain unreciprocated in this disease. Recent discoveries have shown that certain microRNAs (miRNAs) take part in fundamental molecular processes associated with pancreatic cancer initiation and progression including cell cycle, DNA repair, apoptosis,

invasivity, and metastasis. The mechanism involves both positive and negative regulation of expression of protooncogenes and tumor suppressor genes. Various miRNAs are expressed at different levels among normal pancreatic tissue, chronic pancreatitis, and pancreatic cancer and may therefore serve as a tool to differentiate chronic pancreatitis from early stages of cancer. Other miRNAs can indicate the probable course of the disease or determine the survival prognosis. In addition, there is a growing interest directed at the understanding of miRNA-induced molecular mechanisms. The possibility of intervention in the molecular mechanisms of miRNAs regulation could begin a new generation of pancreatic cancer therapies.

Pancreatic cancer remains one of the most aggressive malignancies characterized by an extremely low 5-year survival rate [1,2]. Due to its aggressive nature, most of patients are diagnosed in advanced stages, which limits the The possibility of intervention in the molecular mechanisms of miRNAs regulation could begin a new generation of pancreatic cancer therapies. Pancreatic cancer remains one of the most aggressive malignancies characterized by an extremely low 5-year survival rate [1,2]. Due to its aggressive nature, most of patients are diagnosed in advanced stages, which limits the potential for therapeutic intervention by the time of diagnosis and leads to a poor prognosis [3]. Many miRNAs have been reported to alter pancreatic cancer proliferation and/or migration in vitro and in vivo [12].

In this study, we integrated multiple expression profiles of mRNAs and miRNAs to construct a novel miRNA-mRNA regulatory network in pancreatic cancer. Our data may provide an important contribution to future investigations aimed at elucidating the mechanisms of pancreatic cancer. In this study, we performed a number of available software programs for the purpose of construct of most efficient miRNA-mRNA network analysis and visualization.

3. REVIEW OF LITERATTURE

3.1 Biogenesis of a mature miRNA

MicroRNAs (miRNAs) are small non-coding regulatory RNAs which are involved in regulation of gene expression in a posttranscriptional manner [13]. The first miRNA lin-4, was discovered in C.elegans 1993 [14]. Now it is widely known that miRNAs are evolutionarily conserved across diverse phyla, from nematodes to humans [15]. The human genome has more than 1000 miRNA which regulate over 60% of the total number of human mRNA [16]. Most miRNAs has derived from self-reliant miRNA genes or introns of genes that code for proteins and most of them are transcribed through RNA polymeraseII to generate pri-miRNAs . After pri-miRNAs initially processed by Drosha and DGCR8 which are located in the nucleus, the resulting miRNA precursors (pre miRNAs), then delivered to cytoplasm where the miRNA hairpin structure is processed by Dicer enzyme, that resulting in a miRNA double complex. One of these RNA strands is then loaded into the small RNA-induced silencing complex (RISC) and then subsequently directs this complex to the untranslated regions(UTRs) of the target mRNAs, inducing there repression of target protein expression [17]. miRNAs participated in many physiological processes and can regulate cellular processes such as differentiation, proliferation, and apoptosis [18,19]. Recently, miRNA revealed to play a significant role in the autoimmune processes and autoimmune diseases (ADs) such as systemic lupuse thematosus (SLE) and rheumatoid arthritis(RA) [20,21].

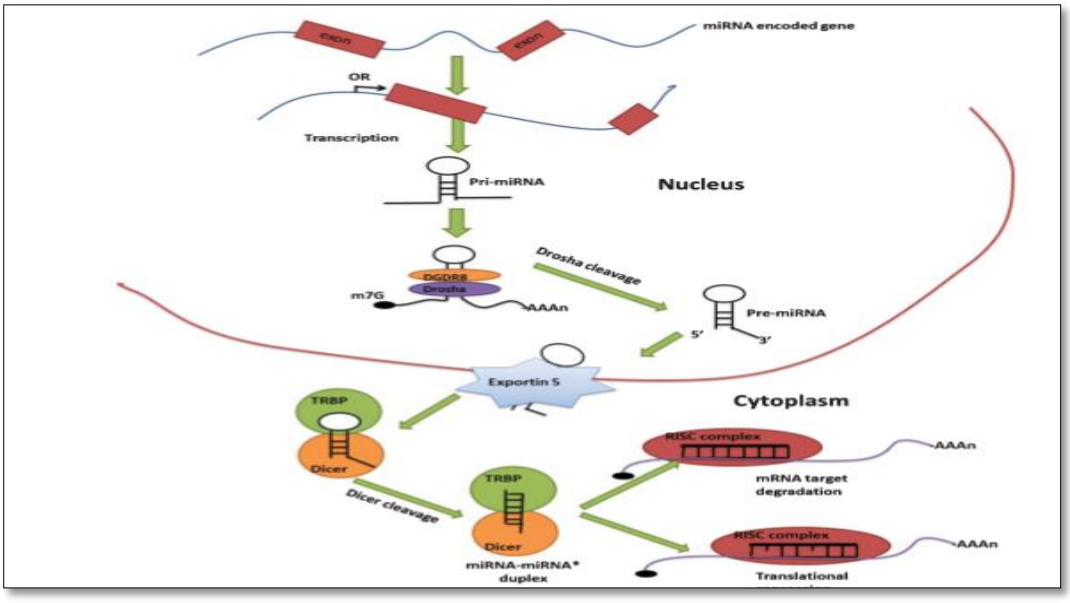

Figure1: The biogenesis of a mature miRNA: The miRNAs are produced from a precursor in an enzymatic procedure. At first step, miRNA genes are transcribed in the form of pri-miRNA. Then Drosha complex processes in the form pre-miRNA which is followed by transport into the cytoplasm by Exportin-5. In cytoplasm, Pre miRNA is processed by Dicer into miRNA duplex. One strand of miRNA duplex is loaded into RISC complex, which functions for either mRNA degradation or translational repression

3.1.1 MicroRNAs can function as tumour suppressors and oncogenes.

In normal tissues, proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability. The overall result is normal rates of cellular growth, proliferation, differentiation and cell death. The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation. A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein . The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation. The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene and lead to cancer progression. Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA ORF, open reading frame.

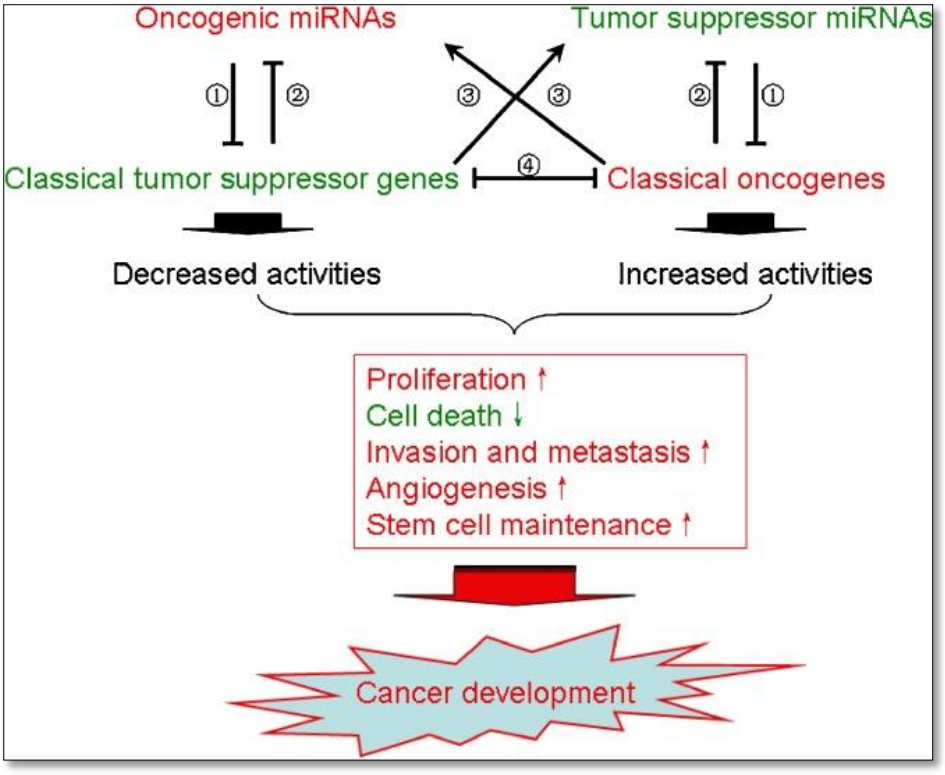

Figure 2. Functions of miRNA in cancer development and progression. Possible interactions among oncogenic and tumor suppressor miRNAs and classical genes and consequences in cancer development are illustrated. Green color, downregulated or attenuated. Red color, upregulated or enhanced. ① Translation inhibition/mRNA degradation; ② Transcriptional

inhibition (direct or indirect); ③ Transcriptional activation (direct or indirect); ④ Transcriptional inhibition/activity inhibition

3.2 PANCREATIC CANCER

Despite the recent medical advances and new diagnostic possibilities, pancreatic cancer (PC) represents a frequent malignancy with disturbingly high mortality rates. Several histology subtypes of pancreatic tumors can be distinguished. The vast majority of them are represented by pancreatic ductal adenocarcinoma (PDAC) occurring at 96.3% of cases followed by less common cystic tumors, lymphomas, and metastases from other primary tumors [22].

The development of pancreatic cancer is associated with increasing cytological atypia forming precursor lesions, which can be divided into four stages of pancreatic intraepithelial neoplasia (PanIN I–PanIN IV) [23]. However unlike other cancers, the detailed knowledge of molecular processes accompanying the pathogenesis of PC has not so far led to identification of a reliable biomarker for early detection of the disease.Diagnostic utility of standard tumor markers is also very limited. The main markers are mucin antigens CA 19-9, CA 242, CA 50, and CA 72-4, but due to their relatively low specificity and sensitivity they are used in monitoring of disease progression rather than diagnosis [24]. Thus the standard diagnosis of PC is based on imaging techniques including an initial ultrasound followed by computed tomography (CT) or magnetic resonance imaging (MRI) and finally endoscopic ultrasound (EUS) which can definitively confirm the diagnosis, especially if the examination is supplemented by a fine needle biopsy (FNB). Unfortunately, due to the absence of specific manifestation, PC is usually diagnosed at the time of nonspecific symptoms such as fatigue, weight loss, dysorexia, abdominal pain, or jaundice (caused by compression of the duodenum) when the disease has already advanced and the prognosis of patients is very poor . Only about 15% of all PC patients are diagnosed at an early stage of the disease when the tumor is operable. In these cases the tumor is surgically resected, which at present provides the only chance for cure. The chemotherapy (most often by gemcitabine) is administered after the pancreatic resection as well as in advanced inoperable stages; alternatively it is also administered in combination with radiotherapy or targeted biological therapy by erlotinib. Suppression of symptoms associated with the disease, such as biliary drainage in the duodenum or use of analgesics, is key in palliative therapy of advanced stages [25,26]. Despite development in the management of the disease, the five-year survival is only about 5% [27].

Efforts towards finding a highly sensitive and specific tool for early diagnosis of pancreatic cancer are currently leading the clinical study of this fatal disease. The role of microRNAs in malignant transformation is gradually becoming more evident [28,29] and increased emphasis is placed on finding and testing microRNAs participating in the development of pancreatic cancer in order to improve diagnosis, assess prognosis, and design new treatment options [30, 31]. MicroRNAs (miRNAs) are endogenous noncoding short RNAs (length of 21–23 nucleotides) encoded by nuclear DNA and their main function is posttranscriptional regulation of gene expression. They bind complementarily to specific sequences of messenger RNA (mRNA), which usually leads to gene silencing via translational repression or target degradation [32,33]. miRNAs play a crucial role in various developmental,

metabolic, and cellular processes including apoptosis, cell proliferation, and differentiation. Some miRNAs regulate levels of protooncogenes or tumor-suppressor genes; therefore their expression is often altered in various tumor tissues including PC (see Figure 1). These miRNAs could serve as useful tumor biomarkers [34].

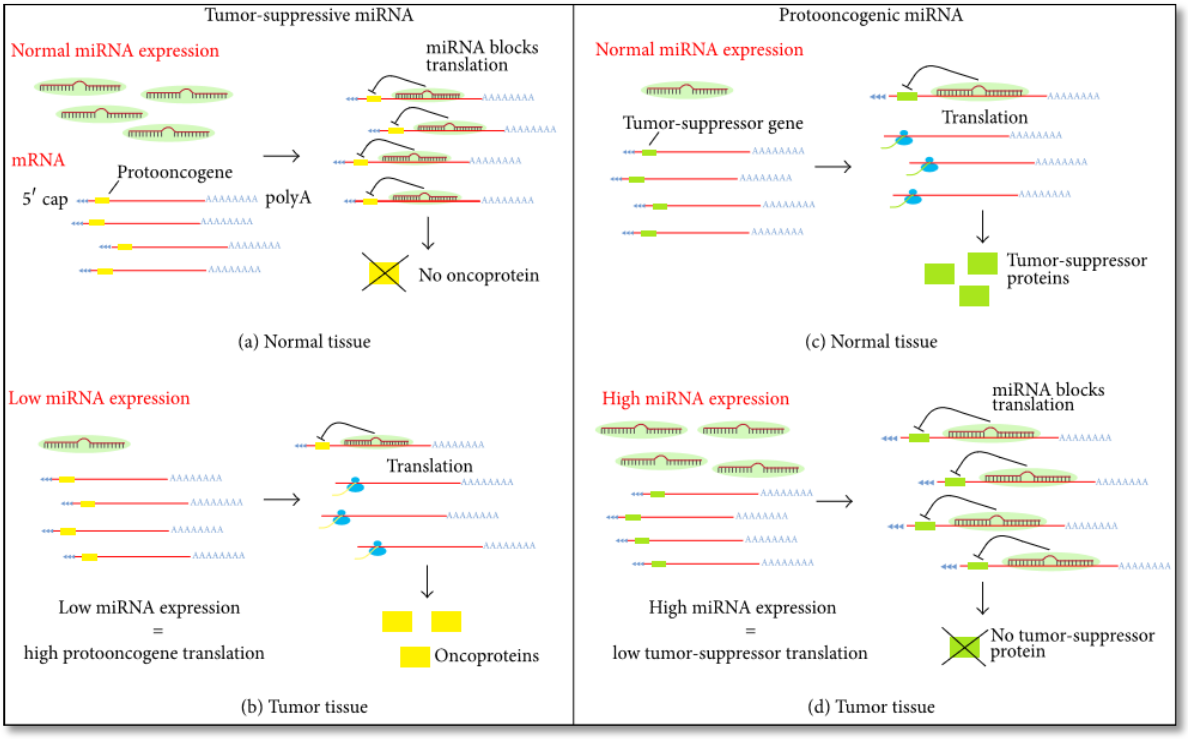

FIGURE 3: Effect of miRNAs expression on the regulation of protooncogenes and tumor-suppressor genes. Some miRNAs act as negative regulators of protooncogenes expression and therefore their role is tumor-suppression (a). In cancerous tissue, reduced levels of these tumor-suppressive miRNAs lead to increased target oncogenes promoting further tumor development (b). Other miRNAs negatively regulate expression of tumor-suppressor genes; hence, their function is (proto) oncogenic (c). In tumor tissue their increased expression results in blockage of translation of tumor-suppressors further assisting the malignant process (d).

3.2.1. Role of miRNAs in Processes of Malignant Transformation of Pancreatic Tissue

The development of malignant transformation consists of many steps that are characterized by the disruption of various cellular processes through the damage of their control mechanisms. These are mainly faulty DNA repair system, dysfunctional cell cycle checkpoints leading to excessive cell proliferation, the failure of apoptosis, loss of contact inhibition, and cellular migration into other tissues to form distant metastases. Current reports on involvement of miRNA in pathogenesis of pancreatic cancer are mostly based on in vitro studies of cell lines derived from malignant cells and therefore some of the results still have to be confirmed using in vivo models.

3.2.1.1 Cell Cycle and Proliferation

Cell cycle checkpoints and kinetics are important regulators of cell proliferation. Various studies conducted in connection with PC have shown several oncogenic miRNA negatively affecting tumor-suppressor genes that act as regulators of the cell cycle progression. One of the most frequently studied miRNAs, the miR-21, affects a tumor-suppressor PTEN (phosphatase and tensin homologue) whose protein product prevents the proliferation of tumor cells and controls the frequency of cell division [35.]. Another known miRNA that contributes to tumour cell proliferation through cell cycle deregulation in PC is miR-203, whose lowered expression leads to advancement from the G1 phase [36]. Several other miRNAs, including tumor-suppressive miR-143 [37], miR-126 [38], and let-7-d [39], regulate expression of a KRAS oncogene, which plays a crucial role by inducing abnormal cellular proliferation through mitogen-activated protein kinase (MAPK) pathways [40].

3.2.1.2 DNA Repair and Apoptosis

In a normal tissue, DNA damage triggers a wide range of cellular processes resulting in either repair of the damaged sections or a programmed cell death, apoptosis. In case of abnormal function of tumor-suppressor genes or protooncogenes, however, the DNA repair pathway as well as apoptotic cascade may be completely disrupted and the cells acquire a malignant potential.

A tumor-suppressor gene TP53 is often studied due to its major role in apoptosis and DNA repair, but it is also heavily involved in regulation of angiogenesis and cellular senescence [41]. miRNA which negatively affects apoptosis appears to be miR-203, whose main function is inhibition of the apoptotic regulator survivin (baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5)). Downregulation of miR-203 results in increased expression of survivin, which inhibits apoptosis [38].

A recent study indicates that several miRNAs may also induce apoptosis. It can be triggered by elevated levels of miR-150* and miR-630, both causing the decreased expression of transmembrane tyrosine kinase receptor IGF-1R (insulin-like growth factor 1 receptor), which has antiapoptotic properties.

3.2.1.3 Invasivity and Metastasis

The tumor cell invasivity and ability to form metastasis are an important factor that affects cancer progression. An essential step in invasivity represents the differentiation of cells through epithelial-mesenchymal transition (EMT). This means that epithelial tumor cells undergo transition to mesenchymal type, which, among others, is capable of crossing the basement membrane and entering the bloodstream. Previously the involvement of miR-143 in pancreatic cancer cell invasivity was tested and its key role in regulation of Rho GTPases signaling was demonstrated [37]. Rho GTPases are G-proteins that control many processes associated with cancer metastasis formation such as cell-cell contact or cell movement. Increased activity of Rho GTPases enhances cellular invasivity and migration. miR-143 has been shown to lower Rho GTPases activity and therefore decrease levels of miR-143, which are observed frequently in pancreatic cancer cells, leading to metastatic phenotype [37].

Another miRNA involved in the formation of metastasis is miR-155. Lower levels of mir-155 in pancreatic cancer cells were found to increase invasive behaviour [42]. The opposite effect was observed for miR-10a. The expression of miR-10a promotes metastatic formation, whereas its repression leads to inhibition of invasive behavior. miR-10a supports the ability of cell to metastasize through the suppression of homeobox transcription factors HOXA1, HOXB1, and HOXB3, which, as demonstrated, may function as metastatic suppressors [43].

3.3 Diagnostic miRNAs

The study of miRNAs as cancer biomarkers is not restricted to tumor tissues only. More recently it was shown that almost all body fluids contain miRNA [44] as a result of either passive release from necrotic or apoptotic cells or due to an active secretion by microvesicles [45]. In comparison to mRNA, whose detection in body fluids is somewhat challenging, miRNAs are stable as they are resistant to cleavage by ribonucleases and survive extreme pH and temperature conditions [46]. With the lack of reliable approaches based on imagining techniques and/or routine tumor markers, the option of detecting miRNA in peripheral body fluids, especially blood serum, has currently a considerable potential for use in clinical practice.

Among others, miR-192 is very promising showing increased levels in serum of PDAC patients compared to healthy controls with sensitivity towards cancer at 76% and specificity at 55% . Another potential biomarker is miR-18a, which occurs at high levels in tumor tissue as well as in plasma of cancer patients. In addition, miR-18a levels were found to be significantly reduced after tumor resection [47]. miRNAs for early blood-based diagnosis of pancreatic cancer has been presented [49].

In order to increase diagnostic accuracy of early stage pancreatic cancer a combination of serum CA19-9 and quantification of miR-16 [49] or combination of CA19-9, miR-16, and miR-196a may be of clinical use.. Indeed, a potential use for diagnostic purposes can be attributed to all miRNAs that are differentially expressed in PDAC compared to healthy tissue and/or chronic pancreatitis [50].

3.4 Prognostic miRNAs

Estimation of prognosis in terms of survival probability has a great significance in clinical management of pancreatic cancer. Patients often show a poor performance status and the effect of treatment is only minor. Systemic therapy or chemotherapy should therefore carefully be considered with regard to the quality of life, especially for unresectable tumors. Finding prognostic markers to assess probable course of the disease prior to treatment is therefore highly desirable. A number of literature reports are devoted to the use of miRNAs as prognostic markers. Many have demonstrated prognostic utility for miRNAs exhibiting aberrant expression in serum or in tumor tissue of PC patients.

8. Role of miRNA in Treatment Response and Potential for miRNA Therapy

Some of prognostic miRNAs also play a role in the efficacy of anticancer therapy and thus present themselves with new therapeutic possibilities. For example, it was found that nanomolar concentrations of antisense miR-21 and miR-221 oligonucleotides effectively

inhibit their targets (oncogenic miR-21 and miR-221) and thus reduce proliferation of pancreatic cancer cell lines and, along with gemcitabine, prevent their growth.
PDAC cells expressing elevated levels of miR-21 are chemoresistant to gemcitabine and reduce the efficiency of apoptosis induction [53, 54]. Addition of phosphoinositide 3-kinase inhibitors (PI3K inhibitors) and mTOR (mammalian target of rapamycin) serine/threonine protein kinase prevented the miR-21 (namely, the pre-miR-21) resistance, thus opening a way to gemcitabine-induced apoptosis. miRNAs can be targeted, for example, by lentiviral vectors (a type of retroviruses) as recently demonstrated for miR-21, wherein PDA-derived cell lines were transduced by lentiviral vector for expression of miR-21 antagonist. Inhibition of mir-21 by its antagonist led to the cessation of tumor growth and the induction of apoptosis in vitro and in vivo (animal model) [55].
Potential drug triptolide acts on pancreatic tumor tissue as an inhibitor of cell proliferation and reduces the levels of the molecular chaperone HSP70. Rather than directly affecting HSP70 it causes increase of the levels of miR-142-3p. Ectopic expression of miR-142-3p in pancreatic tumors caused by the effect of water-soluble precursor triptolide (minnelide) in vivo reduces the expression of HSP70 by direct binding to the 3′UTR region of its transcript. Therefore the miR-142-3p reduces proliferation, induces cell death, and is useable as a proper target for pancreatic cancer therapies [56]. Another study has revealed a relation of miR-142-5p to the therapeutic response to gemcitabine and further states that this miRNA is an important predictive marker in patients treated with gemcitabine after tumor resection, when its higher levels indicate a longer survival . Yet another therapy option comes from a possibility of recovery of function of miR-34a, a potent pro-apoptotic component involved in p53 mediated apoptosis, whose expression is reduced or lost in PDAC cells. .
miR-10a and miR-146a play important roles in pancreatic cancer invasivity and metastasis and represent potential targets for antimetastatic therapies. It has been shown that miR-10a promotes the metastatic behavior of PC and that its expression is regulated by retinoids . The use of retinoic acid receptor antagonists inhibits miR-10a expression and stops metastasis of PDAC cells. In contrast, miR-146a suppresses invasion of pancreatic cancer cells but its expression is lowered in PC compared with normal pancreatic tissue. Finally, use of isoflavones or DIM (3,3′-diinodolylmethane), both nontoxic natural compounds increasing the expression of miR-146a, also presents a promising approach to block the invasivity and metastases.

3.5 Different software used for constructing miRNA-mRNA network

3.5.1 Cytoscape

Cytoscape is an open source software project for integrating biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework. Although applicable to any system of molecular components and interactions, Cytoscape is most powerful when used in conjunction with large databases of protein-protein, protein-DNA, and genetic interactions that are increasingly available for humans and model organisms. Cytoscape's software Core provides basic functionality to layout and query the network; to visually integrate the network with expression profiles,

phenotypes, and other molecular states; and to link the network to databases of functional annotations. The Core is extensible through a straightforward plug-in architecture, allowing rapid development of additional computational analyses and features. Several case studies of Cytoscape plug-ins are surveyed, including a search for interaction pathways correlating with changes in gene expression, a study of protein complexes involved in cellular recovery to DNA damage, inference of a combined physical/functional interaction network for Halobacterium, and an interface to detailed stochastic/kinetic gene regulatory models

The central organizing metaphor of Cytoscape is a network graph, with molecular species represented as nodes and intermolecular interactions represented as links, that is, edges, between nodes. Cytoscape's Core software component provides basic functionality for integrating arbitrary data on the graph, a visual representation of the graph and integrated data, selection and filtering tools, and an interface to external methods implemented as plug-in. Cytoscape is a general-purpose, open-source software environment for the large scale integration of molecular interaction network data. Dynamic states on molecules and molecular interactions are handled as attributes on nodes and edges, whereas static hierarchical data, such as protein-functional ontologies, are supported by use of annotations. The Cytoscape Core handles basic features such as network layout and mapping of data attributes to visual display properties. Cytoscape plug-ins extend this core functionality and may be released under separate license agreements if desired. We have described several projects that Cytoscape has supported to-date:

•Use of the ActiveModules plug-in to identify pathways and protein complexes activated by galactose gene knockouts and by DNA damage

•Inference and attribute-based layout of a combined physical/functional interaction network for Halobacterium

•Access to stochastic/kinetic simulation tools through SBML

3.5.2 KEGG (Kyoto Encyclopedia of genes and genomics)

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information. The genomic information is stored in the GENES database, which is a collection of gene catalogs for all the completely sequenced genomes and some partial genomes with up-to-date annotation of gene functions. The higher order functional information is stored in the PATHWAY database, which contains graphical representations of cellular processes, such as metabolism, membrane transport, signal transduction and cell cycle. The PATHWAY database is supplemented by a set of ortholog group tables for the information about conserved subpathways (pathway motifs), which are often encoded by positionally coupled genes on the chromosome and which are especially useful in predicting gene functions. third database in KEGG is LIGAND for the information about chemical compounds, enzyme molecules and enzymatic reactions. KEGG provides Java graphics tools for browsing genome maps, comparing two genome maps and manipulating expression maps, as well as computational tools for sequence comparison, graph comparison and path computation. The KEGG databases are daily updated and made freely available (http://www.genome.ad.jp/kegg/).

3.5.4 R and Bioconductor

R (http://cran.at.r-project.org) is a inclusive statistical environment and programming language for professional data analysis and graphical display. The related Bioconductor project delivers many additional R packages for statistical data analysis in different life science areas, for example tools for microarray, next generation sequence and genome analysis. The R software is permitted and runs on all common operating systems.

Bioconductor is a free, open source and open development software project for the analysis and understanding of genomic data produced by wet lab experiments in molecular biology. Bioconductor is based mainly on the statistical R programming language, but does contain assistances in other programming languages. It has two releases every year that follow the biannual releases of R. At any one time there is a release version, which corresponds to the released version of R, and a development version, which corresponds to the development version of R. Most users will find the release version appropriate for their needs. In addition there are a large number of genome annotation packages available that are mainly, but not solely, oriented towards different types of microarrays.

Most Bioconductor components are distributed as R packages, which are add-on modules for R. Initially most of the Bioconductor software packages focused on the analysis of single channel Affymetrix and two or more channel cDNA/Oligo microarrays. As the project has matured, the functional scope of the software packages broadened to include the analysis of all types of genomic data, such as SAGE, sequence, or SNP data.

The broad goals of the Bioconductor are to:

1. Provide widespread access to a broad range of powerful statistical and graphical methods for the analysis of genomic data.
2. Facilitate the inclusion of biological metadata in the analysis of genomic data, e.g. literature data from PubMed, annotation data from LocusLink/Entrez.
3. Provide a common software platform that enables the rapid development and deployment of plug-able, scalable, and interoperable software.
4. Further scientific understanding by producing high-quality documentation.

4. Material and Methods

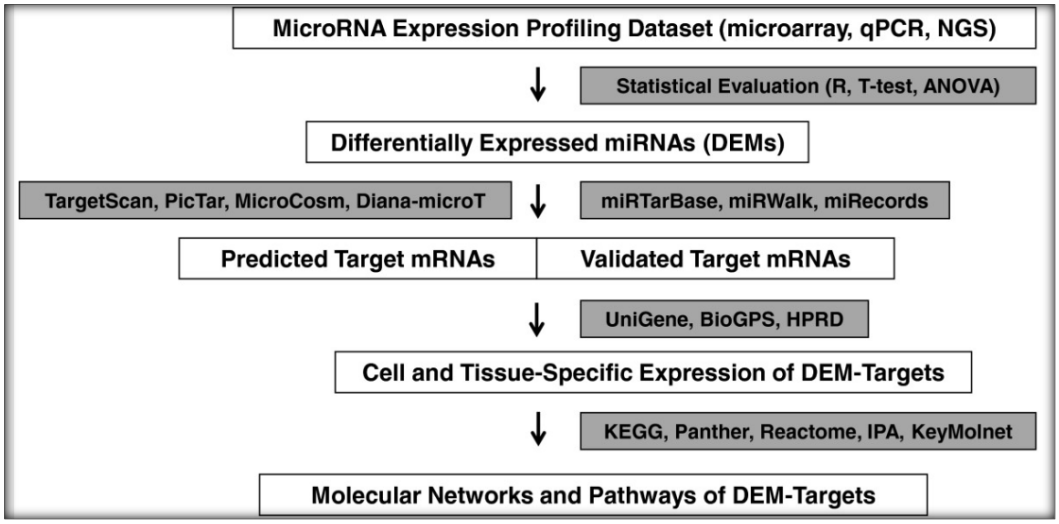

Figure 4: First, differentially expressed miRNAs (DEMs) among distinct samples and experimental conditions that are extracted from microRNA expression profiling datasets based on microarray, experiments by the standard statistical evaluation. Next, predicted targets and/or validated targets for DEMs are obtained by using target prediction programs, such as, PicTar, MicroCosm, TargetScan and Diana-microT 3.0, or searching them on databases of experimentally validated targets such as miRWalk, miRTarBase, and miRecords. Molecular networks relevant to DEM targets are identified by using pathway analysis tools, such as KEGG

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Microarray data analysis:

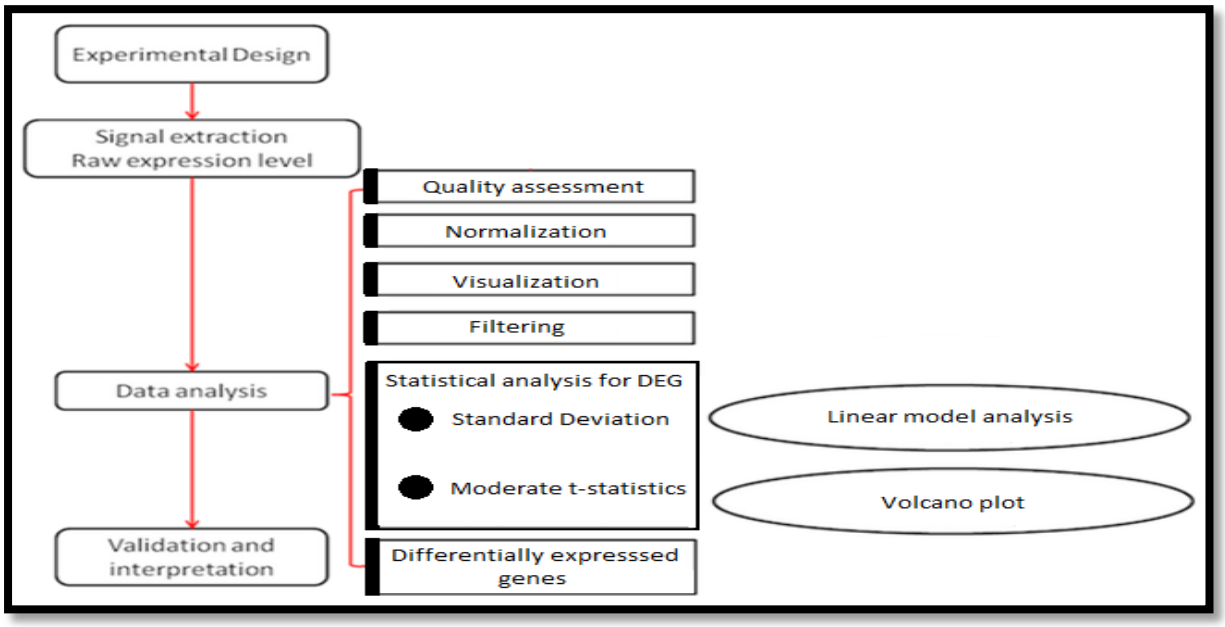

Figure 5: The basic work flow of microarray data analysis.

4.1. Data collection

The micro-array data (supplementary file i.e. raw data) was retrieve from GEO database (NCBI) with the accession number GSE28955 of paper "*A set of 72 differentially express miRNA provide a molecular signature for pancreatic cancer".*

5.1.1. Platform: GPL6955; Aglient-016436 Human miRNA Microarray 1.

4.1.2. Overall design of experiment: 16 pancreatic cancer lines and 4 normal pancreatic RNA samples were hybridized on Agilient vs1 miRNA microarrays.pool of four sample were hybridized on each slide allow normalization between slides.

4.1.3. Experiment type: Expression profiling by array

4.1.4. Organism: *Homo sapiens*

4.1.5. Supplementary file**:** GSE_RAW.tar

4.2 Data Normalization

Normalization was done using robust multi-array average (RMA) method, which transformed he raw data (having probe intensity value) into expression value of each gene.

4.4.1. This involves three steps

4.4.1.1. Background adjustment: It reduces noise and observed intensities require adjustment for accurate measurements of specific hybridization.

4.4.1.2.Normalization: Without this, it is not possible to compare measurements of hybridizations from different array due to many obscuring sources of variation i.e. different efficiencies of transcription (reverse), labeling, reagent batch effects, physical problems of the arrays and laboratory conditions.

4.4.1.3.Summarization: It is needed because all transcripts are re-presented by multiple probes. For each gene, the normalized probe intensities and background adjusted, need to be summarized into expression set with one value.

4.3 Quality Control

Quality control of aglient microarray uses simple graphical exploration methods for quality assessment, before and after the normalization.

Sample were coded with "C" and "N" where cancer sample were coded as C while normal once are coded with N

4.3.1

Boxplots of distribution (log-intensity) are plotted for comparison. The distributions of raw PM log-intensities must not identical but also not totally different. The distributions of normalized probe-set log-intensities must be comparable if they are not identical, normalization make the distributions to be even. These boxplots allows the checking of normalization step.

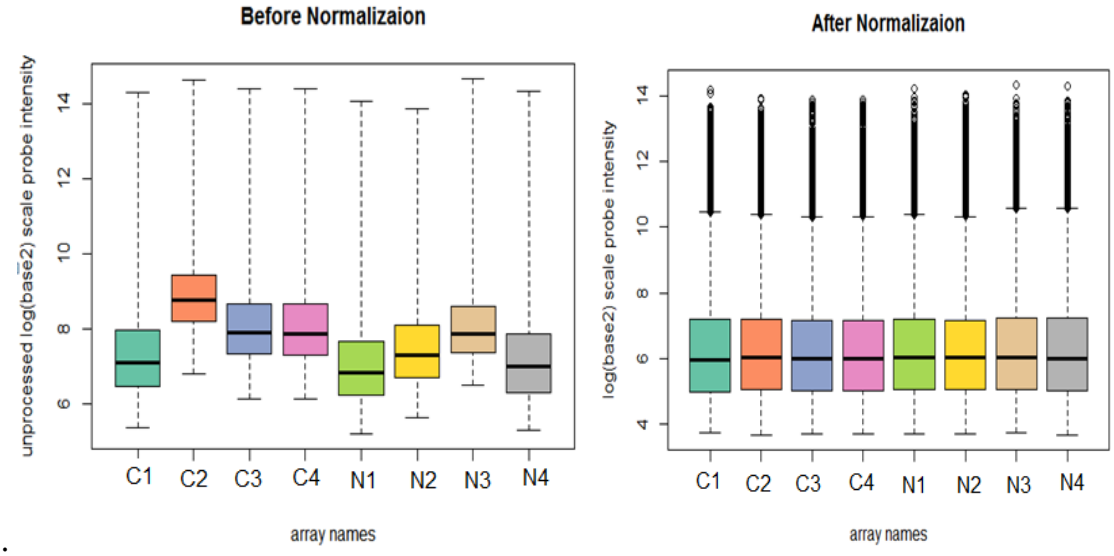

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Figure 6: Boxplot - (A) Plot of raw data i.e. before normalization and (B) plot of expressiondata set after normalization.

4.3.2. Density plot

For better comparison density plots of distribution of all arrays are superposed in a single graph, which allows identification of arrays having weird distribution. The distributions of raw PM log-intensities must not identical but still totally different. The distributions of normalized log-intensities are identical, normalization make the distributions to be even. This density plot allows the checking of normalization step.

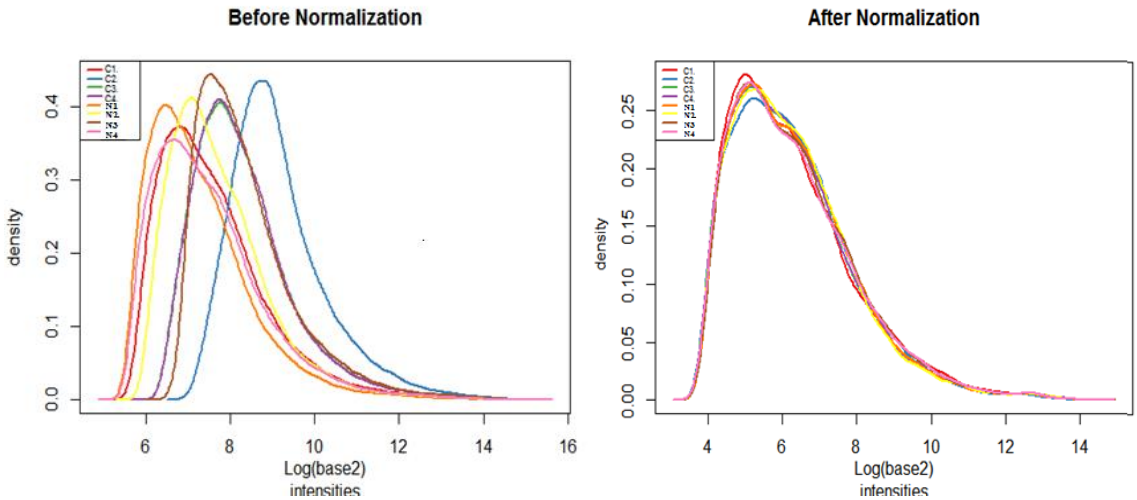

Figure7: Histogram density plot: - (A) Plot of raw data i.e. before normalization and (B) plot of expression data set after normalization

4.3.2. Checking the quality of replication

Hierarchical clustering produces a dendrogram to see whether the samples of the same group are clustered together or not.

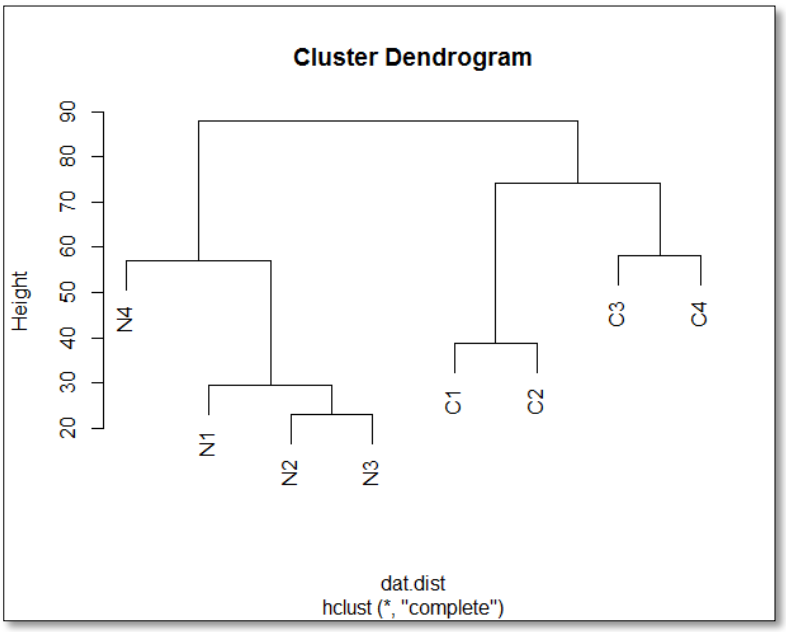

Figure 8: A Dendrogram showing hierarchical clustering of samples

4.4 Statistical analysis for differential expression

Statistical analysis microarray data is still under development. There are no strict guidelines/rules of thumb when to apply or not to apply some tests and certain other tests. Limma is one of the widely used tools (package limma) for the statistical analysis, which implements linear models used for analyzing very complicated datasets).

Linear model analysis

Those genes were determined, who so ever satisfies two criteria useful to biologists: (i) high (absolute) log-fold changes and, (ii) low probability values Hence, Smyth's moderated t-statistic (with its corresponding P-value) was chosen, when the number of arrays are small it provides more stable inferences.

'limma' package in Bioconductor provides a moderated t-statistic. Statistical significance of the genes are precisely estimated by empirical Bayes. The analysis was done by using the lmFit()command followed by eBayes(). The lmFit() get the design matrix, and a data matrix. The analysis was carried out using both the original un-filtered data and the filtered data.

4.5 Volcano plot and heatmap for differential expression

A volcano plot was constructed with the moderated t-statistics. The objects fit.eBayes$t[,2] and fit.eBayes$p.value[,2], contains moderated t-statistics and their corresponding P-values respectively.
In this plot, the 70 miRNA having the lowest P-values are shown in circles (red). Similarly, the 70 genes with the highest log-fold changes are shown as diamonds (blue). Those genes that possess low probability values and high log-fold changes are of interest to biologists. Figure: 13 graphically displays the thirteen miRNA that satisfy this above criteria

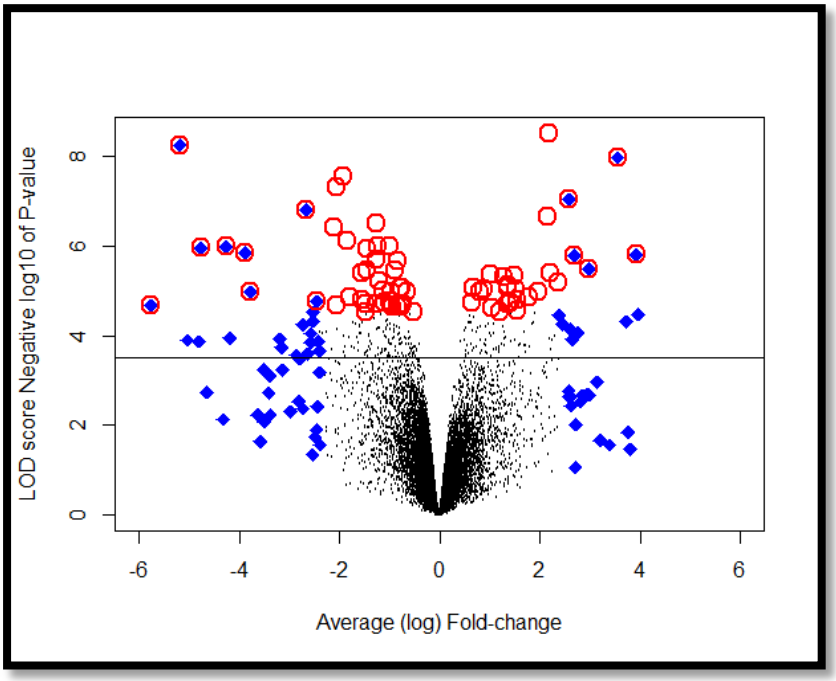

Figure 9: Volcano Plot with moderated t-statistics. Diamonds within circles (Intersects) show interesting miRNA from a biological standpoint.

4.5.2 Heat map of differentially expressed genes
A heatmap is a 2D, colored grid that displays the expression data in the form of a rectangular matrix. The gray scales, of rectangles are determined by the matrix with corresponding entry.

The four control and four tumor arrays cluster tightly in Figure 16, indicating that these arrays on the thirteen intersecting miRNA had similar expression. Dark grey shades are showing down-regulation of the miRNA.
There are 7 common genes in obtained by two different statistical testing i.e. by linear model analysis (filtered dataset) and volcano plot/heatmap (unfiltered dataset) respectively

Figure: 10, shows a heatmap of the thirteen intersecting miRNA with simultaneously low probability values and high fold changes that were displayed in the volcano plot (Figure 9).

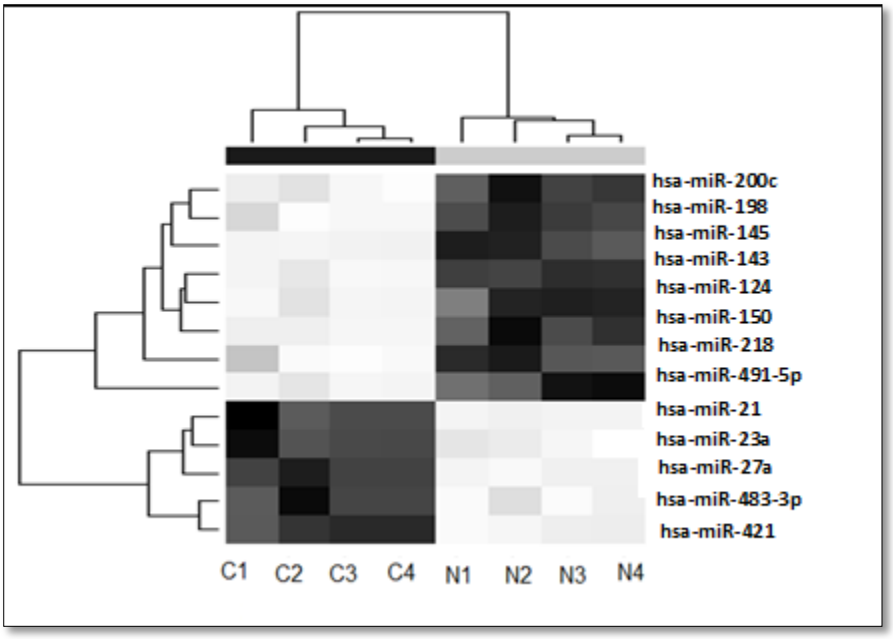

Figure 10: Heatmap of intersecting miRNA.

4.6 **Molecular network analysis of microRNA target mRNAs**

To identify the biologically relevant molecular networks and pathways extracted from high throughput data. we can analyze them by using bioinformatics tools for analyzing molecular interaction based on comprehensive knowledgebase.
Software tools that we are going to use are

- Kyoto Encyclopedia of genes and genomics (KEGG) (www.kegg.gp): (KEGG) pathway analysis were employed to understand the functions and pathways of miRNA target genes. KEGG includes manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases.Currently, KEGG contains 198,560 pathways generated from 428 reference pathways. When importing of Entrez Gene IDs into the Functional Annotation tool of Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (david.abcc.ncifcrf.gov), DAVID identifies the most relevant KEGG pathway and gene ontology (GO) categories, composed of the genes enriched in the given set, followed by an output of statistical significance evaluated by the modified Fisher's exact test.

- Cytoscape: It is an open source software project for integrating biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework. Although applicable to any system of molecular components and interactions, Cytoscape is most powerful when used in conjunction with large databases of protein-protein, protein-DNA, and genetic interactions that are increasingly available for humans and model organisms

4.7 Functional annotation of DEGs

Functional annotation of the DEGs was performed using the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/, DAVID).

To gain insights into the biological functions of these miRNA target genes, we performed the Gene Ontology (GO) classification. To detect the potential pathway of miRNA target genes, we also performed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. KEGG pathway database is a recognized and comprehensive database including all kinds of biochemistry pathways.

5. RESULTS

5.1 Differentially expressed miRNA from linear model analysis

Top ten genes were identified by statistical testing of filtered (standard deviation > 2) expression set (Table: 1). The first column of the table is the miRNA name., The next column (logFC) shows a fold (log2-based) change between the groups. Down-regulated genes are shown with negative sign, and positive values are indicating up-regulation. The t is the moderated t-statistics and the B is the log-odds showing that the gene is differentially expressed. The p-value columns contain the p-value corrected for multiple comparisons (adj.P.Val) and raw p-value (P.Value) using false discovery rate.

Table:1 Output of statistical testing of filtered data based on slandered deviation.

miRNA	logFC	T	P.Value	adj.P.Val	B
hsa-Let-7a	5.18534	9.667457	3.94E-06	0.000138	4.291046
hsa-miR-21	-1.77366	7.873987	2.17E-05	0.000354	2.976725
hsa-miR-23a	5.7582	7.31089	3.95E-05	0.000354	2.485026
hsa-miR-27a	-2.263704	7.288207	4.05E-05	0.000354	2.464325
hsa-miR-124	-3.9184	-6.74579	7.45E-05	0.000448	1.947529
hsa-miR-198	-3.880069	6.719526	7.68E-05	0.000448	1.92141
hsa-miR-224	3.774332	6.102771	0.000161	0.000791	1.277644
hsa-miR-301a	-2.039263	5.967007	0.000191	0.000791	1.127829
hsa-miR-486	-3.96725	-5.91516	0.000203	0.000791	1.069825
hsa-miR-491-5p	4.809125	5.829476	0.000227	0.000794	0.972989

5.2 miRNA target prediction

Table 2: mRNA targets of differentially expressed miRNA in pancreatic ductal carcinoma

miRNA	differentially expression	Validated target gene	Function, target gene	Reference
miR-21	Upregulated	PDCD4, BTG2,PDCD4, TIMP3,PDCD2, NEDD4L,PTEN, RECK,BCL2	Tumor suppressor	57
miR-23a	Upregulated	SORBS2, SMAD4	Tumor suppressor	57
miR-27a	Upregulated	BTG2, BNIP3, SORBS2	Tumor suppressor	57
miR-224	Upregulated	CD40	Antimetastatic	58
miR-421	Upregulated	SMAD4	Tumor suppressor	59
miR-483-3p	Upregulated	SMAD4	Tumor suppressor	60
miR-200c	Downregulated	MUC4, MUC16,ZEB1	Invasion/metastasis	61
miR-143	Downregulated	COX2,KRAS, ARHGEF1, ARHGEF2	Invasion/metastasis	62
miR-145	Downregulate	KRAS, PREB1	Oncogenes	63
miR-198	Downregulate	MSLN, PBX1, VCP	Various	-
miR-124	Downregulate	RAC1	Ras supergene family	64
miR-150	Downregulate	MUC4	Invasion/metastasis	65
miR-218	Downregulate	VOPP1, UGT8	Metastasis	66
miR-491-5p	Downregulate	TP53, BAD	Antiapoptosis	-
Let-7a	Downregulated	RRM2, KRAS	Ribonucleotide reductase	67

5.3 miRNA- mRNA network analysis

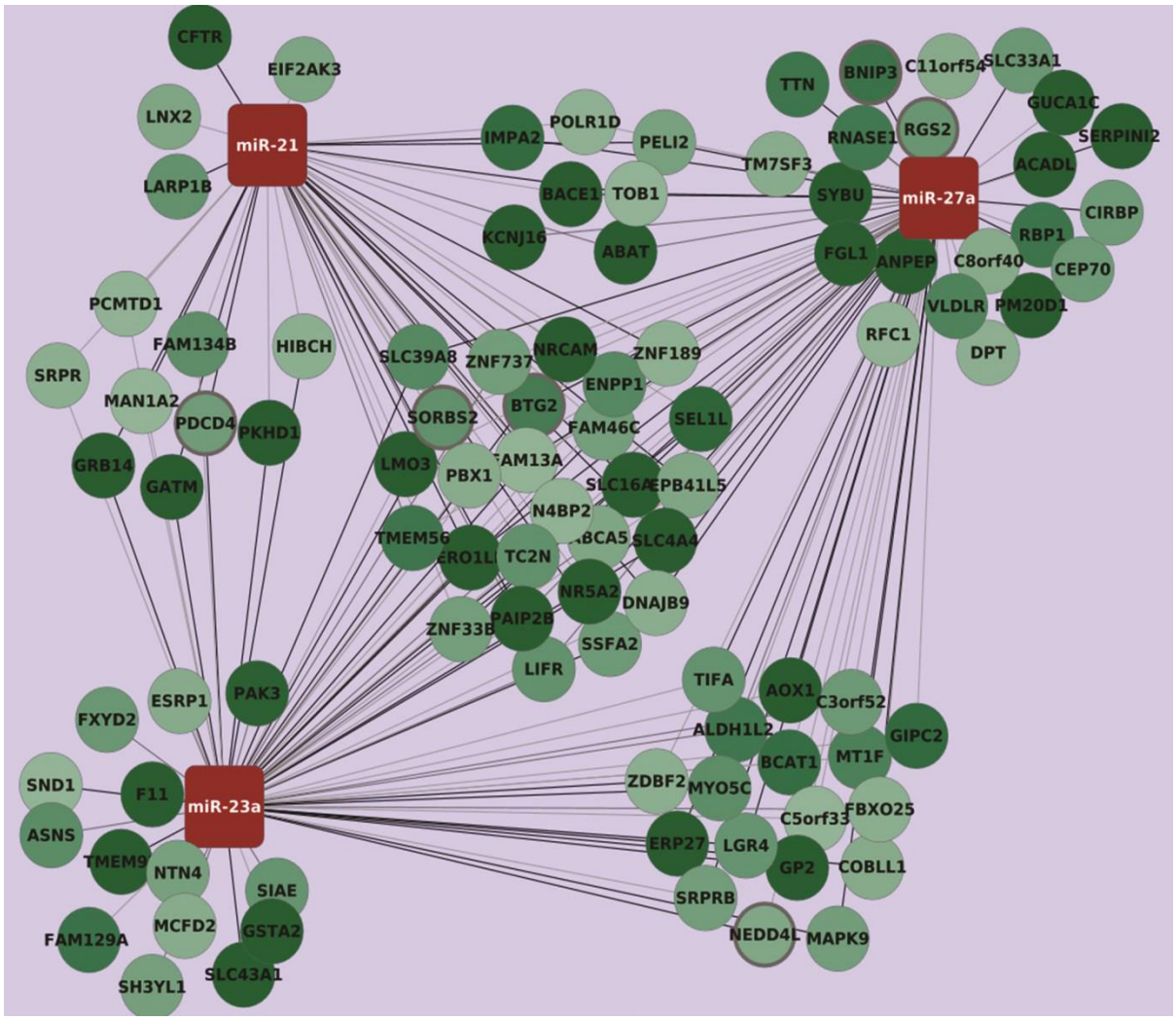

Figure 11: Interaction network for miR-21, miR-23a and miR-27a in PDAC.

Cytoscape visualisation software was used to display putative functional interaction network for miR-21/miR-23a/miR-27a. Nodes: [red] PDAC up-regulated miR-21, miR-23a and miR-27a; [green] PDAC top down-regulated genes

5.4 Functional annotation

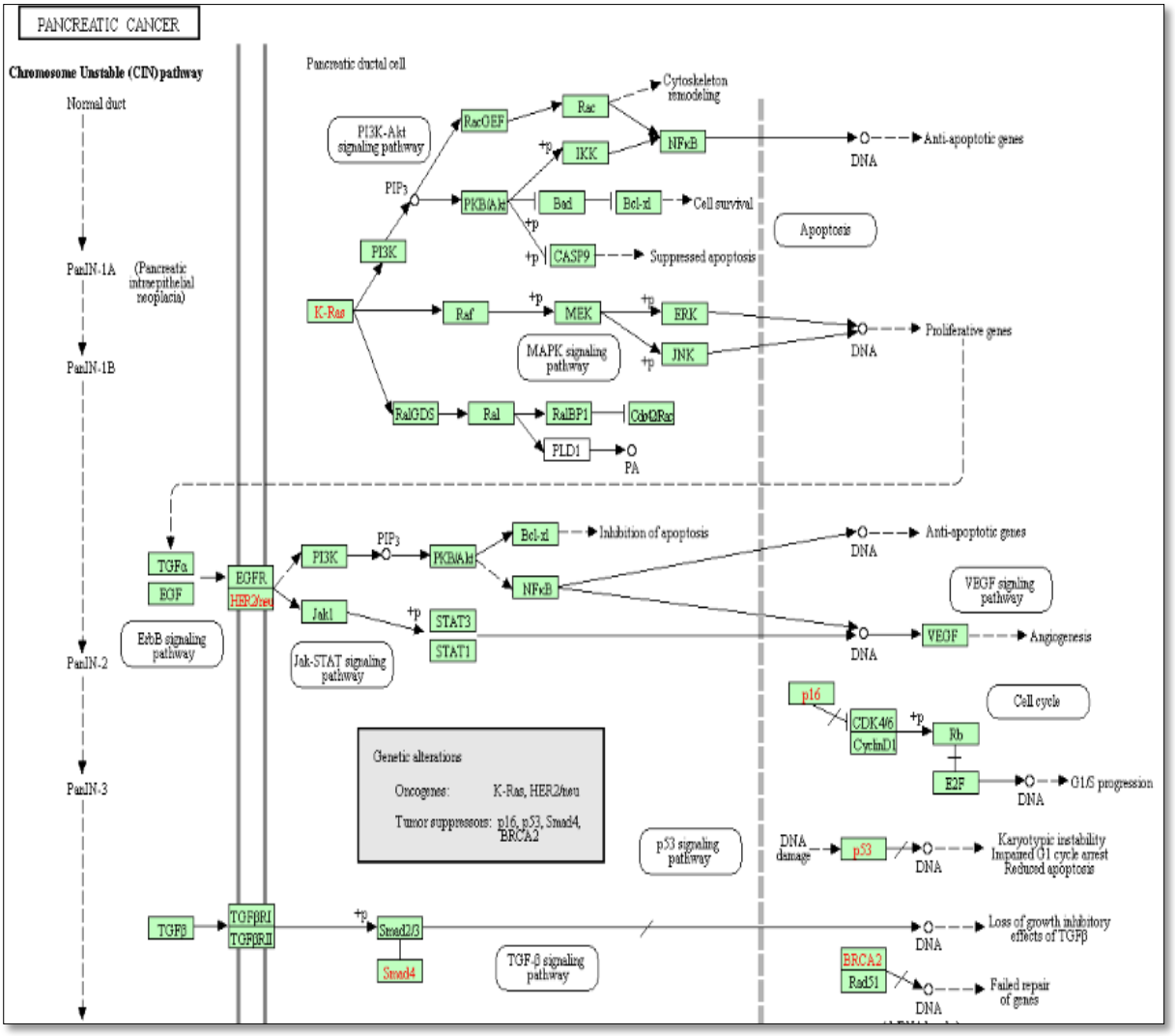

Figure 12: The set of 40 targets for 15 miRNAs in pancreatic cells versus normal cells (GSE28995) were imported into the Functional Annotation tool of DAVID to identify relevant KEGG pathways. Pancreatic cancer specific pathway is shown in figure. The orange nodes represent the genes theoretically upregulated in pancreatic cells.

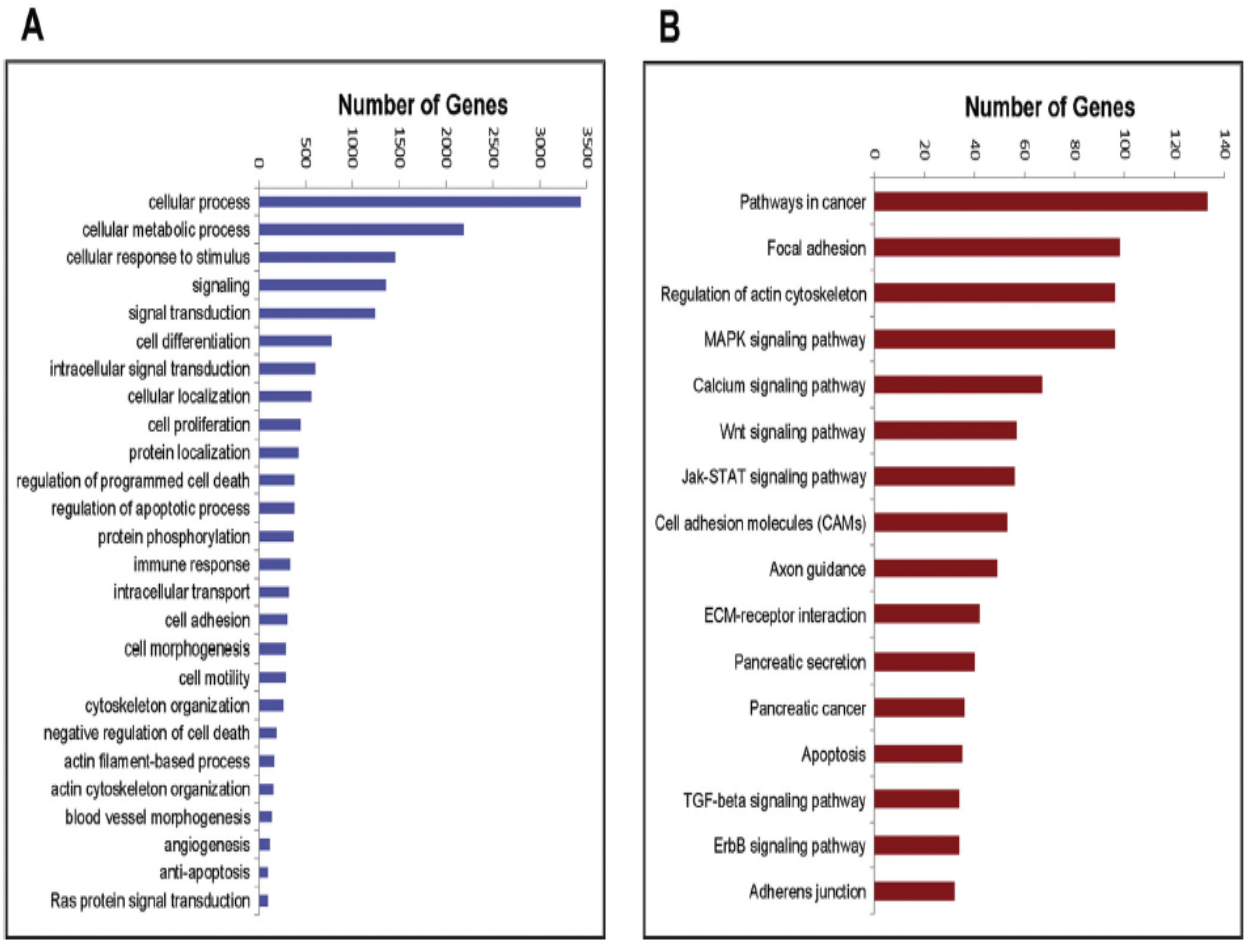

Figure 13: The significantly enriched functional annotation of differentially expressed miRNA target genes. A, The top enriched GO categories for biological process. B, The top enriched KEGG pathways.

6. DISSCUSSION AND CONCLUSION

In this microarray analysis expression profile of samples were used to identify DEG using 8 samples four each of normal and tumor. Raw data was downloaded from GEO database, then the data was read using ReadAffy() function, then the data was normalized using RMA method and the expression values obtained from this was further proceeded using two different statistical method. One method uses stander deviation cutoff over 2 with linear fit model analysis. The other method uses mean and differences (–log10) of normalized expression values of genes to make volcano plot on the bases of their expression value using modern t-statistics with p.value <= 0.001.

The expression of 15 miRNA are found to differ significantly of which 9 genes are down-regulated and 6 genes are up-regulated, in tumor arrays. This differential expression of genes was found using two independent statistical methods.13 miRNA have been previously demonstrated to be involved in have role in pancreatic cancers and 2 miRNAs miR-491-5p and miR-198 are not found in previous study, so these can be potential biomarker and experimential validation needed to be done.

We found that the most relevant pathological event in the whole human miRNA targetome in 'cancer', supporting the general view that the human miRNAome plays a specialized role in regulation of oncogenesis. Many miRNA gene loci are clustered in cancer-associated genomic regions. by drawing connecting networks, it leads us to the main passkeys in regard to the relationship between miRNAs and mRNAs in occurrence or development of cancer under certain conditions. We demonstrate bioinformatics approach to study the key communication between mRNAs and miRNAs.. tumour suppressor network that appears to be targeted by the triple miRNA combination (miR-21/23a/27a) in PDAC, many miRNA mRNA interactions remain to be validated. There can be two possibilities: firstly, the triple miRNA combination is involved in disease progression, by causing increased proliferation, invasion, motility, cell-survival, stemness and other properties required of cells undergoing metastasis, and/or secondly, up-regulation of these miRNAs is able to provide considerable resistance to known PDAC anti-cancer drugs. Therefore this study provides new opportunities for studying novel molecular pathways of pancreatic cancer pathogenesis and for developing new therapeutic approaches.

In this study, we applied the workflow of molecular network analysis of miRNA targetome to datasets of pancreatic cancer . The results supported the view that miRNAs act as a central regulator of oncogenesis. Therefore, the miRNA-based therapy designed to target cancer pathways might provide a rational and effective approach to treating and preventing pancreatic cancer.

7. FUTURE PERSPECTIVE

On growing evidence has suggest the important role of miRNA in development and causes of several diseases. Identifying miRNA functions will reveal further insight into the causes of fatal diseases. Pancreatic cancer is a complex disease with highly remarkable heterogeneity caused by the intricated interplay of various genetic and environmental factors. Molecular network analysis of miRNA-targetome network will enable us to characterize the most relevant networks and pathway involved in the miRNA inSilico that will be providing different views to elucidate the complex regulatory mechanism of miRNA and their relationship with target gene as well as other regulators such as transcription factors (TF) which will be helpful in developing molecular markers and pathways relevant to MS heterogeneity, and promotes us to identify the network-based effective drug targets for personalized therapy of pancreatic cancer. Could also be useful in developing new biomarkers and new therapeutic approaches

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