MYC-microRNA -transcript regulatory Crosstalks in STS oncogenesis

A THESIS SUBMITTED

FOR THE PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DIGREE OF MASTER OF SCIENCE

IN

LIFE SCIENCE

SUBMITTED TO

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

BY

SUBHASHREE PRIYADARSINI

ROLL NO.413LS2047

UNDER THE SUPERVISION

OF

DR.BIBEKANAND MALLICK

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA-769008, ODISHA, INDIA

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA
राष्ट्रीय प्रौद्योगिकी संस्थान, राउरकेला

Dr. Bibekanand Mallick
Assistant Professor

RNAi & Functional Genomics Lab.
Department of Life Science
National Institute of Technology
(*Ministry of H.R.D, Govt. Of India*)
Rourkela - 769 008, Odisha, India

Telephone: +91-661-246 2685 (O)
E-mails: vivek.iitian@gmail.com, mallickb@nitrkl.ac.in
Homepage: http://vvekslab.in

Date: 11. 05. 2015

CERTIFICATE

This is to certify that the thesis entitled "**MYC-microRNA-transcript regulatory Crosstalks in STS oncogenesis**" submitted by **Ms. Subhashree Priyadarsini** (Roll No. 413LS2047) in partial fulfillment 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.

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

(Dr. Bibekanand Mallick)

DEDICATED TO

MY FAMILY AND BELOVED

ONES.....

ACKNOWLEDGEMENTS

I avail this opportunity to express my heartfelt thanks to helping hands behind this work. It is still great at this juncture to recall al the faces and spirits on the form of teachers, friends, near and dear ones. I would consider this work nothing more than incomplete without attending to the task of acknowledging the overwhelming help I received during this research project.

I express my profound sense of gratitude to my supervisor, Dr. Bibekanand Mallick, Department of Life Science, NIT Rourkela for this valuable guidance, kind cooperation and providing all the facilities to work in this department.

I acknowledge with sincere thanks to Dr. Sujit Kumar Bhutia (HOD) and all the faculties of Department Of Life Science, for providing me the necessary opportunities for the completion of my project.

I am overwhelmed with sincere feelings of indebtedness to Devyani Samantarrai, Mousumi Sahu, Bendanta P Mohanty, Jyoti Roy, Mrs. Garima Singh and Chandra Bhushan, Ph.D. Scholars of the Department of Life Science, NIT-Rourkela for their guidance and constant encouragement which helped to sustain my interest and thereby accomplishing this task.

My unfold sense of gratitude and profound indebtedness to my beloved parents for encouragement, love and affection towards me.

I express my special word of thanks to almighty, my friends and lab mates for their intangible support and care.

Date: SUBHASHREE PRIYADARSINI

LIST OF FIGURES

Figure no	Name	Page no
1	Benign tumors and malignant tumors	4
2	Different cancer types	5
3	Different stages of metastasis	8
4	microRNA maturation and function	9
5	General Workflow to find out DE novel genes in STS	
6	Mapping myc regulated miRNA with known miRNAs in STS	22
7	myc regulating miRNA reported in STS	22
8	myc-miRNA regulated network in STS	23
9	Hierarchical clustering of Sarcoma and normal fat samples	24
10	Comparison of myc regulated miRNA targets with DE genes in STS	25
11	Comparison of 12 DE myc-miRNA-targets with DE genes known in STS	25
12	Representing let-7b→ target Interactions	27
13	Representing let-7g→target interactions	27
14	Cycle temperature and time for qRT-PCR	29
15	Melting temperature curve of COL1A2 and RRM2 with respect to ACTB.	29
16	Result of qRT-PCR analysis of COL1A2 and RRM2 gene	30

LIST OF TABLES

Number	Name	Page no
1	Subtypes of sarcoma and their origin	6
2	Number of samples taken for mRNA and miRNA expression analysis	13
3	Primer name and sequence with length and its amplicon size	18
4	Temperature cycle for qRT-PCR	19
5	List of myc regulated miRNAs extracted from TransmiR	20
6	Significant gene ontology terms related to six novel altered genes	26

ABBREVIATIONS

ACTB β	**Actin**
µ	**Micro**
:	**Ratio**
%	**Percentage**
µl	**Micro liter**
PCR	**Polymerase Chain Reaction**
cDNA	**Complimentary DNA**
mRNA	**Messenger RNA**
DEPC	**Diethyl Pyrocarbonate**
Fig.	**Figure**
qRT-PCR	**Quantitative real time PCR**
miRNA	**microRNA**
et al.	**And others**
STS	**Soft tissue sarcoma**
DE	**Differentially expressed**

CONTENTS

Serial no.	Particulars	Page no
1	Introduction	1-3
2	Review of literature	4-10
3	Objectives	11
4	Materials and methods	12-19
5	Results and Discussion	20-30
6	Conclusion	31
7	Future Prospective	32
8	References	33-34

ABSTRACT:

MicroRNAs (miRNA) are a family of small non-coding RNAs (ncRNAs) having capability of regulating gene expression at the post-transcriptional level. MicroRNAs are reported to have role in various biological processes like development of embryo, cell proliferation, differentiation of cells into other forms, cell death, developmental timing, etc. The role of miRNAs as tumor suppressors/oncomiR has already been reported in many cancers. These ncRNAs can alter cellular metabolism by directly interacting with target gene transcripts. Therefore, miRNA profiling is used for detection and progression of cancer in various field of cancer biology such as proliferation, programmed cell death, cancer cell migration and angiogenesis. Our study is mainly based on identification of novel differentially expressed targets of MYC regulated miRNAs that are suspected to play role in fibrosarcoma from microarray data analysis followed by qRT-PCR for experimental validation of selected pair of mRNA (COL1A2 and RRM2). From qRT-PCR study in HT-1080 cell lines, we found that COL1A2 and RRM2 are over-expressed in cancer system and their targeting miRNAs are down-regulated. The altered expression of COL1A2 and RRM2 might be due to down-regulation of hsa-let-7g and hsa-let-7b as these miRNAs have target sites within 3/UTR of COL1A2 and RRM2. We can assume that these target genes and their miRNAs might be playing a role in metastatic in STS by altering the expression of the corresponding mRNA through RNA interference mechanism, which can be a probable therapeutic target in fibrosarcoma.

Keywords: miRNAs, angiogenesis, fibrosarcoma, metastasis, ncRNAs

INTRODUCTION

Living organisms are comprised of billions of cells. Among these, the undifferentiated cells known as stem cells possess unique potential to differentiate into various types of cells while maintaining their self-renewal property. When the body requires cells, stem cells start to divide to produce a number of cells in order to fulfil the requirement of the body. But when these cells keep dividing uncontrollably they create a mass of tissue. Likewise, disorders in somatic cells growth also lead to unnecessary tissue accumulation. This mass of additional tissue is called as tumor that can be formed in all kinds of tissue and can be of benign or malignant in nature. Tumors that are characterized by limited growth are called as benign tumors. These tumors are found to be non-motile i.e. do not spread to different parts of the body. Another type of tumors which are motile and invade nearby tissues and spread to different body parts is called as malignant tumor. Malignant tumors commonly lead to onset of cancer. Cancer is a complex disease that affects millions of people and also causes many deaths over the years. The cancer etiology includes an array of alterations at genetic and epigenetic levels and is characterized by uncontrolled cell growth. At the genetic level, DNA damage has been studied as the main cause of cancer. A normal cell (stem cell/somatic cell) employs numerous mechanisms to repair DNA damage in order to maintain proper balance in cellular integrity and tissue homeostasis. Whereas in case of cancerous cells, DNA damage is not properly repaired causing duplication of undesirable cells instead of being eliminated. In this way, cancer is a multifactor malady created because of molecular modifications in the genome of substantial (stem/somatic) cells.

Oncogenesis or tumorogenesis is the process of formation of cancer and caused by interplay between genetic/epigenetic and environmental factors. There are specific traits which are called hallmarks that have the ability to convert normal cells to cancerous cells. These hallmarks are i) ability to stimulate their own growth and proliferation, ii) ability to withstand the inhibitory signals iii) ability to resist programmed cell death (apoptosis) of their own, iv) ability to induce formation of blood vessels (angiogenesis) to supply nutrients to tumors, v) ability to multiply forever, vi) capability to attack local tissue and spread to distant sites known as. Aside from these six hallmarks, few hallmarks have been added recently to the list. These are- i) unusual metabolic pathways, ii) capacity to avoid the immune system, iii) chromosome abnormalities and unstable DNA, and iv) inflammation Metastasis is the spreading of malignant tumor/cancer. Cancers can be classified depending on the tissue of origin. These are – (i) Carcinoma- cancer of epithelial tissue like skin or tissues that cover internal organs. The subtypes of this category include adeno-carcinoma, carcinoma of basal

cell, carcinoma of squamous cell and transitional cell carcinoma etc., (ii) Leukemia- cancer of blood cells or blood-forming cells such as bone marrow, (iii) Lymphoma- cancer of immune cells, (iv) Sarcoma- cancer originated from mesenchymal cells, (v) CNS cancer- cancer of central nervous system i.e. cancer that originate in the tissue of the brain and spinal cord.

For the metastatic spread of malignant tumors, the supply of vascular network is very important. Angiogenesis and lymhangiogenesis are the processes by which new blood vessels are formed and lymphatic system is established respectively. Both of the above processes have an important role in the growth and maintenance of cancer cells by providing nutrients, oxygen and removing waste products. These phenomena are currently getting special consideration in the field of neoplastic vascularisation. Without the vascular network, the tumors may get to be necrotic and apoptotic. Vascularisation is essentially a four stage process- initially, there will be event of local injury to the basement membranes, then angiogenic component movement creating endothelial cells activation followed by multiplication and stabilization and lastly the angiogenic components keep on affecting the angiogenesis process (Nishida et al., 2006). Metastasis is surely known in diseases of epithelial origin; however it is not clearly seen in tumors of mesenchymal source (sarcoma).

Sarcomas are arised from bone, cartilage or connective tissues and called as mesenchymal malignancies (D'Angelo et al.). World Health Organization defined Adult fibro sarcoma as malignant neoplasm composed of fibroblasts having variable collagen production (Zambo and VeselÃ½). It is also defined by Schultze et al as a tumor originating from mesenchymal cell which is having malignant fibroblasts along with collagen (Schultze et al., 1997). Sarcomas can occur in the form of soft-tissue mass or as a primary or secondary tumor of bone. It is characterized by Schultze et al as a tumor beginning from mesenchymal cell which is made out of malignant fibroblasts with a collagen foundation (Schultze et al., 1997).
There are generally two forms of this disease:

i. Infantile or congenital fibrosarcoma

It is a sort of soft tissue sarcoma (STS) normally seen in youngsters below one year of age (Ainsworth et al.) It displays as a quickly developing mass during childbirth or not long after conception. This type of fibrosarcoma is normally moderate developing, furthermore has a tendency to be more benign than fibrosarcoma found in adults.

ii. Adult form fibrosarcoma

This type of malady can happen in teenagers, generally between 10 to 15 years. It is more dangerous than the infantile form and includes more unpredictable complex treatment.

Despite the fact that the exact cause of soft tissue tumors and fibrosarcoma is not entirely known, certain studies demonstrate that hereditary modifications might be playing a role in these abnormal cellular functioning (Helman and Meltzer, 2003)Constrained studies have additionally demonstrated a conceivable connection between STS and the advancement of other sort of malignancy. A chromosomal alteration has additionally been found in some fibrosarcoma (Sreekantaiah et al., 1994). In spite of the fact that sarcoma may emerge from any place, the most common primary site is considered as the extremity. During metastasis the most well-known site to which the fibrosarcoma spreads is the lungs. As system for controlling metastasis are inadequately seen so it is important to know about the molecular mechanism involved in primary tumor cell propagation and its spread to distance destinations and consequently to distinguish new molecular targets for tumor treatments.

MicroRNAs (miRNAs) are group of small ncRNAs having potential role in cancer biology including STS. miRNAs are mainly 20-24 nucleotides long. These identify their mRNA targets on the basis of sequence complementarity principally to 3/UTR areas and hinder protein interpretation or translation. (Schultze et al., 1997) through mRNA debasement or translational hindrance. Therefore up-regulation and down-regulation of miRNA has been seen to assume a part in disease movement. 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 distinguished in mammals, some of which are communicated in a tissue specific and developmental stage specific way. Lately much advancement has been done in discovery of this regulatory RNA phenomenon.

In normal cell growth, miRNAs play key roles, yet practically it may act either as oncogenes or tumor silencers (Zhang et al., 2007) targeting analogous or tumor suppressor genes. miRNA have the ability to target transcripts directly and impact cell physiology which is found to be involved in cancer etiology.

In this work, we planned to find out efficient regulations of few selected genes (COL1A2 and RRM2) at epigenetic level and that might be associated with soft tissue sarcoma oncogenesis. To fulfil our proposed aim, mRNAs were studied which are significantly altered in STS and experimental validation of selected mRNAs was done by qPCR to predict their correlation of regulations in normal cell line (WI-38) and STS cell line (HT-1080).

REVIEW AND LITERATURE

Cancer

Cancer refers to the excess growth and proliferation of abnormal cells that invades different tissues because of the DNA injury or mutation. Blood vascular system and lymph nodes helps cancer cells to spread throughout the body. The unwanted additional cells aggregate to create mass of cell that is known as tumor. But all type of tumours are not dangerous. According to the nature of tumour they will be classified into 2 types (Figure 1)

i. **Benign tumors**

Noncancerous tumors are called as benign tumors. Benign tumour cells do not spread to different parts of the body. They can be removed from the body easily.

ii. **Malignant tumors**

Cancerous tumors are malignant in nature. These cells affect nearby tissues and migrate to other body parts. The process of spreading of tumor from one site of body to other part is known as metastasis. These tumors have nature of converting themselves into distinctive cancer. There are more than 100 types of tumors found which influence distinctive parts of body. All tumors are unique by its own effects, signs and symptoms and strategies for treatment.

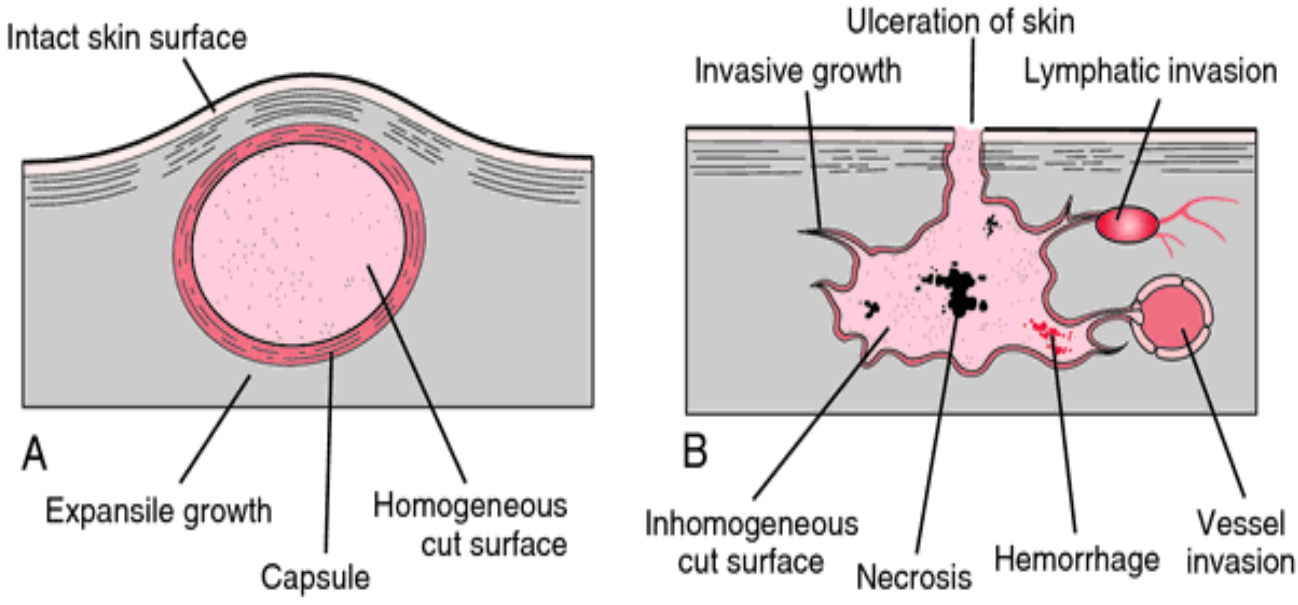

Figure 1: Benign tumors and malignant tumors (Damjanov, 2000.)

On the basis of origin or location where they develop initially cancer can be classified into several types. Thus on the basis of tissues of origin cancers are of the following types (Figure 2).

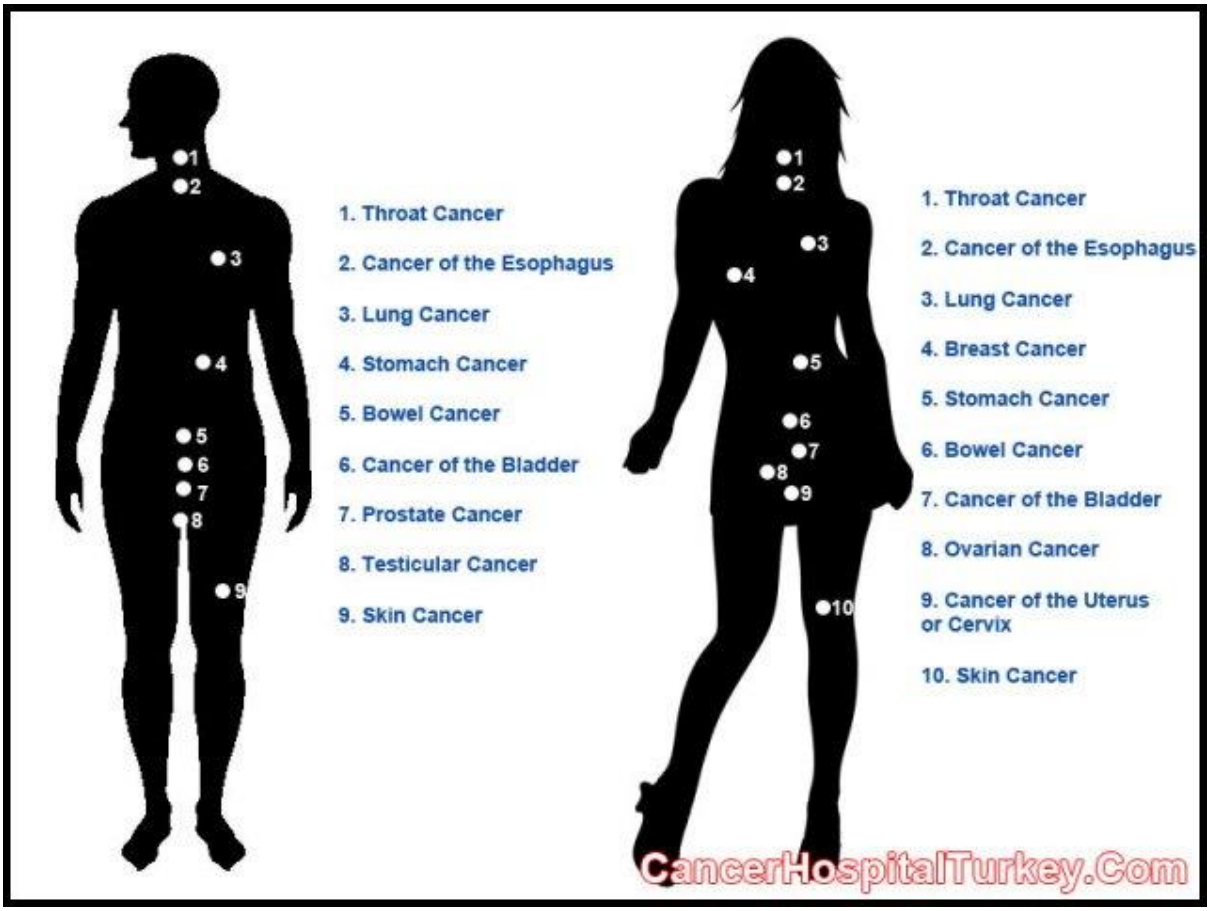

Figure 2: Different cancer types (http://www.cancerhospitalturkey.com/)

i. **Carcinoma**: Cancer of epithelial cells which covers external and internal body surface is called as carcinoma and these are the most common type of cancer.

ii. **Sarcoma**: Cancer arising from mesenchymal cells which support tissues of the body for example bone, fat, connective tissue, cartilage, and muscle.

iii. **Lymphoma:** This type of cancer originates from the nodes or glands of the lymphatic systems and also from tissues of body's defence system.

iv. **Leukemia:** Cancers in which bone marrow is not able to produce new blood cells and thus this type of cancer is also called as blood cancer (Stancu et al.). WBC protects body from infection, RBCs has role in oxygen transport and mainly prevent anemia and platelets help in blood clotting at the time of injury.

v. **Myeloma**: This type of cancer grows in plasma cells of bone marrow(Kyle and Rajkumar, 2009). There are 2 types of myeloma, among them one is Plasmacytoma in which myeloma cells accumulate in a single bone thus forming tumor and second one is multiple myeloma in which myeloma cells accumulate in many bone thus producing many bone tumors.

vi. **Blastoma:** It arises from embryonic tissue. It is found to be more common in children than in adult. e.g. Chondroblastoma occurs due to the precursor of chondrocytes.

SARCOMA

These are the cancers arising from mesenchymal cells which support tissues of the body for example bone, connective tissue, fat and muscle. Sarcoma is quite rare in humans. Metastasis is the major cause of death in sarcoma. 2 types of metastasis are there like nodal metastasis and distant metastasis. In nodal metastasis through lymph node the cancer cell move to distant body parts which is very rare one and other one is distant metastasis which generally spread to lungs.

i. **Soft tissue sarcomas:** It originates from soft tissues e.g. muscles, blood vessels, fat, nerves and fibrous tissue. About 80% of sarcoma arises in soft tissues. According to WHO STS occupies 23rd position among all cancer on the basis of occurrence.

ii. **Osteosarcoma:** It is generally called as osseous sarcoma which begins in bone. It involves 27th position among all tumors on the basis of occurrence. Sarcomas are subdivided into different types based on the cell type which makes up the cancer (Table 1).

Table 1. Subtypes of sarcoma and their origin

Subtypes of sarcoma	**Origin of tumor**
Angiosarcoma	Blood or lymphatic vessel
Chondrosarcoma	Cartilage cells
Ewing's sarcoma	Soft tissue or Bone
Fibrosarcoma	Fibrous tissue
Leiomyosarcoma	Smooth muscles of abdomen and pelvic parts
Liposarcoma	Liposarcoma Fat tissue
Osteosarcoma	Bone
Rahbdomyosarcoma	Skeletal muscle
Pleomorphic sarcoma	Limb or abdomen

SOFT TISSUE SARCOMA (STS)

There are various types of STS, which grows and develop differently. Liposarcoma originates from body's fat which can grow at any part in the body and most commonly affect people aged 50-65. Sarcomas that grow in smooth muscle are called leiomyosarcomas. They are one of the more regular types of sarcoma and can happen any place in the body, particularly in the back of the stomach area (retroperitoneum). Leiomyosarcomas are rare in

the soft tissues of the legs or arms. They mainly happen in grown-ups, especially in the elderly.

Fibrosarcoma is one type of STS. It develops from fibrous tissue which is mesenchymal in origin and is formed of fibroblasts, which are histologically predominant cells (Loh et al., 2002).

METASTASIS

The process of movement of malignant cells through the lymphatic or haematogenous circulatory system from primary tumor to distant organs is called as metastasis. Primary tumor is the main cause of 10% of deaths in patients but 90% of death is caused due to movement of cancer cells (Leber and Efferth, 2009). The sites from where the primary tumor will spread and gives rise to secondary malignant tumor is called site of metastasis. The determination of these metastatic destinations by the tumor cells in essential site is called organ selectivity. Metastasis chooses some useful qualities those aides in invasion and additionally the survivability and stability of disease cells. According to recent studies, metastasis may happen because of instability of genome due to some oncogenic events like DNA damage check points or evasion of growth suppression (Gupta and MassaguÃ©, 2006). Metastasis progression mainly include steps like- loss of cell adhesion, transition, invasiveness, entry to the circulatory system and colonization at a distant tissue sites (Chambers et al., 2002). Recent works based on the genomic-level molecular profiling have unrevealed some key regulatory genes whose expression in primary tumor state and the metastatic state are found to be strongly co-related (Weigelt et al., 2005). The cancer cells acquire the relevant genes to undergo metastasis in different cancer types (Bernards and Weinberg, 2002).Metastasis takes place as a result of progressive implication of traits that enable the cancer cells to go for migration and invasion at the secondary site (Figure 3).All these traits not necessarily should follow this particular order but the factors responsible for each step may facilitate the execution of multiple steps simultaneously(Gupta and MassaguÃ©, 2006). Metastasis is one of the primary reasons for death in cancer patients. Recent reports suggest that microRNA also play an important role in regulating genes involved in different steps of metastasis.

Steps of metastasis

Metastasis consists of the following steps (Figure 3)

i. Local invasion
ii. Intravasation
iii. Transport through circulation

iv. Extravagation

v. Formation of micro-metastasis

vi. Colonization

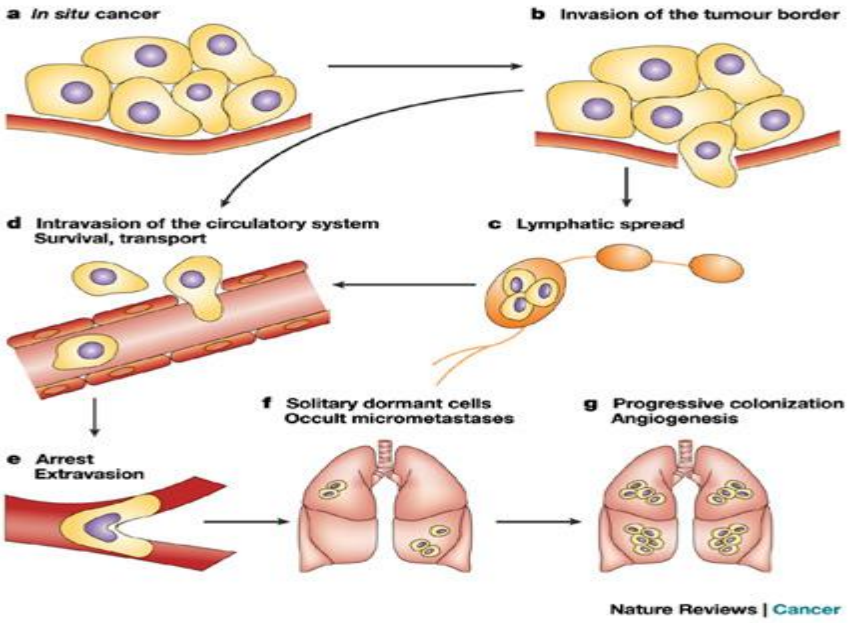

Figure 3: Different stages of metastasis (Steeg, 2003)

MicroRNA

RNAs include coding as well as ncRNAs that interfere in the activity of other coding transcripts. The ncRNAs include small non-coding RNAs (miRNA, siRNA, piRNA, etc.) and long non-coding RNAs (lncRNA). The ncRNAs are involved in specific gene silencing or gene regulation (Gomes et al.). Among the three types of small ncRNAs, miRNA have some pivotal role in normal cell metabolism and its differential expression is supposed to have diagnostic and prognostic value in many cancer cases (Lui et al., 2007).

miRNAs are of single stranded having 20- 22 nucleotide in length, originated from ds hairpin shaped RNA precursors with the help of RNase-III enzyme (Bartel, 2004).miRNAs control expression of genes at post transcriptional level through translational repression or degradation of mRNA (Cai et al., 2009). They can regulate different processes like cell differentiation, growth and cell death (Esquela-Kerscher and Slack, 2006). For various diseases miRNAs are found to be biomarkers and potential therapeutic targets. In cancer cells deregulation of miRNA occurs by different mechanisms like amplification, deletion, mutation and epigenetic silencing (Garzon et al., 2009).

Maturation of miRNAs

Primary miRNA (pri-miRNA) precursor molecule is produced by the transcription of miRNA (Figure 4). It undergoes cleavage producing precursor miRNA (pre-miRNA) which is cleaved to produce miRNA duplex in cytoplasm which contains mature miRNA. Then unwinding of duplex occurs as a result of which mature miRNA are released and assemble with RISC complex. These mature miRNA then direct gene silencing through translational repression or mRNA cleavage.

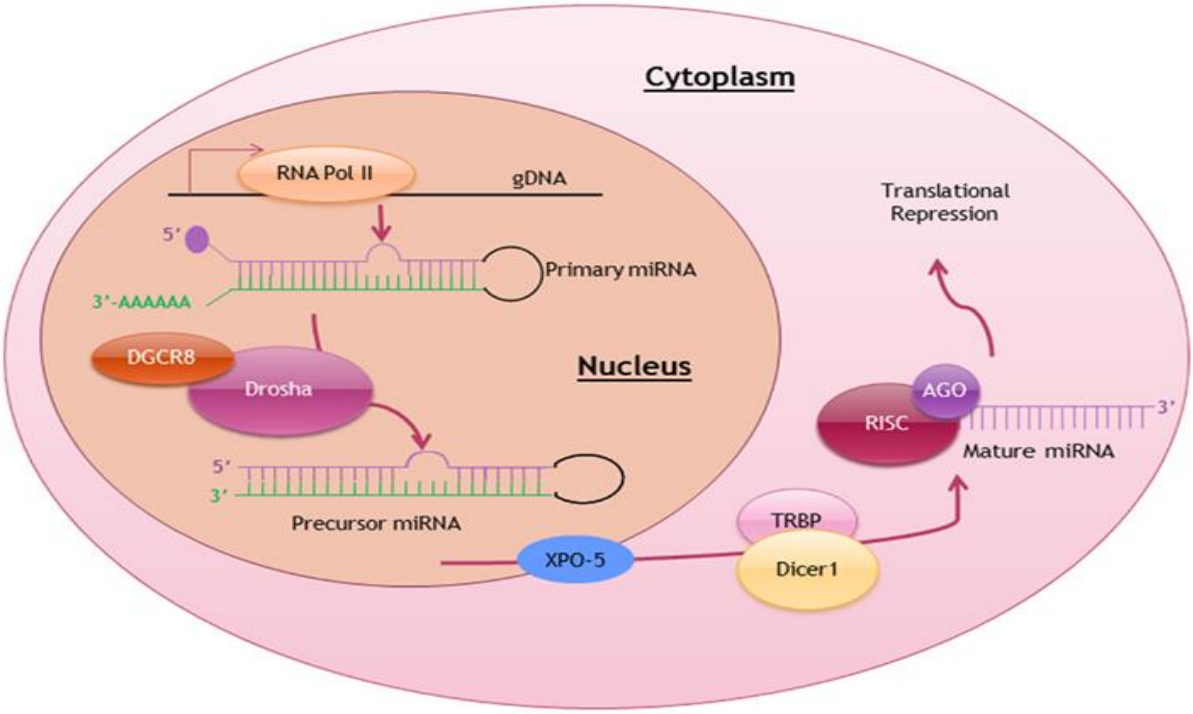

Figure 4: miRNA maturation and function(Yim et al.)

miRNA targeting

miRNA generally targets the mRNA for post transcriptional repression and for that it binds to 3' UTR of mRNA. The complementarily between the miRNA seed region and with that of target mRNA has a pivotal role in miRNA-mediated gene regulation through miRNA targeting. There exist principally three sorts of traditional target binding sites such as canonical sites, marginal sites and atypical sites. In the canonical sites, the coupling mainly occurs between 2-7 or 2-8 nucleotide position on the miRNA, while for marginal sites it lies in the middle of 2-7 or 3-8 nucleotide positions. The supplementary sites are situated at 12-17 position in addition to the seed region with complete Watson-Crick base pairing. At the same time, compensatory base pairing sites are seen in between 12-19 position regarding wobble base pairing in the traditional seed sites in order to compensate the mismatch (Bartel, 2009).

miRNA expression profiling in sarcomas

miRNA expression profiling in sarcomas has helped in the monitoring of the expression of thousands of miRNAs. Presently more than 1000 miRNAs have been identified in human. In different carcinomas, sarcoma and hematologic malignancies, analysis of miRNAs expression disclosed uniform pattern of specific miRNAs. Due to strong association with cancer some miRNAs are also called as oncomiR. For example in gliobalstomas over expression of miR-21 is found or in B cell lymphoma, rhadomyosarcoma and liposarcoma, microRNA 17-92 cluster found to be up-regulated. Some miRNA clusters which are found to be associated with cancer or other disease are seen within chromosomal fragile regions resulting in sequence amplification or deletion. As sarcomas are highly heterogeneous in nature, it is very difficult to validate sarcoma diagnosis. So for proper diagnosis, prognosis and therapeutic intervention of different sarcomas miRNA signature plays an important role.

Role of TFs in cancer system

Transcriptional factors can cause cancer either by activating or repressing the target genes. **MYC (c-myc)** is one of the regulatory gene that codes for a transcription factor. The transcriptional product of this gene is a multifunctional protein that takes part in cell cycle, movement, apoptosis, cell transformation, proliferation, cell-cycle progression, transcription and differentiation (Vita and Henriksson, 2006). myc directly can increase translation by regulating the expression of various members of the protein synthetic machinery like ribosomal proteins and translational initiation factors, including Pol III and rDNA. A transformed form of myc is found in numerous tumors, which affects expression of its downstream molecules. This prompts the unregulated expression of many genes, some of which are included in cell multiplication, and results in the onset of cancer. Malfunctions in myc have likewise been found in carcinoma of the cervix, colon, lung and stomach. myc regulating miRNAs can either induce or suppress the expression of various mRNA which are differentially expressed in STS. myc is consequently seen as a promising target for anti-cancer drugs (Begley).

OBJECTIVES

Objective 1:

To identify the list of miRNAs reported to be regulated by myc.

Objective 2:

To identify myc regulated miRNAs reported in STS and their target genes from different databases and build myc-miRNA regulatory network.

Objective 3:

Microarray expression analysis in fibrosarcoma to identify set of differentially expressed (DE) mRNAs and their comparison with myc-miRNA regulatory network.

i. All myc-miRNA regulated target genes vs. DE genes in fibrosarcoma
ii. DE myc regulate genes vs. known genes in STS

Objective 4:

Identification of anomalous altered expression of novel mRNAs involved in fibrosarcoma and experimental validation by qRT-PCR in HT-1080 cell lines.

MATERIALS AND METHODS

General work flow to find out DE novel genes in STS is shown with the help of flow chart (Figure 5).

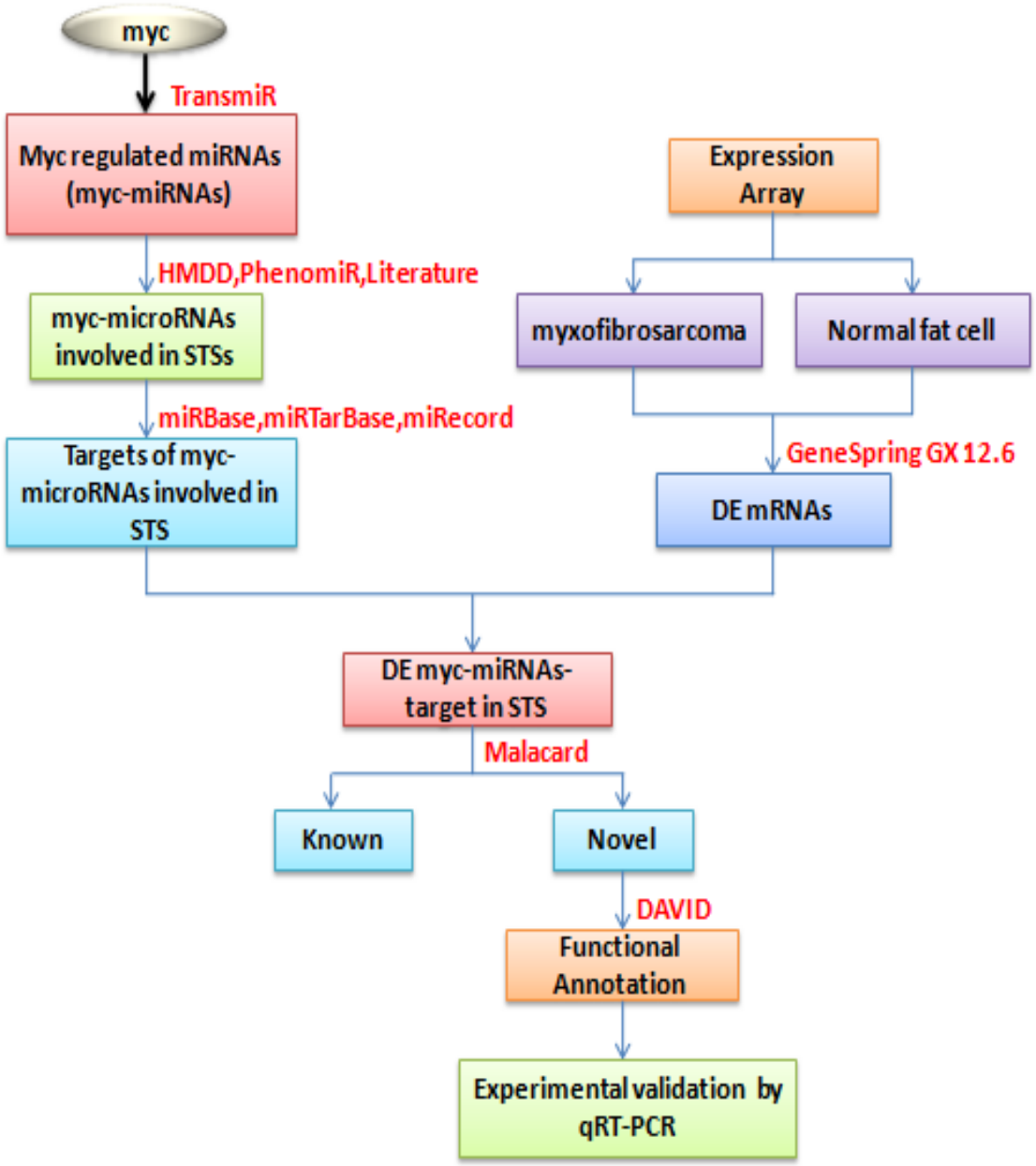

Figure 5: General workflow to find out novel DE genes in STS

1) Myc regulated miRNA

miRNAs transcriptionally regulated by MYC was retrieved from TransmiR database(Wang et al.). TransmiR is a database which gives idea about regulation of miRNA-transcription factor. This database is free for academic uses. Further the miRNAs obtained from TransmiR were mapped to and checked with HMDD database(Lu et al., 2008), PhenomiR(Ruepp et al.) and published literature and only the ones relevant to STS were retained for the study.

2) miRNA-Target Selection

To study the downstream gene targets of Myc-miRNA interaction pathway in STS, the validated targets of those miRNAs which are regulated by myc were selected from miRecords (Xiao et al., 2009), miRBase (Griffiths-Jones et al., 2006) and miRTarbase (Hsu et al.). Total numbers of targets were compiled for those miRNAs from all the three databases and a Myc-miRNA regulatory network was build. The aim was to predict which of these targets were also differentially expressed in STS. From analysis of microarray data, alterations in gene expression were investigated.

3) Microarray analysis of gene Expression Data:

A. Retrieval of mRNA gene expression data

i. All samples of fibrosarcoma and normal control fat (i.e. all GSMs) present in the GSE 21122 were taken for analysis. The details of number of samples taken for mRNA expression analysis are given in (Table 2).The data were taken in multiples to reduce error rate.

ii. The raw files were downloaded in .CEL format.

iii. Then the files were unzipped, extracted and renamed as control and test for mRNA and miRNA accordingly.

Table 2. Number of samples taken for mRNA and miRNA expression analysis

Expression data	Sarcoma	No. of samples
mRNA	MFH:Myxofibrosarcoma	31
mRNA	Normal fat	9

B. Analysis of Gene Expression Data

Agilent's GeneSpring GX 12.6 software is used for gene expression analysis. This software is a powerful microarray expression data analysis tool for fast visualization and analysis of different microarrays (gene, miRNA, exon, genome copy number, etc.). Class of transcripts showing expression pattern which are correlated with experiment variables are identified by using this software. Thus mainly for fulfilling the needs of biologists this tool is designed.

Performing work in GeneSpring GX 12.6 is organized into projects. In a project a number of experiments are involved and again an experiment is composed of samples that are data sources. Samples are grouped as test and control and interpretations are done based on the defined parameters of sample grouping and finally analysis involves a number of statistical steps and corresponding results.

All information on array design along with biological information is found in Genespring GX. So for each new array type which is to be analysed, a new technology must be installed initially. Technologies created include affymetrix for standard arrays, Agilent and Illumina. In GeneSpring following steps are used.

i. In order to minimize the systematic non-biological differences to reveal true biological differences, normalization of data was done, after which profile plot of normalized intensity map values are obtained. To standardize each chip for cross comparison, data is normalized to 75^{th} percentile of signals intensity. The main purpose is to eliminate redundancy and make sure that the data makes sense with minimum number of entities.

ii. A new experiment was created for analysis of gene expression. The experiment type should be specified as Affymetrix. Workflow selected was Guided Workflow. Using choose sample option the unzipped samples (both sarcoma and normal fat) were uploaded to the experiment in GeneSpring from the saved location in the system. Experimental grouping was done to define samples as test and control and assigning a parameter name (e.g. Average).

iii. By using Filter Probsets by Errors, quality control of samples was done. This was done on the raw signal values of all the entities. For filtering, cut off was set at 20 percentile of all intensity values and a profile plot of filtered entities was generated. Using the normalized signal values and grouped samples by active interpretation box whisker plot is generated.

iv. Significance analysis depending upon experiment grouping was done by performing *t-Test* unpaired analysis as 2 groups are there that is control and test along with replicates. For computing *p-values* Benjamini-Hochberg FDR algorithm multiple testing corrections was used. The *p-value* cut off taken was ≤ 0.05. This reduces the number of false positives or false discovery rate. This multiple testing correction is least stringent. Fewer chances of false negative genes are there. A *p-value* of ≤ 0.05 is taken as significant.

v. For identifying differentially genes among the cancerous and non-cancerous samples which are expressed above a definite threshold, fold change analysis is done. It gives the absolute ratio of normalized intensities between the average intensities of grouped sample. Fold change cut off is taken ≥ 2.0.

vi. Further 2D hierarchical clustering of the genes expressed >2.0 fold was carried out taking average linkage to classify the cancerous and normal control samples and a heat map was generated using CLUSTER 3.0 and JAVA tree view. Hierarchical clustering method arranges gene in a tree structure based on their similarity. If the items are similar to each other then they are connected by short branches and if it is dissimilar then it is connected by long braches.

vii. Genes expressed greater than 2 fold were exported from GeneSpring along with normalized signal values, gene symbols, entrez gene IDs etc. The entity list was exported as .txt file and later opened with excel for further analysis.

4) Novel DE gene identification for STS

DE targets of myc-miRNA in fibrosarcoma were screened out from gene expression study. These DE myc-miRNA targets were then compared to known STS genes from Malacard disease database(Rappaport et al.) to find out novel mRNAs with anomalous altered expression in fibrosarcoma.

5) Functional annotation of six novel myc-miRNA targets DE in fibrosarcoma

myc-miRNA-target pathway provides novel genes which are having altered expression in STS. Functional annotation of those genes were done by using Database for Annotation, Visualization, and Integrated Discovery (DAVID), version 6.7 (Huang et al., 2008, 2009) which is generally used for gene ontology (GO) study. This database records function of around 1.5 million unique protein/genes obtained from about 65,000 species.

6) Experimental validation

Human fibrosarcoma cell line HT-1080 was procured from National Centre for Cell Science, Pune, India and the fibroblast cell line WI-38 was obtained from Bose Institute, Kolkata, India. The media used for culturing the HT1080 cell line is DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS (Fetal Bovine Serum) from HIMEDIA and 1% antibiotic solution. The culture flask containing the cell line is kept in CO_2 maintained at 5%. The media contains phenol red, a pH indicator and when the media is utilize by cells the colour of medium changes from red to orange and finally to pale yellow because of change in pH.

Cell Culture

a. Reviving of stored cells

For reviving cells they are first thawed by drastically reducing the stored cells temperature from -80oC to 37oC in a water bath to avoid formation of crystals. 5ml of DMEM was taken

in a T25 flask and the thawed cell specimen was pipetted into the flask. The flask was swirled gently in order to mix the cells with medium. Centrifuge at 1500rpm for 5min, aspirate the supernatant, and then re-suspend the cells in fresh medium. Cells were incubated overnight and then checked for their adherence to the flask and the growth medium was replaced. The cells are grown in a flask until they are confluent enough or their medium has been utilized and they are ready for sub-culture.

b. Trypsinizing cells

Trypsinization is a process which uses the proteolytic enzyme trypsin in order to detach the cells adherent to the surface of a cell cultured vessel. For trypsinization the medium was discarded. Cells were washed with 1X PBS. 1ml 1X trypsin EDTA solution was added. The flask was placed in a CO_2 incubator at 37^oC for 1 to 2 minute or until all cells were detached. Cells were re-suspended in a growth medium having serum. Use medium having the same percentage of serum as used for growing the cells. The serum decreases 21 trypsin activities. Then cells were pipetted gently up and down and transformed to an RNAase free glass or polypropylene centrifuge tube and then centrifugation done at 1500rpm for 5 minute. Then the supernatant was aspirated completely and the cells again re-suspended in 1X PBS and were counted. The appropriate no. of cells were taken for isolation of RNA by again pelleting the cells and removing PBS.

RNA isolation

i. mRNA isolation

For mRNA isolation QIAGEN kit is used.

First at a maximum of 1×10^7 cells were harvested then appropriate volume of buffer RLT was added.

I. To the lysate, 1 volume of 70% ethanol was added and mixed well by pipetting. Immediately proceed to step 3.
II. 700µl of sample including any precipitation was transferred to an RNeasy mini spin column placed in 2ml supplied collection tube. Then the lid was closed and centrifuged for 15 sec at ≥ 8000xg.Then flow through was discarded.
III. To RNeasy spin column 700µl of RW1 was added. Then the lid was closed and centrifuged for 15 sec at 8000xg.Then flow through was discarded.
IV. To RNeasy spin column 500µl of RW1 was added. Then the lid was closed and centrifuged for 15 sec at ≥ 8000xg.Then flow through was discarded
V. To RNeasy spin column 500µl of RW1 was added. Then the lid was closed and centrifuged for 2 minute at ≥ 8000xg.

VI. In a new 1.5ml collection tube the RNeasy spin column was placed. To the spin column membrane 30-40µl RNase free water was added directly and centrifugation was done for 1 minute at ≥ 8000xg in order to elute RNA.

VII. If the expected RNA yield is more than 30µg, then the step-7 was once again repeated by using another 30-50µl of RNase free water or using the elute obtained from step-7.The collection tube were reused from step-7.

VIII. The purity and yield of RNA was measured by using Eppendorf NanoDrop. It is a cuvette free spectrophotometer which eliminates the requirement for other sample containment device. 1µl of sample can be measured with this instrument with high reproducibility and accuracy. 1µl of sample was pipetted onto the end of an optic fiber cable. The instrument is controlled by software and the data is logged in an archive file on PC.

cDNA Synthesis

i. mRNA cDNA synthesis

cDNA Synthesis was done by using Super Script First Standard Synthesis System for RT-PCR by Invitrogen using oligo dT primer.

I. Components were mixed and briefly centrifuged before use.

II. Following components were mixed in a sterile 0.2 or 0.5 tube for each reaction.

III.

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

IV. Mixture of RNA/Primer was incubated at 65^{o}C for 5 minute and then placed on ice for at least 1 minute.

V. Following 2X reaction was prepared in a separation tube by using each component in the indicated order.

Component	1RXn	10RXn
10X RT buffer	2 µl	20 µl
25Mm Mgcl2	4 µl	40 µl

0.1MDTT	2 µl	20 µl
RNaseoutTM(400/µL)	1 µl	10µl

VI. 9µl of 2X reaction mixture was added to each RNA/Primer from step3, mixed gently and collected after brief centrifugation and incubated at 42 ^{o}C for 2 minute.

VII. 1µl of superscript TM II RT was added to each tube and then incubated for 50 minute at 42 ^{o}C.

VIII. The reaction was ended at 70 ^{o}C for 15 minute and then chilled on ice.

IX. By brief centrifugation the reaction was collected. To each tube, 1 µl of RNase H was added and incubated at 37 ^{o}C for 20 minute and then the reaction was used for PCR immediately.

Quantative RT-PCR analysis

In qRT-PCR florescence signal is continuously collected from one or more PCR over a range of cycles. Thus florescence signals coming out from each reaction are converted into a numerical value for each sample. In order to bind DNA florescent maker is used. Thus during the reaction as the number of copies increase, so the florescent intensity increases. SYBR green is a intercalating florescent dye, and it is the cheapest and simplest way in order to monitor a PCR in real time. The dye florescence only on binding to ds DNA. Using dye has one disadvantage, which is lack of specificity .From primer bank Database (Harvard) the gene specific primer sequence were obtained and the primers were synthesized by Sigma. All the primers were desalted.

i. Procedure of mRNA qRT-PCR

In order to perform PCR study, RNA must transcribed into cDNA by reverse transcription reaction. cDNA act as template for RT-PCR along with primers specific to a particular gene (Table 3).

Table 3. Primer name and sequence length and its amplicon size

Gene	Sequence	Amplicon size
ACTB	F- CATGTACGTTGCTATCCAGGC	250
	R- CTCCTTAATGTCACGCACGAT	
COL1A2	F- GAGCGGTAACAAGGGTGAGC	92
	R- CTTCCCCATTAGGGCCTCTC	
RRM2	F- CACGGAGCCGAAAACTAAAGC	129
	R- TCTGCCTTCTTATACATCTGCCA	

In Eppendorf Masterplex Real Time PCR, RT-PCR was carried out. 20ng cDNA was used per reaction. Primer concentration was normalized and gene specific forward and reverse primer pair was mixed. Each primer that is forward and reverse primer concentration in mixture was 9.5µl.

I. Now the experiment was ready and the following PCR program was made on. Threshold frequency was 33%. The temperature cycle were(Table 4) taken as follows:

Table 4. Temperature cycle for qRT-PCR

Stage	Temperature(^{o}C)	Time	Cycle
Stage 1	95	20 sec	1
Stage2	95 59 68	15 sec 15 sec 20 sec	40
Stage3	95 60 95	15sec 15sec 15sec	1

II. cDNA was diluted to 1:20 ratio concentration and then primer was added.

III. 10µl of a real time PCR reaction volume was made.

IV. In each optional tube following mixture was made as follows (Table 5).

Table 5.Reaction mixture for qRT-PCR

SYBR Green Mix(2x)	95 µl
cDNA stock (cDNA:dH2O)	40µl
Primer pair mix(3.5µl each primer)	9.5µl

V. With the help of in-built software, the RT-PCR result was analysed.

VI. The tubes were removed from the machine, after PCR is finished.

RESULTS AND DISCUSSIONS

1. myc regulated miRNA

To identify the transcriptional regulation of miRNAs by myc, 42 myc controlled miRNAs were obtained from TransmiR database and listed in (Table 7). Of these 7 miRNAs were found to be involved in STS and those were selected for further studies (Figure 6). The selected miRNAs were miR-34a, let-7b, let-7g, let-7, miR-22, miR-26a and miR-221(Figure 5).

Table 5: List of myc regulated miRNAs extracted from TransmiR

TF	miRNA	miRNA tumor involvement	Action type	Reference
MYC	mir-106a	Y	Activation	17943719
MYC	mir-17	Y	Activation	17943719 19066217
MYC	mir-18a	Y	Activation	17943719 19066217
MYC	mir-19a	Y	Activation	17943719 19066217
MYC	mir-19b	Y	Activation	17943719 19066217
MYC	mir-20a	Y	Activation	17943719 19066217
MYC	mir-221	Y	Activation	17943719
MYC	mir-92a	Y	Activation	17943719 19066217
MYC	mir-195	Y	Repression	18066065
MYC	mir-22	Y	Repression	18066065
MYC	mir-29a	Y	Repression	18066065
MYC	mir-29c	Y	Repression	18066065
MYC	mir-26a	Y	Repression	18713946 19197161
MYC	let-7a	Y	Repression	19211792
MYC	let-7b	Y	Repression	19211792
MYC	let-7c	Y	Repression	19211792
MYC	let-7d	Y	Repression	19211792
MYC	let-7e	Y	Repression	19211792
MYC	let-7f	Y	Repression	19211792
MYC	let-7g	Y	Repression	19211792
MYC	let-7i	Y	Repression	19211792
MYC	mir-15a	Y	Repression	19211792
MYC	mir-16-1	Y	Repression	19211792
MYC	mir-34a	Y	Repression	19211792

MYC	mir-23a	Y	Repression	19219026
MYC	mir-23b	Y	Repression	19219026
MYC	mir-200	Y	Regulation	19745813
MYC	mir-429	Y	Regulation	19745813
MYC	mir-141	Y	Regulation	19745813
MYC	mir-9	Y	Activation	20173743 20173740
MYC	mir-141	Y	Regulation	20053927
MYC	mir-17	Y	Regulation	20008931
MYC	mir-19a	Y	Regulation	20008931
MYC	mir-19b	Y	Regulation	20008931
MYC	mir-17	Y	Repression	20878079
MYC	mir-20a	Y	Repression	20878079
MYC	mir-20b	Y	Repression	20878079
MYC	mir-106a	Y	Repression	20878079
MYC	mir-106b	Y	Repression	20878079
MYC	mir-93	Y	Repression	20878079
MYC	mir-17	Y	Upregulation	20643754
MYC	let-7	Y	Repression	22388088
MYC	mir-29b	n/a	Repression	22249264
MYC	mir-26a	n/a	Repression	21941025
MYC	mir-26b	n/a	Repression	21941025
MYC	let-7a-1	n/a	Repression	21903590
MYC	let-7f-1	n/a	Repression	21903590
MYC	let-7d	n/a	Repression	21903590
MYC	miR-106b	Y	down-regulation	23028803
MYC	miR-25	Y	down-regulation	23028803
MYC	miR-93	Y	down-regulation	23028803

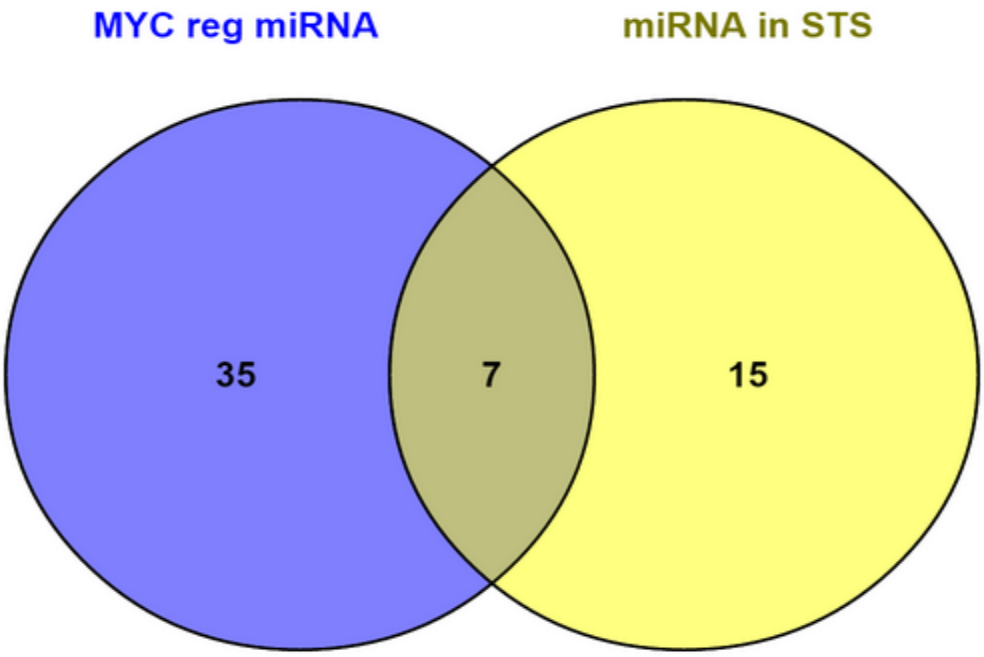

Figure 6: Mapping myc regulated miRNA with known miRNAs in STS

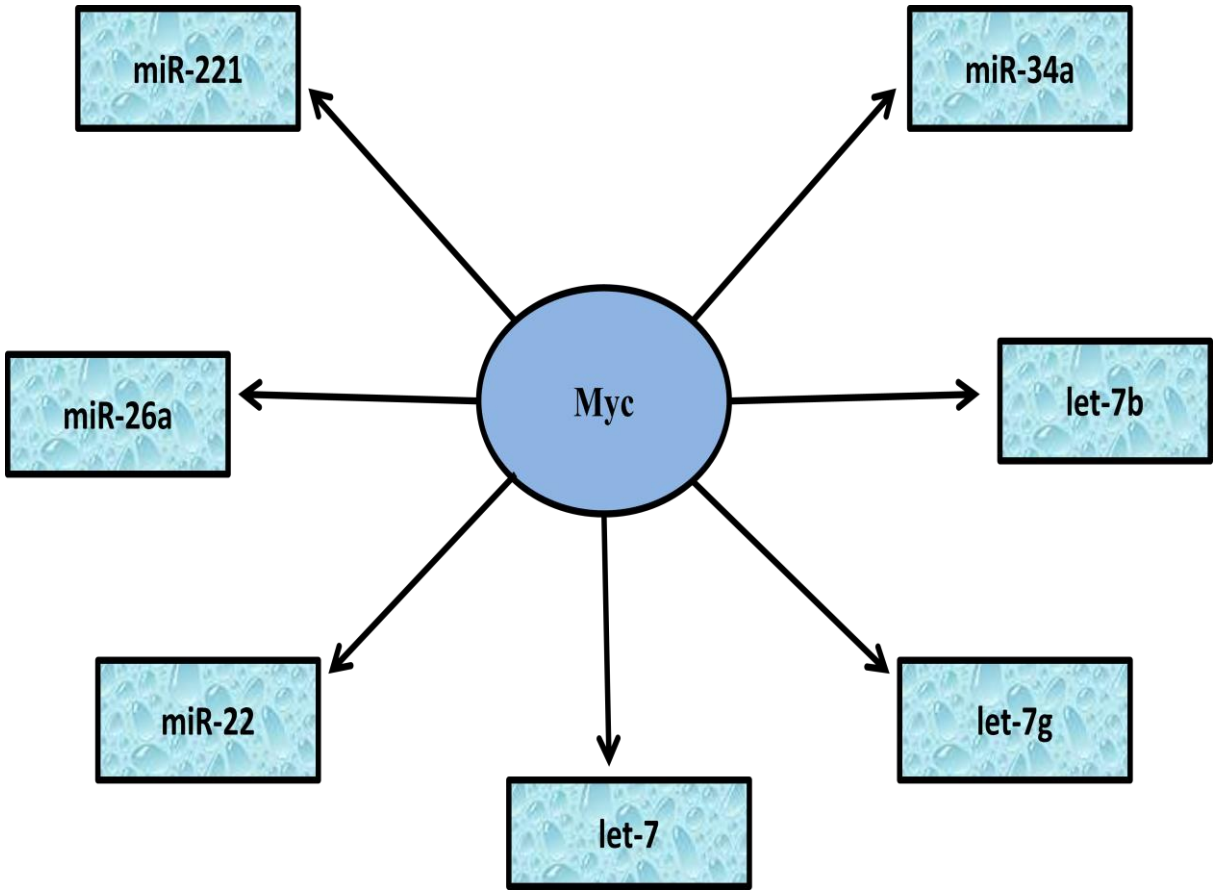

Figure 7: myc regulating miRNA reported in STS

2) miRNA-Target Selection

To study the downstream effects of myc-miRNA interaction, the validated targets of these seven miRNAs from miRecords, TransmiR and miRTarBase were selected. A total of 265 targets were found (Figure 8). We compared these target gene list with DE genes in STS obtained from microarray analysis.

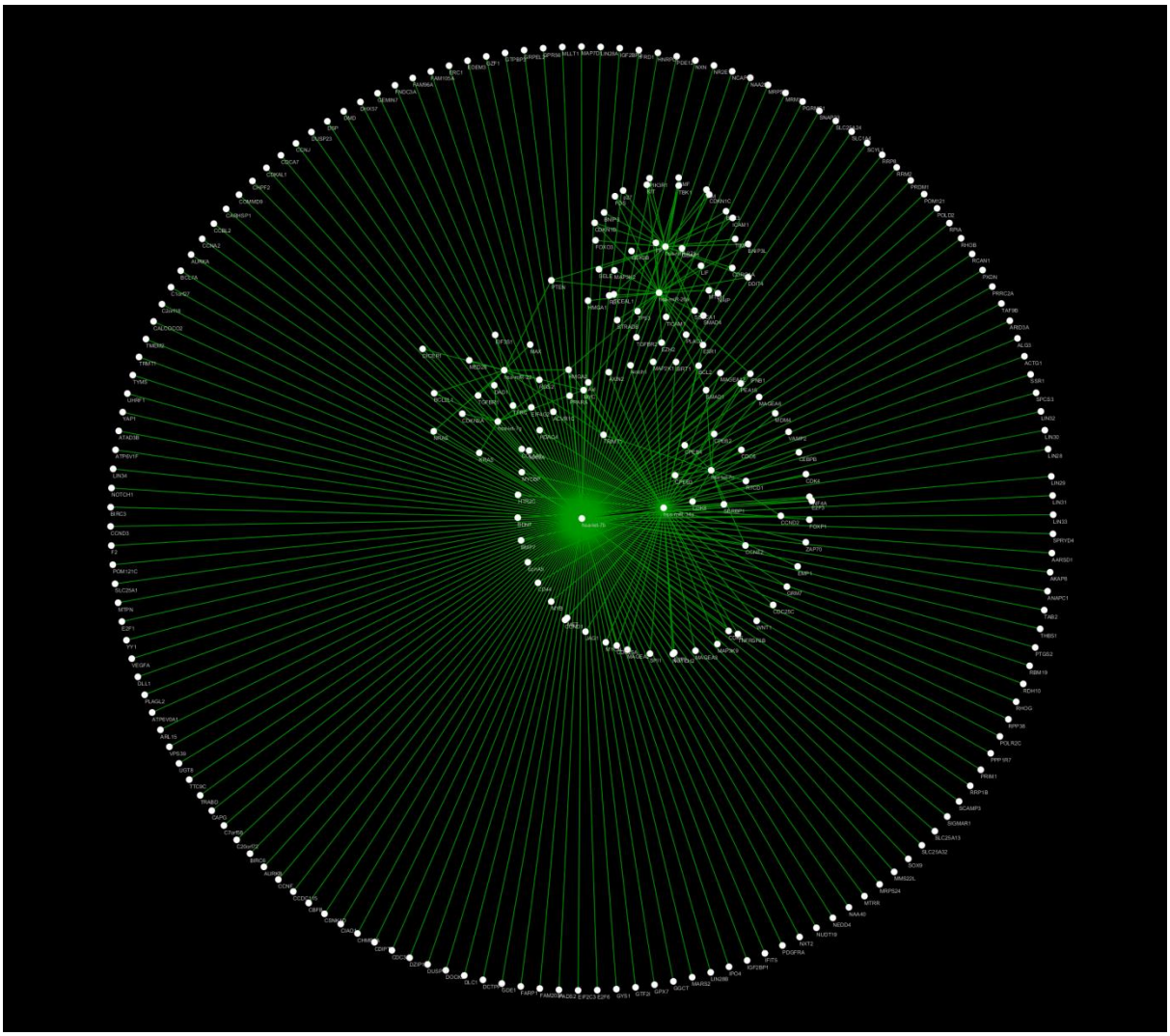

Figure 8: myc-miRNA regulated network in STS

3) Expression Data Analysis

Microarray Data analysis - 1068 differentially expressed genes (DE) were obtained between myxofibrosarcoma and the control normal fat samples. Of these, 558 genes were found to be up-regulated and 510 genes were down-regulated in myxofibrosarcoma vs. control normal fat

samples. Hierarchical clustering of the DE genes showed the relatedness among the samples as all control samples clustered together and all sarcoma samples clustered together (Figure 9)

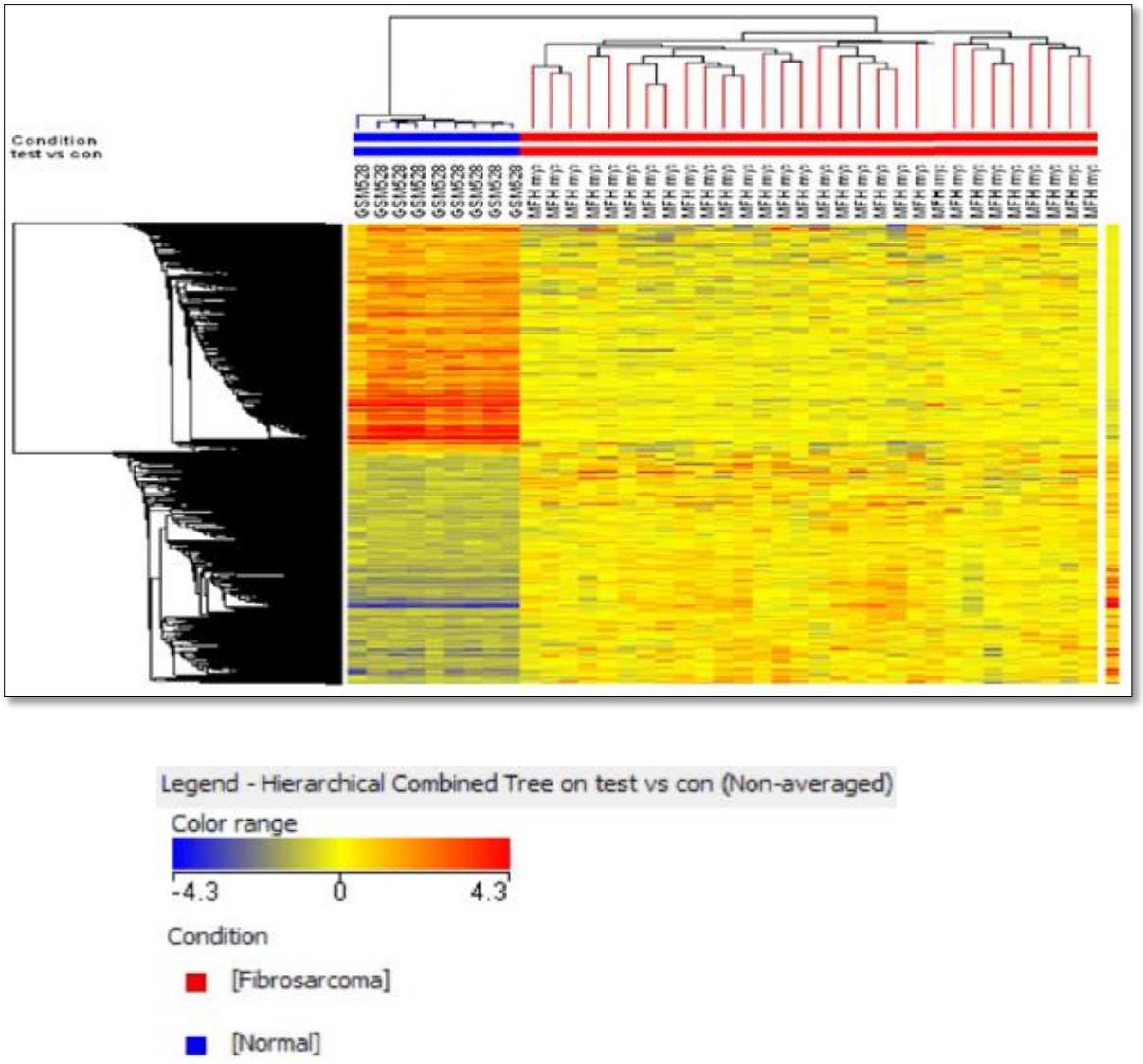

Figure 9: Hierarchical clustering of Sarcoma and normal fat samples

4) Novel DE gene identification for STS

We obtained 265 genes which were the targets of those 7 miRNAs regulated by **myc** transcription factor. From microarray analysis we obtained the list of 1068 genes which were differentially expressed in Sarcoma than normal fat samples. By comparing those 2 sets of genes, we found out 12 target genes which were differentially expressed in STS (Figure 10). Those 12 genes are **COL1A2, MYC, RRM2, TYMS, EMP1, CEBPB, MAOA, PTEN, EZH2, CDKN1C, FOS** and **ICAM1.**

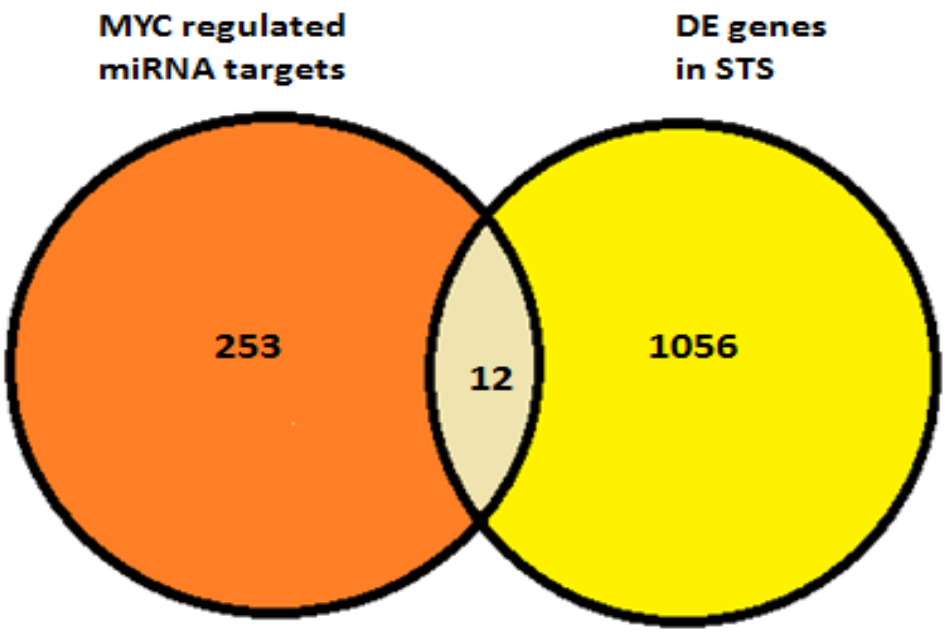

Figure 10: Comparison of myc regulated miRNA targets with DE genes in STS

These 12 DE genes which were the targeted by 7 STS related myc-miRNAs were then compared with the list of known genes DE in STS obtained from Malacard database (Rappaport et al.). To this end 6 genes were short listed for their association with STS and having altered expression in myxofibrosarcoma compared to normal fat cells (Figure 11). From myc-miRNA-target pathway enrichment study 6 novel genes were obtained, having altered expression in STS. Functional annotation of these genes were done and shown in (Table 6).

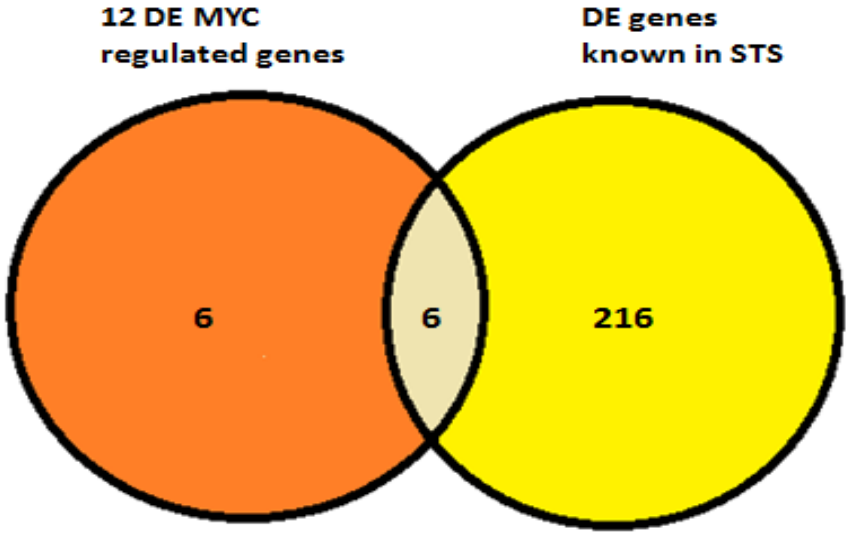

Figure 11: Comparison of 12 DE myc-miRNA-targets with DE genes known in STS

Table 6: Significant gene ontology terms related to six novel altered genes

Gene Name	Enriched Database	Predicted Functional Association
CEBPB	BIOCARTA	IL 6 signalling pathway
	GOTERM_BP_FAT	regulation of cytokine production, transcription
	GOTERM_MF_FAT	transcription activator
	SP_PIR_KEYWORDS	transcription regulation, differentiation, DNA binding
COL1A2	GOTERM_BP_FAT	Skeletal system development, blood vessel development, vasculature development
	GOTERM_CC_FAT	extracellular matrix,
	SP_PIR_KEYWORDS	chromosomal rearrangement,
EMP1	GOTERM_BP_FAT	cell proliferation, regulation of cell size, epidermis development, cell growth,
	GOTERM_CC_FAT	cell fraction, membrane fraction
	INTERPRO	Epithelial membrane protein EMP-1
MAOA	GOTERM_BP_FAT	regulation of neurotransmitter levels, cellular amino acid derivative metabolic process
	COG_ONTOLOGY	Amino acid transport and metabolism
	GOTERM_CC_FAT	mitochondrial envelope, mitochondrial outer membrane
	GOTERM_MF_FAT	amine oxidase activity, electron carrier activity, oxidoreductase activity
	SP_PIR_KEYWORDS	Neurotransmitter degradation, oxidoreductase, transmembrane,
RRM2	GOTERM_BP_FAT	DNA metabolic process, DNA replication, nucleotide biosynthetic process
	KEGG_PATHWAY	Purine metabolism, Pyrimidine metabolism,
	SP_PIR_KEYWORDS	DNA replication, iron, metal-binding, oxidoreductase
FOS	BBID	T_cell_receptor
	BIOCARTA	Oxidative Stress Induced Gene Expression Via Nrf2, B Cell Survival Pathway, BCR Signalling Pathway, IL2 signalling pathway, IL3 IL6 signalling pathway, Insulin Signalling Pathway,
	GOTERM_BP_FAT	DNA modification, DNA alkylation, DNA methylation, regulation of transcription
	KEGG_PATHWAY	Pathways in cancer, Colorectal cancer

These 6 genes are **COL1A2, RRM2, EMP1, CEBPB, MAOA** and **FOS.** The miRNA target interactions found were **hsa-let-7g→COL1A2**, **hsa-let-7b→RRM2**, **hsa-miR-34a→EMP1**, **hsa-miR-34a→CEBPB**, **hsa-miR-34a→MAOA** and **hsa-miR-26a→FOS**. Out of these 6 targets, we experimentally validated **COL1A2** and **RRM2** as they are up-regulated in STS and their targeting miRNA were found to be down-regulated. Rest 4 genes were down-regulated in

STS. Interactions of miRNA hsa-let-7g and hsa-let-7b are shown in Figure 12 and Figure 13 below.

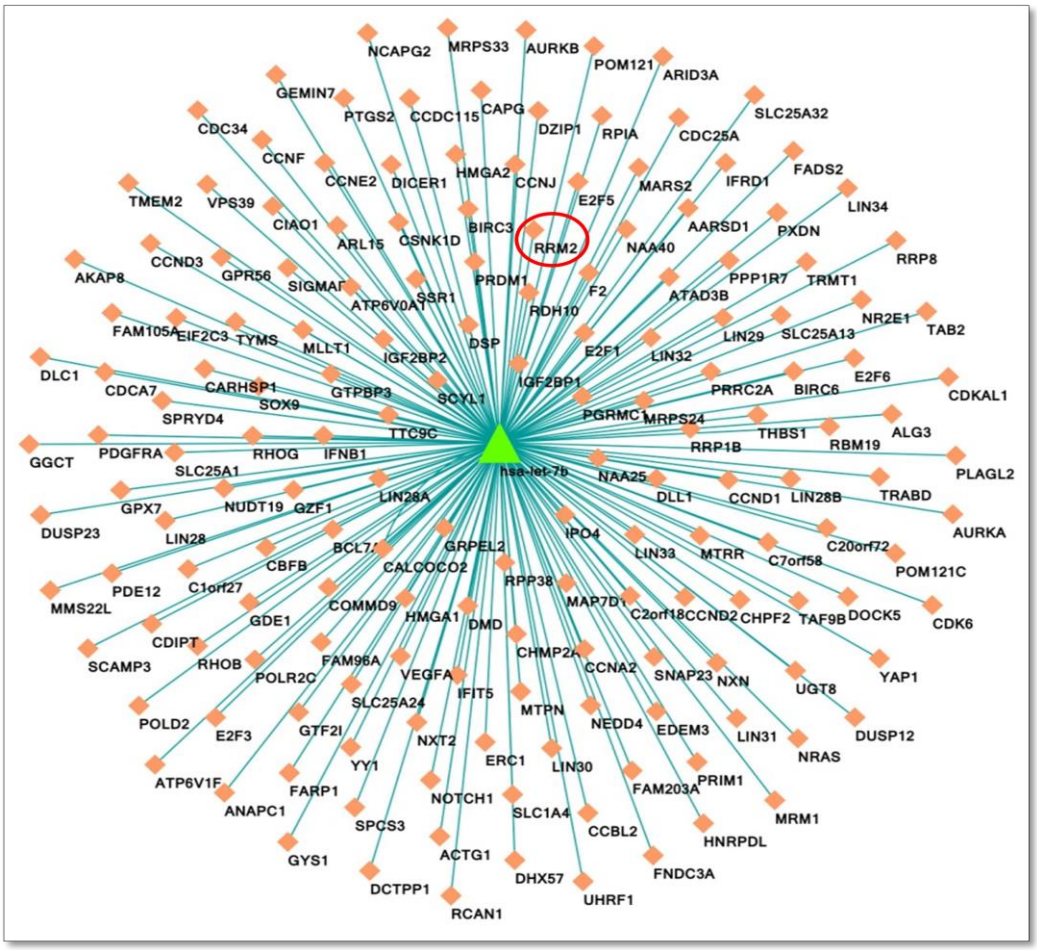

Figure 12: Representing let-7b→ target Interactions. RRM2 has been highlighted in red.

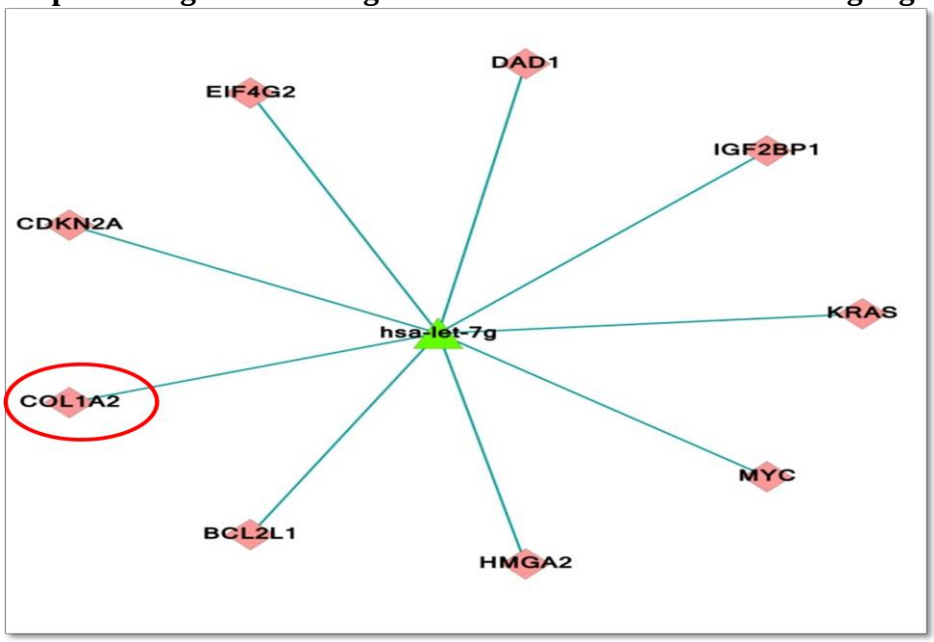

Figure13: Representing let-7g→target interactions. COL1A2 has been highlighted in red.

Further literature studies were done to explore genomic status and function of COL1A2 and RRM2 in detail. COL1A2 gene encodes one chain of type I collagen alpha-2(I) protein and found in most of the connective tissue. It is located at position 22.1 on chromosome number 7.Up-regulation of this gene is already reported in various cancers like dermal fibroblasts, gastric cancer, colorectal carcinoma etc. Expression of this gene correlates with endogenous Tbx2 (T box family of transcription factor) in several fibroblast cell line, induce tumor angiogenesis and oncogenesis (Davis et al., 2008). Its suppression leads to decrease in vascular growth and its expression is necessary for angiogenesis. It can organize new blood vessels and may serve as important biomarker (Senger et al., 2002). RRM2 (ribonucleotide reductase M2) encodes ribonucleoside-diphosphate reductase subunit M2 which catalyses the formation of deoxyribonucleotides from ribonucleotide. Increase in RRM2 can induce VEGF (vascular endothelial growth factor). Thus it is predicted to be regulating the invasive and metastatic potential of tumors. Thus it helps in angiogenesis and critical to tumor malignancy (Zhang et al., 2009).

5. Experimental Validation

RNA Isolation

260/280 ratio: It referes to the absorbance of RNA and DNA at 260 nm and 280 nm respectively. It is used to access the purity of DNA and RNA. Approximately 1:8 ratios is generally expected and accepted as “pure” for DNA and approximately 2.0 ratios is as “pure” form RNA. In either case, if the ratio is significantly lower it may indicate the presence of phenol, protein or other contaminants that absorb at or near 280nm.

260/230 ratio: The 260/230 values for “pure” nucleic acid is generally higher than the respective 260/280 values. Expected 260/230 values are normally lies in the range of 2.0-2.2. If the ratio is significantly lower than the expected ratio, then it indicates that contaminants absorbing at 230 nm are present.

qRT-PCR

The qRT-PCR program used 59º as annealing temperature as the amplification for ACTB, COL1A2 and RRM2 was best at that temperature (Figure 14).

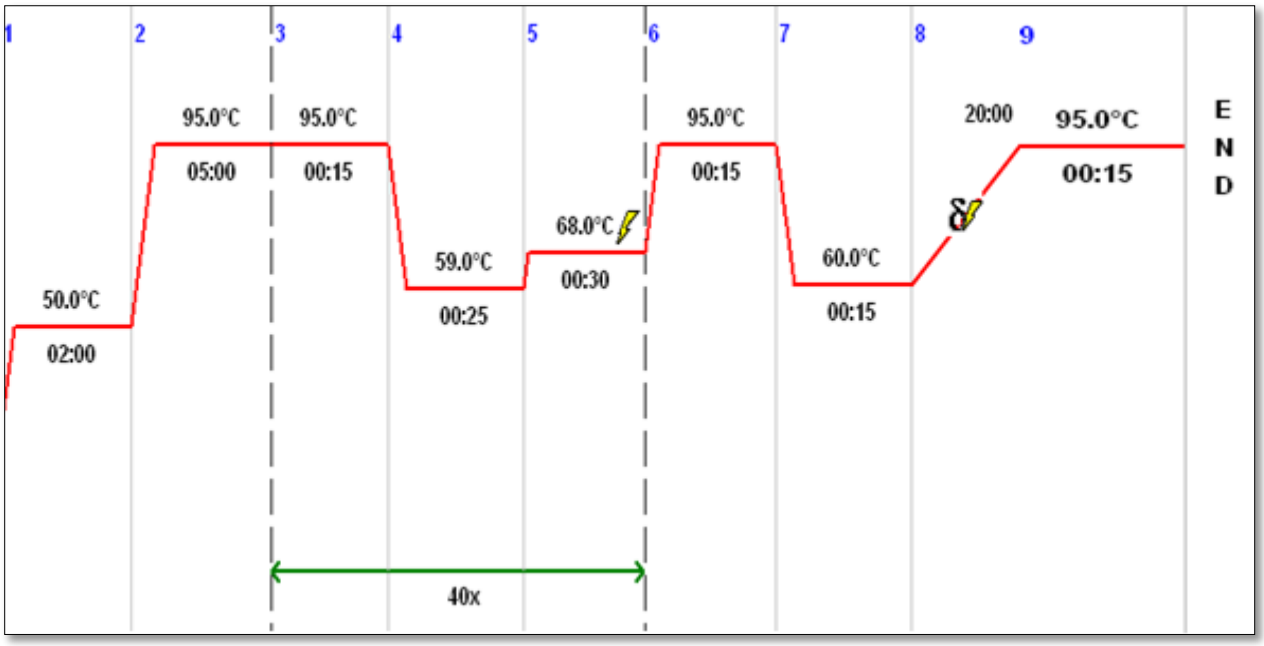

Figure 14: Cycle temperature and time for qRT-PCR.

qRT-PCR melting curve analysis showed a single amplification product was formed for the COL1A2, RRM2 and ACTB as only a single peak was obtained for the respective genes. There was no nonspecific amplification detected (Figure 15). COL1A2 and RRM2 showed elevated expression w.r.t ACTB (Figure 16).

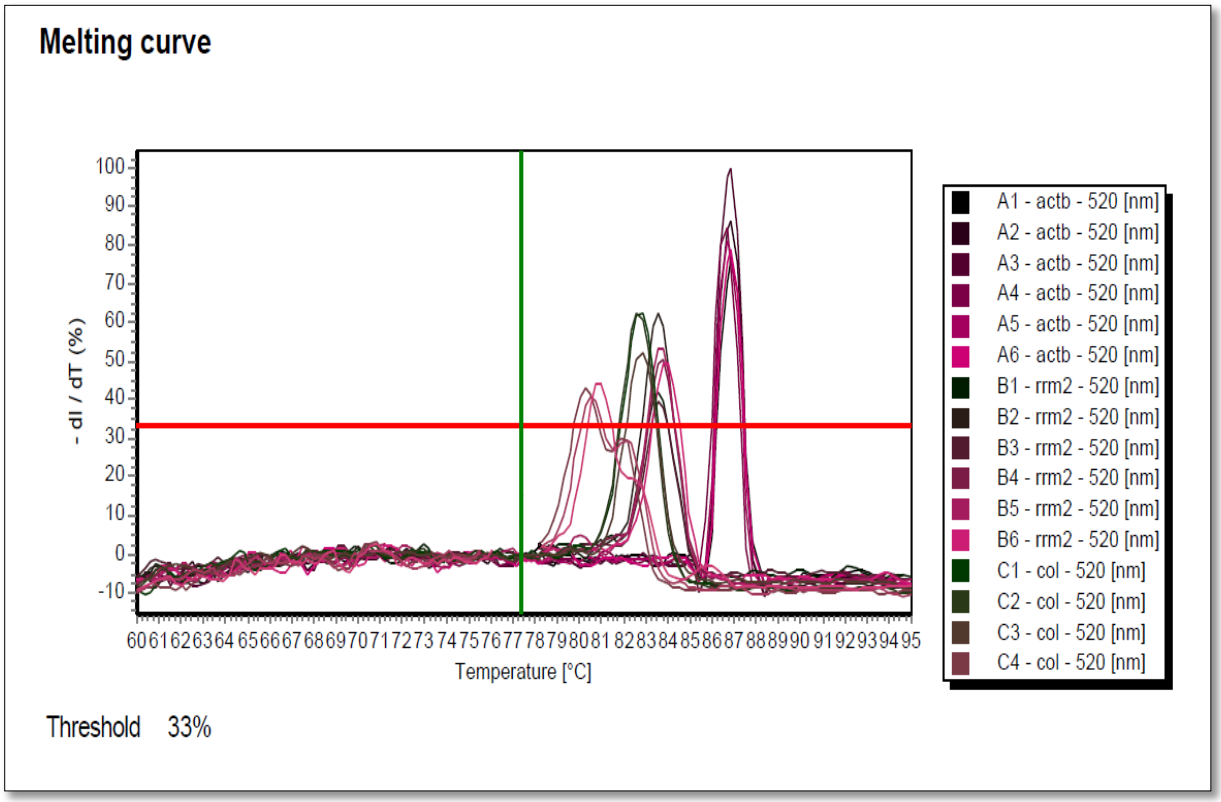

Figure 15: Melting temperature curve of COL1A2 and RRM2 with respect to ACTB.

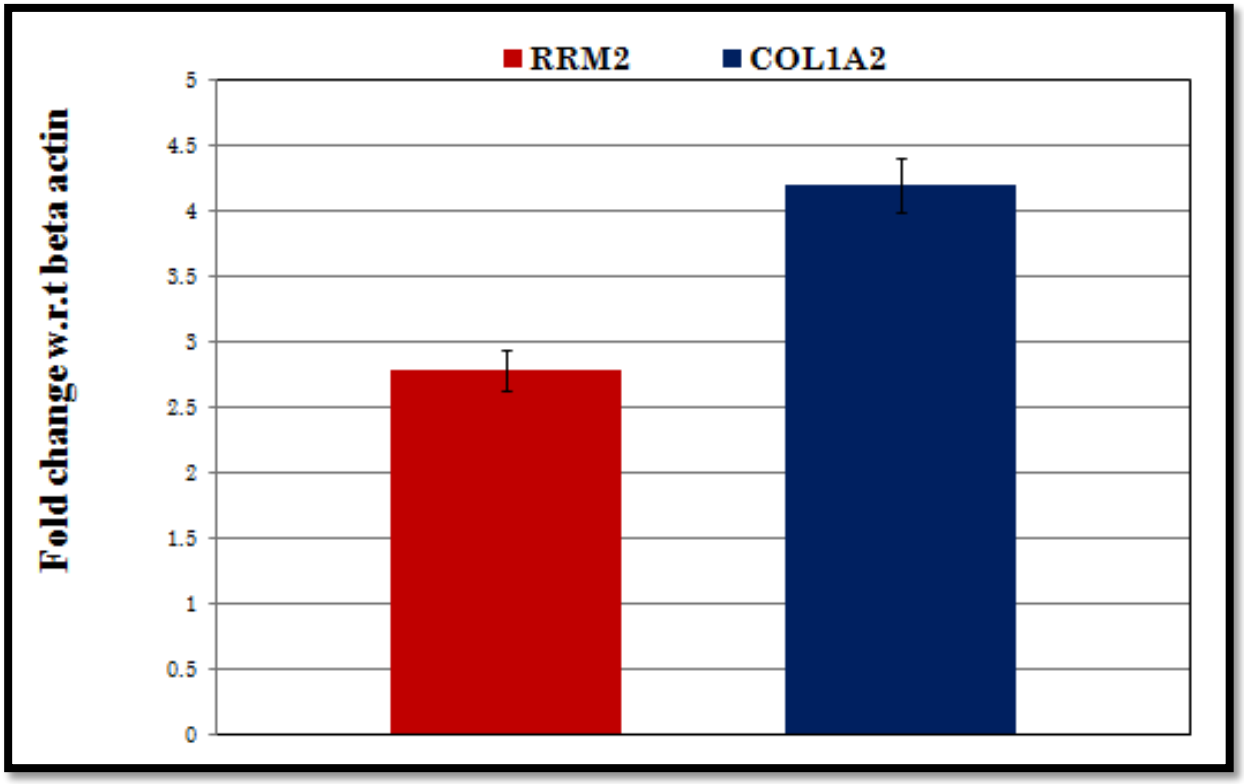

Figure 16: Result of qRT-PCR analysis of COL1A2 and RRM2 gene.

CONCLUSIONS

From myc targeting miRNA table we found out 7 miRNAs which are already reported in STS and obtained 265 targets of those miRNAs. Out of those 265 targets, 12 genes are having altered expression in STS and among them 6 genes are known and rest 6 are not yet validated in STS but they are having altered expression. From these 6 genes, 2 genes i.e. COL1A2 and RRM2 were selected for confirming their regulations in sarcoma cell lines through experimental validation. COL1A2 and RRM2 are reported to be over-expressed in other cancer systems. Through qRT-PCR, COL1A2 and RRM2 gene were found to be over-expressed in STS. That signifies the study as targeting miRNAs of COLIA2 and RRM2 i.e. hsa-let-7g and hsa-let-7b respectively, were previously reported to be down-regulated in STS. As COL1A2 and RRM2 were reported earlier as an angiogenesis inducing factor in most cancer systems and critical to tumor malignancy, so we hypothesize that the over-expression of this gene in STS may contribute to its metastatic spread and lethality and this over-expression can be attributed to altered expression of miRNA hsa-let-7g and hsa-let-7b that might have resulted in abnormal expression of MYC oncogene.

FUTURE PROSPECTIVES

Based on the current findings, further studies can be performed to understand and confirm the role of COL1A2 and RRM2 genes in regulating STS thereby legitimizing its role in angiogenesis and metastasis. Understanding the basis of regulation of these mRNA and their targeting miRNA may provide a clue towards discovering RNA-based therapeutic approach for STS. The effectiveness of miRNA based fibrosarcoma therapy can be a land mark in fibrosarcoma studies. Therapeutic value of this study can be further assessed by over expressing miRNAs in sarcoma cell lines thereby checking the proliferation rate along with the expression of the target genes. After successful execution of these in cell lines, it can be extended further to validate in animal models in order to check the level of angiogenesis. This novel approach can thus prevent the cancer progression at the initial stages and hence can reduce the adverse effect of STS. Subsequent analysis of this novel miRNA-target pair will enhance our understanding to manipulate pathways for treatment of fibrosarcoma through microRNA mediated therapeutics.

REFERENCE

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Begley, S. Archive for 2013.

Bernards, R., and Weinberg, R.A. (2002). Metastasis genes: a progression puzzle. Nature *418*, 823-823.

Cai, Y., Yu, X., Hu, S., and Yu, J. (2009). A brief review on the mechanisms of miRNA regulation. Genomics, proteomics & bioinformatics *7*, 147-154.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Ä□â,¬ Dissemination and Growth of Cancer Cells in Metastatic Sites, Ä□â,¬ Nat. Rev Cancer *2*, 563Ä • .

Esquela-Kerscher, A., and Slack, F.J. (2006). Oncomirsâ€"microRNAs with a role in cancer. Nature reviews cancer *6*, 259-269.

Garzon, R., Calin, G.A., and Croce, C.M. (2009). MicroRNAs in cancer. Annual review of medicine *60*, 167-179.

Gomes, A.Q., Nolasco, S., and Soares, H. Non-coding RNAs: multi-tasking molecules in the cell. International journal of molecular sciences *14*, 16010-16039.

Griffiths-Jones, S., Grocock, R.J., Van Dongen, S., Bateman, A., and Enright, A.J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic acids research *34*, D140-D144.

Gupta, G.P., and MassaguÃ©, J. (2006). Cancer metastasis: building a framework. Cell *127*, 679-695.

Helman, L.J., and Meltzer, P. (2003). Mechanisms of sarcoma development. Nature reviews cancer *3*, 685-694.

Hsu, S.-D., Lin, F.-M., Wu, W.-Y., Liang, C., Huang, W.-C., Chan, W.-L., Tsai, W.-T., Chen, G.-Z., Lee, C.-J., and Chiu, C.-M. miRTarBase: a database curates experimentally validated microRNAâ€"target interactions. Nucleic acids research, gkq1107.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2008). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols *4*, 44-57.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research *37*, 1-13.

Kyle, R.A., and Rajkumar, S.V. (2009). Treatment of multiple myeloma: a comprehensive review. Clinical Lymphoma and Myeloma *9*, 278-288.

Leber, M.F., and Efferth, T. (2009). Molecular principles of cancer invasion and metastasis (review). International journal of oncology *34*, 881-895.

Loh, M.L., Ahn, P., Perez-Atayde, A.R., Gebhardt, M.C., Shamberger, R.C., and Grier, H.E. (2002). Treatment of infantile fibrosarcoma with chemotherapy and surgery: results from the Dana-Farber Cancer Institute and Children's Hospital, Boston. Journal of pediatric hematology/oncology *24*, 722-726.

Lu, M., Zhang, Q., Deng, M., Miao, J., Guo, Y., Gao, W., and Cui, Q. (2008). An analysis of human microRNA and disease associations. PloS one *3*, e3420.

Lui, W.-O., Pourmand, N., Patterson, B.K., and Fire, A. (2007). Patterns of known and novel small RNAs in human cervical cancer. Cancer research *67*, 6031-6043.

Rappaport, N., Nativ, N., Stelzer, G., Twik, M., Guan-Golan, Y., Stein, T.I., Bahir, I., Belinky, F., Morrey, C.P., and Safran, M. MalaCards: an integrated compendium for diseases and their annotation. Database *2013*, bat018.

Ruepp, A., Kowarsch, A., Schmidl, D., Buggenthin, F., Brauner, B., Dunger, I., Fobo, G., Frishman, G., Montrone, C., and Theis, F.J. PhenomiR: a knowledgebase for microRNA expression in diseases and biological processes. Genome biology *11*, R6.

Sreekantaiah, C., Ladanyi, M., Rodriguez, E., and Chaganti, R.S. (1994). Chromosomal aberrations in soft tissue tumors. Relevance to diagnosis, classification, and molecular mechanisms. The American journal of pathology *144*, 1121.

Stancu, A.L., Smith, M., and Almasan, A. New agents for the treatment of lymphoid leukemia and lymphoma: focus on recent FDA approvals. Discoveries *2*, e14.

Steeg, P.S. (2003). Metastasis suppressors alter the signal transduction of cancer cells. Nature reviews cancer *3*, 55-63.

Vita, M., and Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. Paper presented at: Seminars in cancer biology (Elsevier).

Wang, J., Lu, M., Qiu, C., and Cui, Q. TransmiR: a transcription factorâ€“microRNA regulation database. Nucleic acids research *38*, D119-D122.

Weigelt, B., Peterse, J.L., and Van't Veer, L.J. (2005). Breast cancer metastasis: markers and models. Nature reviews cancer *5*, 591-602.

Xiao, F., Zuo, Z., Cai, G., Kang, S., Gao, X., and Li, T. (2009). miRecords: an integrated resource for microRNAâ€“target interactions. Nucleic acids research *37*, D105-D110.

Yim, R.L.-H., Kwong, Y.L., Wong, K.Y., and Chim, C.S. DNA methylation of tumor suppressive miRNAs in non-Hodgkinâ€™s lymphomas. Frontiers in genetics *3*.

Zhang, B., Pan, X., Cobb, G.P., and Anderson, T.A. (2007). microRNAs as oncogenes and tumor suppressors. Developmental biology *302*, 1-12.