UNDERSTANDING THE REGULATION OF ISOMIR AND MIRNA IN CML

Thesis submitted to the Jawaharlal Nehru University
for the award of the degree of

DOCTOR OF PHILOSOPHY

PAMCHUI MUIWO

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067,
INDIA
2017

SCHOOL OF LIFE SCIENCES
JAWAHAR LAL NEHRU UNIVERSITY
NEW DELHI-110067
INDIA

CERTIFICATE

The research work embodied in the thesis entitled **"Understanding the regulation of isomiR and miRNA in CML"** has been carried out in School of Life Sciences, Jawaharlal Nehru University, New Delhi.

This work is original and has not been submitted so far, in part or in full, for award of any degree or any diploma of any university.

PROF. ALOK BHATTACHARYA
(Supervisor)

ASSOC. PROF. ROHINI MUTHUSWAMI
(Co-Supervisor)

PAMCHUI MUIWO
(Candidate)

for PROF. S. K. GOSWAMI
19/7/17
(Dean)

ACKNOWLEDGEMENT

Over my six year long association with the following roll of indispensables, I have had the most wonderful experiences one could have in a PhD student's life. Each of the following mentioning deserves more than what my mere words could express. I could have had no better training had anyone of them been excluded out of the long, arduous, but memorable course of my research work here in Jawaharlal Nehru University campus in New Delhi.

Prof Alok Bhattacharya, my guide.
Working under the guidance of Alok sir has been a privilege. Every bit of his advice, encouragement, criticisms and his ability to stimulate scientific aptitude helped me immensely in achieving my goals. His commitment, wisdom and workmanship inspired and motivated me. I was a student to his logical thinking and his unique outlook for research problems. Besides the research skills, I learned two other things from him: the art of bird watching and interest in cooking. Apart from science I also learned a lot from him about life, history and philosophy. I am extremely fortunate to have had an opportunity to work under his supervision.

Dr. Rohini Muthuswami, who has been my co-supervisor ever since Prof Alok Bhattacharya retired, has given precious feedbacks and advices all along. She has been a continuous source of encouragement and support.

Prof Sudha Bhattacharya is an epitome of hard work and perseverance. I thank her for offering valuable suggestions and constructive criticism during lab-meetings. She is a great scientist and good mother – a role model for modern women.

Dr. Yogendra Kumar Gupta, Department of Pharmacology, AIIMS, New Delhi and **Dr Lait Kumar**, Department of Oncology, AIIMS, New Delhi, for providing the patient samples and all kinds of inputs during the course of study.

This letter of acknowledgement has a special place reserved at its core for mentioning the people who have made this abode, our Laboratory, a joyful place to be in. It has been a pleasure working with the humblest, among whom are the following:
Dr. Hafiz Ahmad
No words could ever match up or justify the favour he has bestow upon me. Without Hafiz sir's help it would be impossible to be in the position I am today. I thank him for helping me with laying the background work, for teaching me all the techniques patiently despite those mistakes I made innumerable times, and for his kindness and unconditional support at work. I am indeed very lucky to have worked under a senior like him. He had always been my defender in the wars of banter in our lab. He believed in me, and I am thankful for that.

My wonderful seniors – **Somlata, Saima, Arpita and Sahid** – who have been the source of encouragement, advice, company and all the laughs. They are more like a friend than a senior.

Dr. Suganthi Ramachandran
In a Lab where the talk was mostly on Amoeba, I had Suganthi with whom I could have great discussions on miRNAs. Her dedication toward her work was inspiring indeed. She was like our Lab doctor.

Priyatama Pandey
I thank her for doing all the bioinformatics work. I enjoyed working on miRNA project with her. She was very helpful and simplest of all.
Lab 118 members – **Somlata, Mrigya, Ravi, Aruna, Shalini A, Madhu, Sabir, Shalini S, Janhawi and Kapila**. I am indebted to them all. They made my stay in this Lab a joyful one. There was no junior-senior differentiation. We all, as friends, had a great time pulling each other's leg at every opportunity we got all day long. The members of Lab 118 are very hard working, supportive, ever ready to help, great discussant and have a sound scientific aptitude. I will always remember the chai time every afternoon with Lab 118 members and Gunjan – the best way to kill stress. It has been a joy to you met you all and I shall always remember you guys with a smile. You guys have been my second family. Thank you for that.

My colleagues from the SES Lab – **Amit, Ashwini, Devinder, Deepshikha, Shashi, Sarah, Mridula, Shraddha, YP, Mannit** and **Aparna** had been helping in troubleshooting in my RNA related work. I am grateful to them also for all the scientific discussions, and most of all their friendship.

I sincerely acknowledge **Jeevan Ji, Manoj Ji, Rinku Bhaiya, Ganesh ji and Umesh ji** for their help and making things available as and when required. Also my thanks are due to Om **Prakash Ji and Madhu** for their technical assistances. **Tiwari sir** deserves a special mention for his ever helping nature. I thank the CIF members, especially **Tripti,** for the confocal work in SLS, and other staffs at **AIRF, JNU** too.

I would like to convey my heartfelt thanks to **Jawaharlal Nehru University**, for providing me with the ideal environment to grow as a person. I would like to acknowledge **Department of Biotechnology (DBT)** for the fellowship during my Ph.D., and **Department of science and Technology (DST)** for providing scholarship in 9B.

Besides my scientific world, I want to thank my family for their unconditional support and prayer. Their love and prayer kept me going. I am what I am because of them. I cherish the sweet little times I got to spend with them in this phase. And I am sorry for the times I couldn't be around when you folks needed me, but I hope it was a worthy sacrifice for us all. Thank you for everything. I am also thankful to Ngachonshem Jagoi for sharing the burden of the path I took, for being the emotional cushion, for the encouragements, for the patience and for believing in me. Thank you for proof reading all my writings too. Thank you Apei and Kim. Sabarmati hostel has been colourful because of you two; always a joy to be with you guys. The feminism classes from Apei during our evening walks will always be remembered.

Above all, I thank God for making all these happen. He leads me beside green pasture and nurtures me. It has been Your art work all this time.

Pamchui Muiwo
SLS, JNU
New Delhi

Table of Contents

GENERAL INTRODUCTION

1.1.0 MicroRNA introduction

MicroRNA is a small regulatory non-coding RNA molecule of 21-24 nucleotides that modulates the expression of a genes by binding mostly at their 3' UTR. The first miRNA, Lin-4, was discovered in *Caenorhabditis elegans* by Ambros et al in 1993 (Lee, Feinbaum et al. 1993). Lin-4 was found to negatively regulate Lin-14 protein expression, a gene that controls the development timing in *C. elegans*. However, it was only after 7 years later that another miRNA Let-7 was discovered, which controls the transition of late larvae to adult cell in the same system. This miRNA was reported to be conserved in a wide range of animal species (Pasquinelli, Reinhart et al. 2000). Since then there has been a mass of research on miRNA in diverse species. In 2009, 940 distinct miRNAs were reported in human genome (Macfarlane and Murphy 2010). The latest release, *miRBase* (*release 21,* June 2014), contains 28,645 entries from 223 species. In this release, 1881 precursor and 2588 mature miRNAs (http://www.mirbase.org) have been reported for humans. Most of the annotated miRNAs are experimentally validated and only few are predicted sequences homologs of miRNAs verified in a related organism (Griffiths-Jones, Grocock et al. 2006). MiRNAs are involved in various biological process like developmental timing, cell proliferation, signal transduction, hematopoiesis, apoptosis, and tumorigenesis (Sempere, Freemantle et al. 2004). They are known to regulate 60% of the total protein coding genes in human and each miRNAs can regulate many target gene (Bartel 2009). MiRNAs seems to be the key player in cancer development and progression. Deviant expressions of these miRNAs are found in various human cancers, both solid tumours and hematological malignancies. And their roles in pathogenesis of cancer, prognosis and treatment response are being extensively studied (Lu, Getz et al. 2005, Lin and Gregory 2015).

1.2.0 MiRBase

miRBase database is an archive of miRNA sequences and annotations. MiRBase provides three services. **MiRBase Registry** – this service provides a consistent and unique names for all novel miRNAs to prevent overlapping of a miRNA name. It follows an officially accepted nomenclature for naming a miRNA. The naming is in the form of XXXX-miR-YYY, where XXXX represents three or four letter species code; miR or mir represent the mature and precursor hairpin respectively; YYY represents the order of their discoveries. If an identical mature miRNA, sequence arise from two different genomic loci. They are given numerical suffixes, such as has-mir-10-1, has-mir-10-2 *(Fig. A.).* MiRNA sequence with

only one or two nucleotide changes are assigned suffixes in the form of, hsa-miR-34a and hsa-miR-34b. Also the 5p or 3p denotes from which arm of the hairpin the mature miRNA arises, as in miR-24-5p and miR-24-3p (Griffiths-Jones 2004). **MiRBase sequence** is a primary repository database for all published miRNA – the sequence, the precursors and annotation related to their discovery, genomic locations, structure and function. **MiRBase target** provides an automated pipeline for prediction of the target genes of miRNAs. (Griffiths-Jones, Grocock et al. 2006).

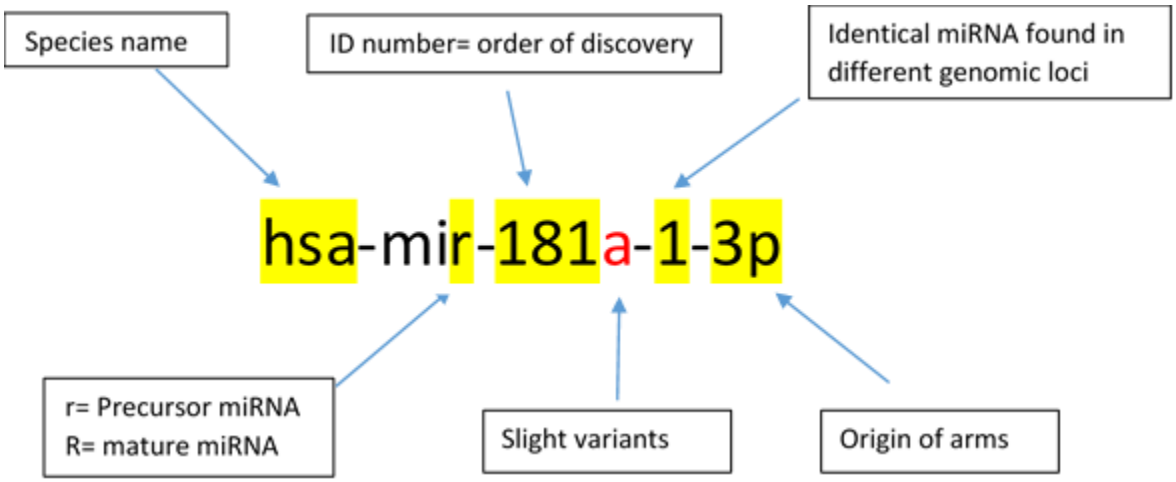


Figure A: Schematic representation of miRNA nomenclature.

1.3.0 MicroRNA genome

The miRNA genes is one of the most abundant gene families, evolutionary conserved and are extensively distributed in animals, plants, protists and viruses (Griffiths-Jones, Grocock et al. 2006). They regulates around 60% of the human genes (Friedman, Farh et al. 2009). MiRNAs are found frequently in the intergenic regions and introns of protein-coding genes; less commonly found in exons and antisense of a transcripts. MiRNA genes are un-uniformly distributed in the genome and around 30% of the miRNA genes are clustered in human genome (Laddha, Nayak et al. 2013). They are often transcript as one unit i.e. a polycistronic transcription unit (Lagos-Quintana, Rauhut et al. 2003, Rodriguez, Griffiths-Jones et al. 2004). A study has also showed that miR-548 and miR-1302 originates from transposable element (Ahn, Gim et al. 2013). Often multiple miRNA loci with related sequences were found in the genome of many species that arise due to gene duplication. And they generally have identical seed sequences (2-8 nt) and belong to same ‘miRNA family’.

For instance, 14 paralogous loci that belong Let-7 family has been found in human (Bartel 2009).

Earlier, miRNA gene discovery relied on classical methods such as cloning size fractionated RNA, Sanger sequencing, conservation across the species and bioinformatics to locate the gene in the genome (Lee and Ambros 2001). Later, a web interface was used to scan the genome for the presence of a stem loop like structure for the identification of miRNA genes, some of these tools are CID-miRNA (computational identification of miRNA), miRscan and MiRseeker (Lai, Tomancak et al. 2003, Tyagi, Vaz et al. 2008). With the advent of next generation sequencing (NGS) the identification of miRNA has become much easier, more sensitive and quicker. The reads of the small RNA sequencing are aligned with the reference miRNA database (*miRBase)* to look for expressed miRNA. This has the advantage of identifying the lowly expressed miRNAs, the novel miRNA and the variants of miRNA such as isomiRs (Vaz, Ahmad et al. 2010, Vaz, Ahmad et al. 2013).

1.4.1 MicroRNA biogenesis

MicroRNA is transcribed mostly by RNA polymerase II-associated transcription factors and epigenetic regulators. The conventional biogenesis pathway consist of two endonuclease cleavage events, one in nucleus and other in cytoplasm. The RNA pol II and sometime pol III transcribed miRNA genes to produce primary miRNA (pri-miRNA), it is several kilo-bases long, capped, sliced and polyadenylated (Lee, Ahn et al. 2003), (Lin and Gregory 2015). Some miRNA like viral miRNA and miRNA derived from tRNA are transcribed by RNA pol III (Lin and Gregory 2015). The intronic miRNAs are transcribed long with the host genes whereas intergenic miRNA has their own promoter and they are mostly polycistronic with their own distinct 5’ and 3’ boundaries, 7-methyl guanylate (m7G) caps and poly(A) tails (Lagos-Quintana, Rauhut et al. 2003). An overview of canonical miRNA synthesis pathway is shown in figure B.

1.4.2 Microprocessor complex: The transcribed pri-miRNA usually contains a stem of 33-35 bps, a terminal loop and single stranded nucleotides at both 5’ and 3’ ends. This pri-miRNA is processed to ~70 nucleotide long hairpin structure (pre-miRNA or precursor miRNA) by nuclear RNase III type protein, Drosha, with the help of DiGeorge syndrome critical region gene 8 (DGCR8) protein, together known as the microprocessor, a

heterotrimer composed of one Drosha and two DGCR8 molecules (Pasha in *D. melanogaster* and *C. elegans*) (Lee, Ahn et al. 2003, Han, Lee et al. 2004, Li and Patel 2016). DGCR8 known as the "molecular ruler" helps Drosha to recognize ssRNA-dsRNA junction as well as the distance from terminal loop region in pri-miRNA and the nuclease Drosha which has two RNase III domain (RIIIa and RIIIb), cleaves approximately 11 base pairs away from basal junction and approximately 22 base pairs away from apical junction linked to terminal loops to produce pre-miRNA with an overhang of either 1 or 2 nucleotides at the 3′ end (Han, Lee et al. 2006, Burke, Kelenis et al. 2014). Drosha has a weak dsRNA binding domain at the C terminal which is generally augmented by DGCR8. DGCR8 also gives stability to Drosha (Li and Patel 2016). Recently it has been shown that some sequence motifs are present in the terminal loop (UGUG motif) and the ssRNA-dsRNA junction (UG and CNNG motif) which are found in 79% of human pri-miRNA. They help in proper positioning and direct cleavage by microprocessor complex in the nucleus (Ha and Kim 2014). Many other accessory proteins are involved in processing of pri-mRNA to pre-miRNA such as ADAR1&2, ALL/AF$, Ars 2, KSRP, Lin-28 p53, etc. (Siomi and Siomi 2010). Other non-canonical pathway also occurs, a small group of miRNA (mirtrons) have been discovered in introns, which are transcribed with the host gene and are later spliced out by forming a lariat structure (Winter, Jung et al. 2009). Another set of miRNAs known as "*5' capped pre-miRNA*" including miR-320 and miR-484 have been reported. They are microprocessor independent miRNAs. The 5' end coincide with the transcription start site, and 3' are thought to be generated by transcription termination. These pre-miRNA are exported by Exportin 1 and further processed by Dicer (Xie, Li et al. 2013). After Drosha processing, the pre-miRNAs are transported to the cytoplasm for maturation. The transportation is carried out by the protein Exportin 5 (XPO5) complex that also has a GTP binding nuclear protein RanGTP (Lund, Guttinger et al. 2004, Okada, Yamashita et al. 2009). Following translocation through the nuclear pore, GTP is hydrolysed and this results in disassembly of the complex and release of the pre-miRNA into the cytosol (Okada, Yamashita et al. 2009).

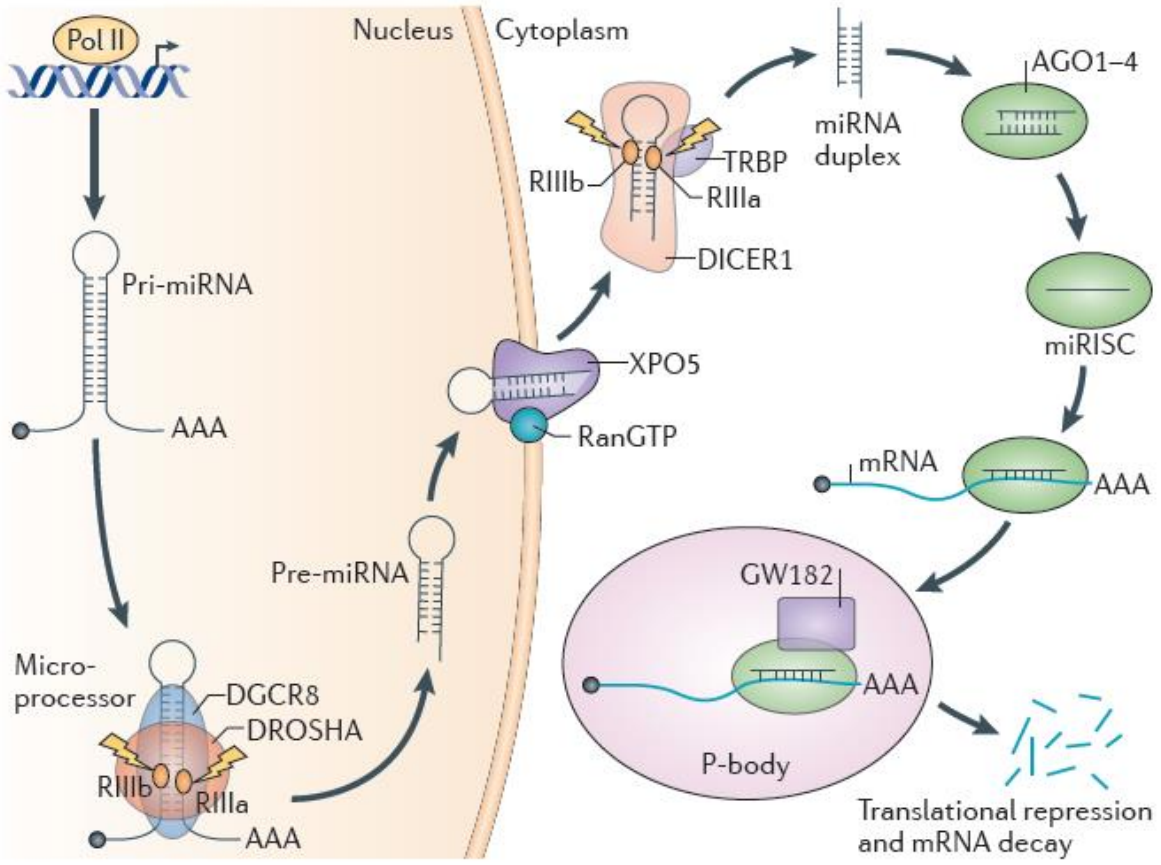


Figure B: An overview of microRNA biogenesis pathway. It is a two-step process occurring both in nucleus and cytoplasm by two different RNase III endonuclease enzyme – Drosha and Dicer (Image adpated from Shuibin Lin et al 2015)

1.4.3 Dicer processing in cytoplasm: In the cytoplasm, pre-miRNAs are processed by Dicer, releasing ~22-nt miRNA duplexes (Yates, Norbury et al. 2013). Dicer is found to contain two pockets that bind pre-miRNAs, the 3' end binding pockets in PAZ domain and the 5' end binding pocket located in both PAZ and platform domain (Macrae, Zhou et al. 2006, Park, Heo et al. 2011). The PAZ and RNAIII domain are important for determining the length and cleavage of the substrate (Kurzynska-Kokorniak, Koralewska et al. 2015). Dicer has been shown to interact with trans activating response RNA binding protein (TRBP) (Chendrimada, Gregory et al. 2005) and PACT (also known as PRKRA) (Lee, Hur et al. 2006). These interacting partners are not required for processing activity, but they contribute to RISC (RNA-induced silencing complex formation) (Lee, Hur et al. 2006). TRBP is known to alter the kinetics and the cleavage site of Dicer (Fukunaga, Han et al. 2012, Lee and Doudna 2012). Some pre-miRNA are processed independent of Dicer but depend on Drosha for pri-miRNA processing. Mature miR-451 is known to be generated from pre-miR-451 by catalytic activity of Ago2 (Cifuentes, Xue et al. 2010). Other factors interacting with Dicer

are lin-28, a KH-type splicing regulatory protein (KSRP), TAR DNA-binding protein-43 (TDP-43) and ADAR family protein, MCPIP1 (monocyte chemoattractant protein [MCP]-1-induced protein 1), some of these may be specific for processing of few pre-miRNA (Kurzynska-Kokorniak, Koralewska et al. 2015).

1.4.4 RISC complex: The small RNA generated by Dicer are then loaded onto Ago protein to form a complex called RISC (RNA Induced Silencing Complex)(Yates, Norbury et al. 2013). In humans, the Dicer, TRBP and Ago 2 form the core of the RISC-loading complex (RLC). The small regulatory miRNA guides the RISC complex to its target mRNA. The Dicer and TRBP are found to stimulate target RNA processing by Ago 2, while some other studies suggested dissociation after the RNA duplex is loaded onto Ago 2 (Kurzynska-Kokorniak, Koralewska et al. 2015). Both the strands (5p or 3p) can be loaded into Ago 2 and be functional (Ro, Park et al. 2007).

1.5.0 MiRNA-mRNA interaction

There are many factors that influence the interaction of miRNA-mRNA in animals. Understanding this interaction is required to predict the target gene of the miRNA with minimum false prediction. Here are few parameters that should be considered for accurate target prediction.

1. **Seed pairing/ canonical sites:** The 2 to 8 nucleotides in the 5' ends of miRNA, known as the seed region or 6mer site, play an important role in determining the target gene (Lewis, Shih et al. 2003, Stark, Brennecke et al. 2003). The seed region binds the 3' UTR of the target gene with perfect complementary, which requires little or no 3' pairing. The 7mer site is seed region in addition to 8^{th} nucleotide of miRNA matched with the target (7mer-m8 site) or seed region with A at the mRNA position complementing the 1^{st} nucleotide of the microRNA (7mer-A1 site). In 8mer site the seed region is flanked by both the match at 8 nt of miRNA and A at the position 1 (Grimson, Farh et al. 2007, Agarwal, Bell et al. 2015). The base pairing around the seed region is considered to be the most essential parameter in miRNA-mRNA interaction (Lewis, Shih et al. 2003, Grimson, Farh et al. 2007, Yue, Liu et al. 2009). Apparently, there is a positive correlation between the GC content and base pairing, the more GC content the stronger is the binding (r- 0.91), few targets pairs with the seed region when the GC content is low (Wang 2014).

2. **Non-canonical sites:** Often there is a bulge, mis-match or wobble G:U pairing at the seed region that leads to partial complementary. Such phenomena is strongly complemented by a pairing at 3' end (Vella, Choi et al. 2004, Brennecke, Stark et al. 2005). A study has shown that interruption of the 3' pairing of miRNA but with intact seed region, loses the capability to down regulate the target gene (Didiano and Hobert 2006). The central sites (4 to 15nt) interactions are known to happen more frequently than expected and are evolutionary conserved (Martin, Wani et al. 2014). Most of the miRNA target gene interaction exhibit a non-canonical interaction (Helwak, Kudla et al. 2013, Martin, Wani et al. 2014, Wang 2014).
3. **Evolutionary conservation:** In many cases the orthologous sites of the seed binding region, especially the 3' UTR, are conserved across the species (Lewis, Burge et al. 2005, Friedman, Farh et al. 2009), and to some extend the offsite seed match and 3'-compensatory sites are also conserved (Friedman, Farh et al. 2009, Ghoshal, Shankar et al. 2015). The seed binding sites in mRNA are often flanked by Adenine residues (Lewis, Burge et al. 2005). Many tools like TargetScan, Diana and PicTar rely on this parameter.
4. **Thermodynamic energy:** The free energy of the miRNA-mRNA duplex predicts that if the interactions are stable then they are likely to be a binding partner. It searches for a minimum free energy hybridization (Rehmsmeier, Steffen et al. 2004, Ghoshal, Shankar et al. 2015).
5. **The secondary structure of target site:** The RISC complex assembly and the unpairing of intra pairing of mRNA for new miRNA-mRNA pairing can be effected by the secondary structure of the transcript (Vella, Reinert et al. 2004, Robins, Li et al. 2005).

6. **Site accessibility:** A study has shown that insertion of ~200bp fragment near the binding site decreases the repression comparable to the seed region mutation (Kertesz, Iovino et al. 2007). The energy of miRNA-mRNA hybrid, ΔG duplex, shows poor correlation to repression ($r = 0.36$), but taking site accessibility into consideration in this complex, $\Delta\Delta G$, equal to the difference between the free energy gained by the binding of the miRNA to the target, ΔG duplex, and the free energy lost by unpairing the target site nucleotides, ΔGopen, shows high correlation to target repression ($r = 07$) (Rehmsmeier, Steffen et al. 2004, Hofacker 2007, Kertesz, Iovino et al. 2007, Ghoshal, Shankar et al. 2015). In animal genomes some target genes are preferentially found in highly accessible region, and this may be one reason why the target strength varies (Kertesz, Iovino et al. 2007).

7. **Proximity of the target sites:** The number of binding sites in the transcript is positively correlated to the levels of mRNA destabilization (Grimson, Farh et al. 2007). And if the two seed regions are closer (between 13 and 35 nt), the repression is greater (Grimson, Farh et al. 2007, Saetrom, Heale et al. 2007), and these sites are favourably co-conserved (Grimson, Farh et al. 2007).
8. **Combinatorial effect:** Mixture of different miRNAs could more efficiently repress the target gene compared to a single miRNA, and the miRNAs that are co-expressed are likely to regulate the same target gene (Krek, Grun et al. 2005).
9. **Expression database:** Expression data from high throughput screening has contributed in quicker and easier target prediction. For instance, incorporating the expression data of miRNA and mRNA like GenMir++, taking advantage of the fact that their expression should be inversely correlated (Huang, Babak et al. 2007), the miRNA overexpressing transcriptome like *Targetscore* (Li, Goldenberg et al. 2014) and the cell type specific expression of miRNA or the gene (Ovando-Vazquez, Lepe-Soltero et al. 2016). However, the Argonaute protein cross-linking and immunoprecipitation (Ago-CLIP) can detect the specific sites involved in the Ago-miRNA-mRNA complex (Khorshid, Hausser et al. 2013). Also a study also has reported the need of incorporating Ago protein in the target recognition process. MiREN, an algorithm for building and scoring the 3D structure of a tertiary complex, miRNA-mRNA bound to Ago. Scoring is done based on number of base pairing, the clashes between RNA and protein and atom outside the binding site of Ago, and the estimated energy. The scores are used to predict the target gene (Parker, Parizotto et al. 2009, Leoni and Tramontano 2016).

Using these known information many algorithm have been generate to predict the target gene with minimum false positive, few of the software are given below.

Table 1: List of few tools for miRNA target prediction

Tools	**Organism**	**Principle**	**specificity**	**reference**
PicTar	Vertibrates, fly, nematode	Seed match, conservation of seed site, free energy, combinatorial effect	76%	(Krek, Grun et al. 2005)

RNA22	Human, mouse, fly, worm	Multiple, distinct, statistically significant pattern in target site	81%	(Miranda, Huynh et al. 2006)
PITA	Human, fly, mouse, worm	Site accessibility, thermodynamics	-	(Kertesz, Iovino et al. 2007)
DIANA microT	Human, mouse, worm	Thermodynamic	65%	(Paraskevopoulou, Georgakilas et al. 2013)
miRanda	human, worm, rat, fly, mouse	Complementary binding, canonical and non-canonical binding	76%	(Enright, John et al. 2003, Betel, Wilson et al. 2008)
miRTarBase	Any	Experimentally validated miRNA-target interaction	-	(Chou, Chang et al. 2016)
ComiR	Human, fly, worm, mouse	miRNA expression data, thermodynamic and machine learning techniques, PITA, TargetScan, miRanda	-	(Coronnello and Benos 2013)
GenMiR++	Any	Expression dataset based on Bayesian algorithm		(Huang, Babak et al. 2007)

1.6.0 Mechanism of gene regulation

Based on computer modelling miRNA can regulate 60% of the coding genes in mammalian genome (Macfarlane and Murphy 2010). They have been recognised as a major regulator of cellular processes. MicroRNA expression are dysregulated in many diseases including cancer. They regulate the gene expression by two mechanisms, translation repression and mRNA degradation. MiRISC arrests translation initiation by Ago 2 competing with eIF4E for 5' capped region (Mathonnet, Fabian et al. 2007). Though Ago2 does not bind the cap directly, it is mediated by some other factor (Eulalio, Huntzinger et al. 2008) . A study has

shown that miR-125b and let-7 can accelerate the deadenylation of the poly (A) tail and this decay is proposed to be the consequence of the interaction with miRNA complex (Wu, Fan et al. 2006) leading to removal of the 5' cap and then expose to exonucleolytic enzymes, and prevent circularization of mRNA needed for translation to occur. These complexes are stored in P bodies. P bodies can both store and degrade the repressed miRNA (Pillai, Bhattacharyya et al. 2005). A study also has shown that the stages of translation repression of the target gene (initiation phase or post-initiation of protein synthesis) depends on the promoter of the gene (Seggerson, Tang et al. 2002, Kong, Cannell et al. 2008). Animals can induce degradation of the target gene even if it is a partial complementary between the miRNA-mRNA complexes. The Ago protein, miRNA and the target gene are found to be sequestered in P bodies, which may be degraded eventually. The degradation depends on the slicer activity of the Ago 2 and reduces abundance of mRNA. Other mechanisms such as deadenylation, decapping, and exonucleolytic digestion are also involved (Wahid, Shehzad et al. 2010).

1.7.0 MicroRNA in Cancer

Cancer is always assumed to be the caused by genetic and/or epigenetic changes to the oncogenes or the tumor-supressor. But recent studies on the miRNAs that regulates the genes involved in cancer has revealed an additional layer of complexity to the disease development. MicroRNAs curbed the disease by inhibiting the expression of the proto-oncogenes, relieving the inhibition of tumor-supressor genes and sometime act like a transcription factor to regulate a diverse signalling pathways. In cancer cells the expression of miRNA are deregulated (Vaz, Ahmad et al. 2010, Vickers, Sethupathy et al. 2013). A number of studies have correlated miRNA abundance with cancer progression that is, more miRNAs more tumorigenesis. Jun Lu et al 2005 had analysed 217 mammalian miRNAs from 334 samples, including multiple human cancers, and came to the conclusion that tumors showed general downregulation of miRNAs compared to healthy tissues (Lu, Getz et al. 2005). Another study has shown that miRNA cluster miR-379/miR-656 which consist of 50 miRNAs are downregulated in multiple cancers (Laddha, Nayak et al. 2013). In our previous studies we have showed that expression of total miRNAs are restricted in CML. The aberrant expression of miRNAs in many cancer cells may be due to gene deletion or amplification, genetic mutation or epigenetic mechanism. Human miRNA gene are often found in fragile site of the genome. A study showed that 50.25% of miRNA genes are in

cancer-associated genomic region (translocation breakpoints, repetitive element and CGp island) moreover miR-15a and miR-16a genes are deleted in B cell chronic lymphocytic leukemia (Calin, Sevignani et al. 2004, Lagana, Russo et al. 2010). Also chromosome 13q14 is often deleted in ~50% of the mantle cell lymphoma, in 60% of prostate cancers and in 40% of multiple myeloma (Calin, Sevignani et al. 2004). Almost half (45%) of the epigenetically regulated miRNAs are found to be involved in different types of cancer (Kunej, Godnic et al. 2011).

Besides the genetic alterations of the miRNA genes, the impairment of miRNA biosynthetic machinery are also often reported in cancerous cells and they play an important role in carcinogenesis in many tumor (Horikawa, Wood et al. 2008, Ahmad, Muiwo et al. 2017). The global repression of the miRNA processing machinery (Drosha, DGCR8, DICER1, TRBP and XPO5) promotes transformation and tumorigenesis (Kumar, Lu et al. 2007, Melo and Esteller 2011). Drosha mutation (Zhang, Hou et al. 2016), low expresion (Torrezan, Ferreira et al. 2014) and deletion (Kim, Kim et al. 2016) has been reported in many cancer and they are poorly correlated with survival (Czubak, Lewandowska et al. 2015). Dicer downregulation was correlated with decreased survival and poor prognosis in CLL, non-small cell lung cancer, hepatocarcinoma and breast cancer (Dedes, Natrajan et al. 2011, Zhu, Fan et al. 2012) . High expression of Dicer was also reported in prostate adenocarcinoma (Chiosea, Jelezcova et al. 2006) and AML (Acute myeloid leukemia) (Martin, Payton et al. 2009). Also miRNAs are also regulated by transcription factors like p53, c-Myc and fos B (Melo and Esteller 2011, Ahmad, Muiwo et al. 2017). A study showed that 25% of the differential miRNA expression could be attributed to change in transcription in B cells (Kuchen, Resch et al. 2010).

MicroRNAs are well studied in CML (Chronic Myeloid Leukemia, details in chapter 3). The oncogenic miR17/92 cluster is known to be overexpressed in CP (chronic phase) but not in BC (blast crisis), and they have been found to be involved in BCR-ABL dependent pathways (Venturini, Battmer et al. 2007). But other studies have shown upregulation of m*iR-17*, *miR-19a*, *miR-19b*, and *miR-20a* during the BP (blast phase) of CML (Machova Polakova, Lopotova et al. 2011). The expression of miR-150 is known to impact the survival rate of CML patients. It is downregulated in CML, but in response to Imatinib treatment, miR-150 level goes up; an indication that miR-150 could be a diagnostic marker for CML (Fallah, Amirizadeh et al. 2015). MiR-150 expression is not specific for CML, it is known to be

widely deregulated in many cancers such as gastric cancers, colorectal cancer, breast cancer and endometrial cancer (Wang, Ren et al. 2015). The promoter of miR-203 is hypermethylated in several hematopoietic tumors including CML and ALL. MiR-203 is also known to target the 3'UTR of the ABL and BCR-ABL transcripts, thus acting as a tumor suppressor (Bueno, Perez de Castro et al. 2008, Fu, Zhang et al. 2016). In relation to imatinib resistance a study has found that miR-181c is downregulated in imatinib resistance when compared to imatinib responder. Mir-181c targets genes like *PBX3, HSP90B1*and *NMT2* which are involved in drug response (Mosakhani, Mustjoki et al. 2013).

1.8.0 isomiR introduction

1.8.1 IsomiR definition:

IsomiR, a term coined by Morin et al (Morin, O'Connor et al. 2008), are variants of canonical miRNAs that differ by 1 or 2 nucleotide at the 5' end, 3' end or within the miRNA body (Soifer, Rossi et al. 2007, Shah, Leidinger et al. 2010, Profumo and Gandellini 2013). IsomiRs are synthesized as the canonical miRNAs with few modification mostly after the dicer processing (Cloonan, Wani et al. 2011, Fukunaga, Han et al. 2012, Neilsen, Goodall et al. 2012, Vaz, Ahmad et al. 2013). There are different types of isomiR *(Fig. C).* The templated isomiRs are those isomiRs whose sequence variants are derived by altering the cutting site of the pre-miRNA, variations can be at 5' end or the 3' end. Non-template isomiRs are those with nucleotide added mostly at the 3' end of the canonical miRNA, non-templated 5' end addition being rare. In rare case internal isomiRs sequence can vary and they care called polymorphic isomiRs, these arise due to RNA editing (Cloonan, Wani et al. 2011, Fukunaga, Han et al. 2012). The 5' isomiRs are known to occur less frequently (5-15%) compared to 3' isomiRs (~40-50%) in three stem cell libraries and only 22% of the target genes were common between the 5 'isomiRs and canonical miRNA (Tan, Chan et al. 2014). The abundance of 3' isomiRs is likely because of the most of the nucleotidyl transferases adds nucleotide in 5' to 3' directions. The shift in the seed region (in case of 5' isomiR) is known to impact the target gene selection. The existence of the variants of miRNAs were not known until the advent of next generation sequencing, and it was initially discarded as sequencing artefacts. But recently a number of research papers have been published reporting their existence.

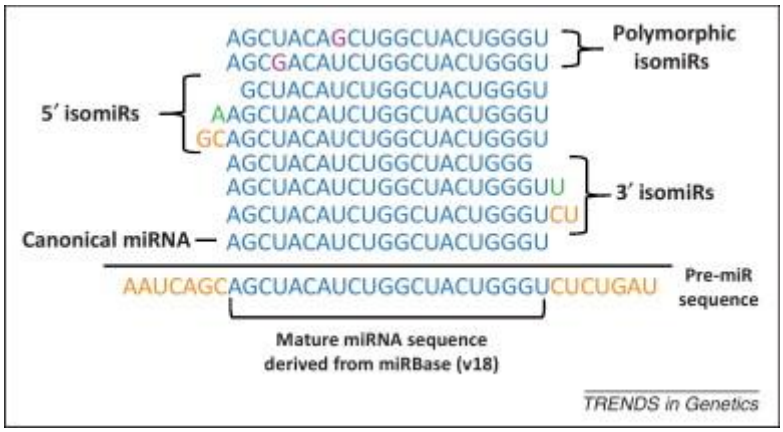


Figure c: Schematic representation of isomiR species. From a single parent pre-miRNA different types of isomiRs could be genereated. (Neilsen, Goodall et al. 2012).

1.8.2 IsomiR biosynthesis

As single miRNA locus can give rise to many isomiRs that different in length and in sequence composition. The imprecise cleavage by Drosha and Dicer usually creates mature templated 5' and 3' isomiRs of varying lengths (Fernandez-Valverde, Taft et al. 2010, Starega-Roslan, Galka-Marciniak et al. 2015). However, other secondary processes are also involved in its generation. The structure of the precursor sequence gives rise to length (Starega-Roslan, Krol et al. 2011, Ma, Wu et al. 2016). The sequence feature around the Drosha and Dicer cleavage sites helping in diversifying miRNA populations. Dicer RNase IIIA domains avoid discharging miRNAs with G nucleotide and favours to generate miRNA with U nucleotide at the 5' end (Starega-Roslan, Galka-Marciniak et al. 2015). Dicer is known to processe different substrates at rates that vary by ~100-fold and TRBP could enhance the dicing of the substrate. TRBP alters the kinetics and selection of cleavage site activity of Dicer (Fukunaga, Han et al. 2012, Lee and Doudna 2012). A study has shown that TRBP helps in generation of isomiR that is one nucleotide longer than the reference miRNA (Lee and Doudna 2012). The non-template isomiRs are likely to be generated due to nucleotidyl transferases (PAPD4, PAPD5, ZCCHC6) or exonucleases such as PARN, Nibbler and QIP or the RNA editing activity of enzymes, such as ADAR that may or may not be specific for a particular miRNA (Fernandez-Valverde, Taft et al. 2010, Wyman, Knouf et al. 2011). A study has shown that eukaryotic MID domain of Ago2 could interact with the 5' end of miRNA and efficiently discriminate the different monophosphate (AMP, CMP, GMP and UMP) by binding assay. Ago2 could bind AMP and UMP with 30 fold higher affinity than GMP and CMP (Frank, Sonenberg et al. 2010, Seitz, Tushir et al. 2011).

Exonucleases: A 3'-5- exonuclease enzyme, Nibbler is known to trim the miRNAs bound to Ago 2 complexes in Drosophila (Liu, Abe et al. 2011). The knockdown of Nibbler has been reported to associate with the loss of many 3' isomiRs, showing the involvement of nuclease in synthesis of these variants.

Nucleotidyl transferases: These are template independent polymerases that adds ribonucleotides to the ends of the RNA molecule. In human, twelve nucleotidyl transferases are known, out of which seven are associated with isomiR synthesis (Neilsen, Goodall et al. 2012). Most of the polymerases shows uridyltransferase and/or adenyltransferase activity and also majority of the 3'isomiRs observed are uridylation and adenylation. In breast cancer cell line, oncogenic miR-21 is posttrancriptionally adenylated by PAPD5 (PAP associated domain containing 5) and then trimmed by PARN (poly(A)-specific ribonuclease). The degradation of miR-22 by tailing and trimming is disrupt in many cancer cells (Boele, Persson et al. 2014).

RNA editing: The deamination of Adenine to Inosine (A-I) is also prevalent in eukaryotes. Both pri-miRNA and pre-miRNA are targets for RNA editing enzymes. A study shows that 20% of the human pri-miRNAs are subjected to A-I edited (Kawahara, Zinshteyn et al. 2007). Moreover, another reports has shown that a tissue-specific A-I editing within the seed region of miR-376 resulted in predominant isomiR generation and this new isomiR could target a different gene (Choudhury, Tay et al. 2012). But the frequency of editing in mature miRNA is very less or difficult to distinguish from background sequencing error (Nishikura 2016). A comprehensive analysis of over 700 million human small RNA reads shows 44 novel miRNA editing sites (including 11 within seed regions) and an additional ~2500 editing sites within 3′ UTRs that may affect miRNA binding sites (Peng, Cheng et al. 2012). But little is known about the edited miRNAs.

Single nucleotide polymorphism (SNPs): Bioinformatics analysis of the human genome using public SNPs data a group has shown that miRNAs has a low density of SNPs in their seed region but target genes has many SNPs (Saunders, Liang et al. 2007). Approximately 400 SNPs were found in experimentally verified target genes (at the binding site) and around 250 SNPs that potentially creates a novel target sites for miRNAs. In our earlier studies 31 SNPs were found in the 3'UTR of the oncogenic gene NET1 (neuro-epithelial transforming gene 1) and one particular SNP was mapped to the binding region of miR-22. This SNP could relief NET1 from miR-22 repression (Ahmad, Muiwo et al. 2014).

1.8.3 IsomiR as effector

IsomiRs are biologically active molecules. They are known to interact with the RISC complex and also with translational machinery polysomes, showing that they interacts with transcripts (Cloonan, Wani et al. 2011, Llorens, Banez-Coronel et al. 2013, Loher, Londin et al. 2014). 5' isomiR is shown to target independently of the canonical miRNA due to its shift in seed region though there are some common targets (Humphreys, Hynes et al. 2012, Manzano, Forte et al. 2015). The level of target repression seen in SNPs at the seed region can be compared with that of the 5'isomiRs (Jones, Quinton et al. 2009, Chan, Lin et al. 2013, Karali, Persico et al. 2016).

The 5' isomiRs like miR-142 and miR-133a has differing target gene and both are validated by luciferase assay (Humphreys, Hynes et al. 2012, Manzano, Forte et al. 2015). The functional synergy of the reference miRNA and its 5' isomiR helping the progression of breast cancer is also reported (Salem, Erdem et al. 2016) . The canonical miR-34/449 and 5' isomiR-34/449 showed drastic change in target gene recognition in human airway epithelial cells (Mercey, Popa et al. 2017). As mentioned above, most of the miRNA target gene interaction exhibit a non-canonical interaction (Helwak, Kudla et al. 2013, Martin, Wani et al. 2014, Wang 2014). The 3' portion of the miRNA can complement the central miRNA pairing of 13-17 nucleotides with continuous Watson-Crick pairing or compensate for single nucleotide bulge or mismatch in the seed region. The 3' pairing is insensitive to thermostability but rather sensitive to pairing geometry (Lewis, Burge et al. 2005). For instances, let-7 regulates the expression of *lin-41 in* worms. The Let-7 family consist of *let-7, miR-48, miR-84,* and *miR-24* and they have same seed sequences. But to prevent the repression by *miR-48, miR-84,* and *miR-24* which are expressed before let-7, *lin-41* sites has acquired two features: imperfect seed pairing with let-7 family and compensatory site for 3' of let-7 miRNA (Brennecke, Stark et al. 2005, Lewis, Burge et al. 2005, Bartel 2009). The adenylation and uridylation are thought to increase the stability of the miRNA and also strengthen miRNA-target interaction in plants, mice and Drosophila (Katoh, Sakaguchi et al. 2009) (Fernandez-Valverde, Taft et al. 2010). For miR-21, adenylation and degradation is the normal occurrence in tumour tissues for maintaining mature miRNA levels (Boele, Persson et al. 2014). The uridylation at the 3' end of miR-26 enhances the repression of the IL-6, showing that isomiRs are functional and the degree of regulation differs with that of the reference miRNA (Jones, Quinton et al. 2009).

1.9.0 Why miRNA study is important?

The expression of microRNAs are altered in various diseases. They are either overexpressed or under-expressed in a particular diseased condition, this can help us to use miRNA as a biomarker (Wang, Chen et al. 2016). MicroRNA can successfully classify the stages of a tumor making them a good candidate as a diagnostic marker. MiRNAs are known to be stable in formalin fixed paraffin embedded tissue samples from lung cancer, renal cancer, pappilary thyroid cancer, pancreatic cancer and in plasma (Tetzlaff, Liu et al. 2007, Szafranska, Davison et al. 2008, Barshack, Lithwick-Yanai et al. 2010, Melo and Esteller 2011) making it easy to characterized the disease state. Biomarkers suggested for different cancers are miR-203 for colorectal cancer (Fu, Zhang et al. 2016), miR-505 and miR-193b for CML (Ramachandran, Muiwo et al. 2017), miR-22 and miR-20a for gastric cancer (Jafarzadeh-Samani, Sohrabi et al. 2017), miR-196a in pancreatic ductal adenocarcinoma (PDAC) (Melo and Esteller 2011).

A circulating miRNAs and exosomal miRNAs are also known to rise from tumor tissues. The miRNAs are protected from nuclease activity. The exosome containing miRNAs are taken up by the neighbouring or distant cells and regulate the recipient cells (Zhang, Li et al. 2015). In 2007 a circulating miRNA from serum was first used as a biomarker to detect diffused large B-cell lymphoma (Lawrie, Soneji et al. 2007). Also serum levels of miR-141 have been used to differentiate advance prostate cancer from normal individuals (Mitchell, Parkin et al. 2008), the ration of miR-126 and miR-182 in urine sample are used to detect bladder cancer (Mitchell, Parkin et al. 2008).

MiRNAs can function as oncogenes and tumor suppressor, this can be exploited by using miRNAs as interesting candidate as therapeutics (miRNA mimics) or as inhibitors (antimiRs) (Rupaimoole and Slack 2017). In table2, list of few miRNAs that are undergoing clinical trials are given (Christopher, Kaur et al. 2016). The number of miRNAs patent for therapeutics has increased over the decade. The annual number of US and Europe issued patents on miRNA is around 500 (Christopher, Kaur et al. 2016).

The miRNA mimics and antimiRs are often degraded by nucleases in bloodstream and poorly delivered to the target site. For this purpose synthetic siRNA oligos and chemically modified oligo ribonucleotide (addition of 2’ O methyl group or Locked nucleic acid) has been developed. A targeted delivery of miRNAs using nano particles (neutral lipid emulsion or dendrimer complexes) are also developed recently (van Rooij and Kauppinen 2014,

Christopher, Kaur et al. 2016, Rupaimoole and Slack 2017). Synthetic siRNA oligos are effective in *in vivo* for almost a couple of week in a mouse model, to overcome this a lentiviral vector containing hairpin RNA cloned in tissue specific promoter is also used (Askou, Aagaard et al. 2015). A sponge vector is also used to eliminate the endogenous miRNA from inhibiting their natural target gene (Ebert and Sharp 2010).

Table 2: MicroRNA therapeutic status

Company	Targeted miRNA	Diseases	Technology/ chemistry	Mechanism/effect	Stage
Regulus Therapeutics	miR-122	HCV infection	Anti-miR	Block HCV infection	Preclinical
	miR-10b	Glioblastoma	Anti-miR	Reduces proliferation by blocking cell cycle progression and triggering cell death	Preclinical
	miR-221	HCC	Anti-miR	Delayed tumor progression resulting in a survival rate	Preclinical
	miR-21	Renal fibrosis	Anti-miR	Reducing the expression of extracellular matrix proteins	Preclinical
	miR-33	Atherosclerosis	Anti-miR	Regulation of cholesterol and fatty acid homeostasis via decrease in very LDL triglycerides and an increase in high-density lipoprotein	Completed preclinical
Santaris Pharma	miR-122	HCV	Anti-miR	Prolonged mean reductions in viral plasma RNA levels from baseline	Phase IIa
Mirna Therapeutics	miR-34	Primary liver cancer or solid cancers with liver involvement	mimic	Reduction in the expression of oncogenes, tumor regression, enhanced the survival, and inhibited the growth of other nonhepatic tumors	Phase I
	miR-155	Hematological malignancies	Anti-miR	Restores normal function and reduces the aberrant cell proliferation	Completed preclinical
miRagen Therapeutics	miR-92	Peripheral artery disease	Anti-miR	Enhances blood vessel growth and improves functional recovery of damaged tissue	Preclinical
	miR-15	Myocardial infarction	Anti-miR	Reduces heart muscle cell death and promotes heart muscle cell regeneration	Preclinical

miRNA=MicroRNAs, HCV=Hepatitis C virus, HCC=Hepatocellular carcinoma, LDL=Low-density lipoprotein

(Christopher, Kaur et al. 2016)

AIMS AND OBJECTIVES

2. Aims and objectives:

- Identification of miRNAs whose isomiR profile has altered after PMA treatment
- To study the characteristic features of isomiRs produced
- Analysis of expression profile of different enzymes which are likely to be involved in generation of isomiRs.
- Functional analysis by overexpression of isomiRs in K562 cells and analysis of alteration in gene expression networks.
- A biomarker studies in CML for diagnostic and prognostic purpose.

MATERIALS AND METHODS

3.1. Materials and methods

3.1. Source and materials: Cell culture medium RPMI-1640 and FBS (Fetal bovine serum) were purchased from GIBCO, Invitrogen. Trizol and DNase1 was purchased from Ambion, Invitrogen. 2XSYBR-green PCR master mix were purchased from Applied Biosystems. PMA, PD98059 and molecular biological reagents were purchased from Sigma (USA), MBI Fermentas (Canada), Merc, Qualigens (India). RNA oligos for miR-22 and isomiR were purchased from Sigma. Clone for 4EBP1 was purchased from Origene. Monoclonal antibodies were purchase from Cell Signalling Technologies.

3.2. Cell culture and treatment*:* The K-562 cell line was obtained from National Centre for Cell Sciences, Pune and grown in RPMI 1640 medium. This medium was supplemented with 10% FBS (fetal bovine serum) and penicillin-streptomycin, and the cells were grown at 37°C in 5% CO_2 in incubator chamber. PMA (20µM) was added to K562 cells and cultured for 48h. DMSO was used as a control for PMA. PMA was purchased from Sigma (P1585). To inhibit the MAP kinase pathway we have used inhibitor PD98059 (sigma P215). K562 cell was treated with PD98059 (50µM) 30 min before PMA treatment.

3.3 PBMC isolation*:* From the patient 5ml of blood was drawn. The blood sample was layered over Histopaque 1077 (sigma) in 1:1 ratio in a 15 ml falcon tube and centrifuged at 400g at RT for 30 mins. The plasma on the top was transferred to a tube and stored in -80°C. At the interphase of the Histopaque and plasma a white buffy coat formed was collected in 15 centrifuge tube. To this, 2/3 volume of RBC lysis buffer was added and incubated for 5 min and centrifuged at 250g at RT for 5 min. The cells were washed with PBS.

3.4 Total RNA Isolation*:* K562 cells treated with DMSO and PMA, and growing in log phase were harvested at 1200 × g for 5 mins at room temperature, and the cell pellet was washed with PBS pH 7.4. The pellet was resuspended in 1 ml Trizol® reagent (Invitrogen). Then the lysed cells was kept in the room temperature for 5 mins, after which 200 µl of chloroform was added and vortexed for 15-30s. It was then followed by incubation for 3 mins at room temperature. For phase separation the tubes were centrifuged at 12000 × g for 15 min at 4ºC. The top aqueous phase which contained RNA was transferred to a fresh microfuge tube and 500 µl of isopropanol was added for RNA precipitated and incubated at room temperature for 10 mins. The tube was then centrifuged at 12000 × g for 10 mins at 4ºC to collect the RNA pellet. The RNA Pellet was washed with 1 ml of 80% ethanol in

DEPC treated water and then centrifuged at 7500 × g for 5 mins at 4ºC. The pellets were air-tried at 37ºC for 5-10 mins and resuspended in 30 µl DEPC treated water and stored at -80 ºC for further use.

3.5 Analysis of RNA: 1.2% denaturing agarose gels with 2.2 M formaldehyde were prepared in 1X MOPS buffer (20mM MOPS, 2mM sodium acetate and 1mM EDTA). The glass wares were treated with 0.1% (v/v) DEPC (Diethyl pyrocarbonate solution in water) for 10-16h at 37 ºC and baked at 180 ºC for 8h (Sambrook et al). 3% (w/v) hydrogen peroxide was used to clean the electrophoresis tank and washed extensively with DEPC treated water.

3.6. Small RNA sequencing*:* For small RNA sequencing, 20 µg of total RNA were sent to C-camp. The samples were checked to confirm the quality of RNA fit for running with the specified RIN (RNA integrity) value of >8. Small RNAs were collected by separating by denaturing PAGE, and then adapters were ligated at both ends of small RNA. Then cDNA libraries were constructed from the adaptor ligated small RNA. The cDNA constructs were then gel purified, size selection targeting miRNA's in the range of 20 to 35 nucleotides for loading on the illumina cluster station. Then PCR was carried out on a solid surface coated with primers complementary to adaptor sequence, and by bridge amplification, high density clusters were generated. Small RNA sequencing was carried out at the Center for cellular and Molecular Platforms (C-Camp) on Illumina HiSeq 1000 platform (dataset 1 & dataset 2).

3.7 Real time RT-PCR

1 µg of total RNA was treated with DNase I (purchased from Thermo cat no EN0521) and incubated at 37ºC for 30 mins. Then the RNA was reverse transcribed using Revert Aid Reverse transcriptase (cat no EP0441) from thermo using the random hexamer as primer (for target gene analysis) and isomiR specific stem loop primer for cDNA synthesis. To this, 0.5 µl Ribolock RNase inhibitor at 40U/µl (Fermentas) was added to inhibit any RNase activity. The reaction mixture was incubated at 25 ºC for 30 mins (16 ºC for isomiRs) for primer annealing and extension was carried out at 42 ºC for 60 mins for total gene cDNA synthesis, and 30 min for isomiR profiling. The enzyme was inactivated by incubation at 70 ºC for 15 mins. cDNA preparation was diluted 5 fold and 4 µl of it was used for real time

PCR. Real time RT-PCR was done on an Applied Biosystems 7500 Real Time PCR instrument. The primers sequence are given in appendix I.

PCR were carried out using the following parameters-

Stage 1. 50 ºC/2min, cycles-1

Stage 2. 95 ºC/10min, cycles-1

Stage 3. 95 ºC /15s,

60 ºC/1min

Cycles- 40.

3.8 Western Blot: RIPA buffer was used to make cell lysate. Lysate was incubated on ice for 45 mins. And then centrifuge to remove the debris. 25-100 µg of total cell lysate was used for western blotting depending on the protein. Lysate was run on SDS PAGE and transferred on a PVDF membrane (Millipore, USA). Membrane was blocked in 5% BSA made in 0.5% PBST. Antibody for was purchased from CST and used according to manufacturer's protocol. For loading control we used β-Actin/β-tubulin antibodies (BD Biosciences). Antibodies for Drosha (#3364), Ago2 (#2897) and DICER1(#5362) were purchased from Cell Signaling®.

3.9. Transfection*:* The miRNA oligonucleotides AAGCUGCCAGUUGAAGAACUGU (miR-22.1), AAGCUGCCAGUUGAAGAACUGUA (miR-22.2) and AAGCUGCCAGUUGAAGAACUGG (miR-22.3) were obtained from Sigma. Transfection of oligonucleotides were done using siPORT NeoFX® transfection reagent (Ambion). MiR-22 and its isomiRs and the scrambled miRNA were used at a concentration of 30nM. Cells were harvested after 48h.

3.10. Confocal Microscopy: Cells were treated with DMSO and PMA and grown for 48h. Cells were then adhered on poly L-Lysine treated coverslip for 1h at 37 ºC. Cell were washed with PBS and 3.7% paraformaldehyde was used to fix the cell for 30 mins. Then BSA (1% w/v) in PBS was used for blocking and then incubate with primary antibody for 1h at 37 ºC. Then stained with TRITC conjugated secondary antibody for 30 mins at 37 ºC. The nucleus was stained with DAPI for 15 mins. Coverslip were mounted on the slide with DABCO and visualized using Olympus fluoview FV1000 Laser scanning microscope.

3.11. miRNA target Prediction*:* The target prediction for miR-22 was mentioned in our previous paper (Ahmad, Muiwo et al. 2014). The online tools used were DIANA-microT,

MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargertScanS. The target which was predicted by at least five programs and the expression profile that matched with PMA treated K562 microarray data were considered as potential targets.

3.12. Statistical analysis: SEM was calculated using Graphpad prism 6 to determine the error bars for qRT-PCR. Statistical significance was calculated using unpaired t-test. Those with p-values less than 0.05 were considered statistically significant. (t-test (*) $p< 0.05$; (**) $p<0.01$; (***) $p<0.001$; (****) $p<0.0001$; (ns) $p>0.05$. Each experiment was repeated independently at least three times. For densitometry analysis AplhaErase FC was used. The density of the blot was normalized with that of the loading control. Using the control sample as reference the change in density of other experimental samples was measured.

3.13. Sequencing run and data quality: This bioinformatics was performed by Priyatama from SIT. The sequencing data of the four samples were provided in the fastq format by the sequencer. The adaptor sequences in the reads were trimmed by illumina scripts. We performed the quality check of the fastq reads using the FastQC tools. Illumina 1.9 Encoding was used to encode the quality score of the fastq files. The reads with phred score >30 were considered.

3.14. Bioinformatic analysis*:* NGS small RNA reads have been used (2 pairs of samples named as 1 and 2) for the analysis. The sequences with less than 16 and greater than 25 nucleotides (read length) were removed. A custom Perl code has been used to count the abundance of every unique read. Raw fasta files were then converted into modified files, where header of fasta sequence contains just counting of reads, length of sequence and the abundance of their reads. These modified files were used for further analysis. To obtain isomiRs for every known miRNA, an alignment of the reads to the pre-miRNA hairpins is necessary. This alignment facilitates identification of isomiRs of mature miRNA sequences derived from both, 5′ and 3′ region of the pre-miRNA hairpin. So the filtered reads were aligned against the precursor sequences. A PERL code was used for exact matching of aligned reads with mature miRNA to select known mature miRNAs which were present in our library. Since isomiRs can have few additions or truncations at their 5′ or 3′ end, or internal single-base differences, we selected only those sequences which included upto 3 flanking edges on either side of known miRNAs (till i-3 and i+3 if it is starting position of

reference miRNA). Small RNA sequences were matched against precursor miRNAs (release 21), downloaded from miRBase database using Bowtie software at default parameter which allows upto 2 mismatches. The count of samples was normalized using a simple trimmed per million (TPM) method using in house PERL code. The exact match of bowtie output with the reference mature miRNAs (release 21 downloaded from miRBase) was done using a PERL code. To select isomiRs of interest we applied a filter using in house MATLAB code which eliminates isomoRs (miRNA-offset RNAs) and loops. Allowing 3 letter variations on the edges of reference miRNAs, the percentage of total IsomiRs was calculated. The relative percentage of an isomiR was calculated as, the TPM of an isomiR divided by the total TPM of the respective miRNA multiplied by 100.

Differentially expressed miRNAs were identified by calculating fold change. Known miRNAs in normal samples were compared to those from the cancer cell line. MicroRNAs showing more than 2 fold differences as compared to both the normal cells were considered as differentially regulated. The sequences with less than 10 TPM in both normal and cancer samples were ignored (Vaz, Ahmad et al.). For isomiRs we considered miRNAs with total TPM values (counting all isomiRs) greater than 100, as there was a possibility of noise when miRNAs with lower values were used.

3.15 Appendix-1

The primers used are as follow:

F NET1, 5’-TGGTCACATTCTCGTGAGCTGGTTAC-3’;

R NET1, 5’-CAATATAGCATCCTCCAGAAGCTGAACATC- 3’;

F YARS, 5’-CCAGCTCAGCAAAGAGTACACACTAGATG-3’;

R YARS, 5’-AATACTCTTCATCCAAAGCCTGCAGTC-3’;

F MTHFR, 5’-CCGCTGGGGCAATTCCTCTTC- 3’;

R MTHFR, 5’-CAGGCAAGTCACTTTGTGACCATTCC-3’;

F IPO7, 5’ -CAGCTCAATGAAGCACACAAGTCTCTG-3’;

R IPO7, 5’-TGAATGCATGTAGTAAGCTGTACCCTGATG-3’;

F GAPDH, 5’-GGTCGGAGTCAACGGATTTGGTC-3’;

R GAPDH, 5’-GAGGGATCTCGCTCCTGGAAG-3’.

RT Primer for miR-22.1

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGTTC

RT Primer for miR-22.2

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGTACACAGTTC

RT Primer for miR-22.3

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGCCACAGTTC

Forward miR-22 GCGGAAGCTGCCAGTTGAAG

Universal Reverse primer GTGCAGGGTCCGAGGT

CHAPTER 1

STUDY OF ISOMIRS EXPRESSION PATTERN

4.1.0 Introduction

IsomiR, are variants of canonical miRNAs that differ by 1 or 2 nucleotide at the 5' end, 3' end or within the miRNA body (Soifer, Rossi et al. 2007, Shah, Leidinger et al. 2010, Profumo and Gandellini 2013). IsomiRs are synthesized as the canonical miRNAs with few modification mostly after the dicer processing (Cloonan, Wani et al. 2011, Fukunaga, Han et al. 2012, Neilsen, Goodall et al. 2012, Vaz, Ahmad et al. 2013). IsomiRs are known to interact with the RISC complex and translational machinery polysomes (Cloonan, Wani et al. 2011, Llorens, Banez-Coronel et al. 2013, Loher, Londin et al. 2014). The 5' isomiR is shown to target independently of the canonical miRNA due to its shift in seed region though there are some common targets (Manzano, Forte et al. 2015, Salem, Erdem et al. 2016) . The 3' portion of the miRNA can complement the central miRNA pairing of the nucleotide 13-17 with continuous Watson-Crick pairing or compensate for single nucleotide bulge or mismatch in the seed region (Brennecke, Stark et al. 2005, Lewis, Burge et al. 2005, Bartel 2009). The 3' isomiRs are mostly associated with the stability of miRNAs in plants but it is not clear in animals (Katoh, Sakaguchi et al. 2009, Fernandez-Valverde, Taft et al. 2010).

For this study we have used K562 cell line. K562 cell is a model cell line for chronic myeloid leukemia (CML) (CML will be elaborated in the chapter-3). It is a immortalised myelogenous leukemia line derived from a CML patient from blast crisis (Lozzio and Lozzio 1975). It has a Ph chromosome (explained in chapter-3). The fusion protein BCR-ABL (breakpoint cluster region- Abelson Murine Leukemia virus) leads to continuous activation of tyrosine kinase activity and thus the pathogenesis of CML (Klein, Ben-Bassat et al. 1976). The overexpression of the fusion protein increases the surface adhesion property (Karimiani, Marriage et al. 2014). K562 cells appears to be similar to early-stage erythrocytes, granulocytes and monocytes with respect to different markers (Lozzio, Lozzio et al. 1981). K562 cells being a myeloid progenitor cell, it can be differentiated into terminal mature cells like erythroids, monocytes, and megakaryocytes depending on the stimulus received (Duncan, DeLuca et al. 2016).

PMA (phorbol 12-myristate 13-acetate) treatment can differentiate K562 cell into a committed mature megakaryocytes (Herrera, Hubbell et al. 1998, Ahmad, Muiwo et al. 2017). And this requires MAP kinase signalling (Shelly, Petruzzelli et al. 1998). PMA is known to activate MEK ERK pathway by protein kinase C (PKC) in a manner dependent on Raf and independent of Ras (Marquardt, Frith et al. 1994, Ueda, Hirai et al. 1996). MAP

kinase activates a cascade of proteins and eventually relaying the signals to the nucleus as shown in the model (*Fig D).* The final effect of the signalling are mostly: alteration of translation process, alters the transcription of genes involved in cell cycle, leading to changes in cell proliferation (Avruch, Khokhlatchev et al. 2001, Pende, Um et al. 2004).

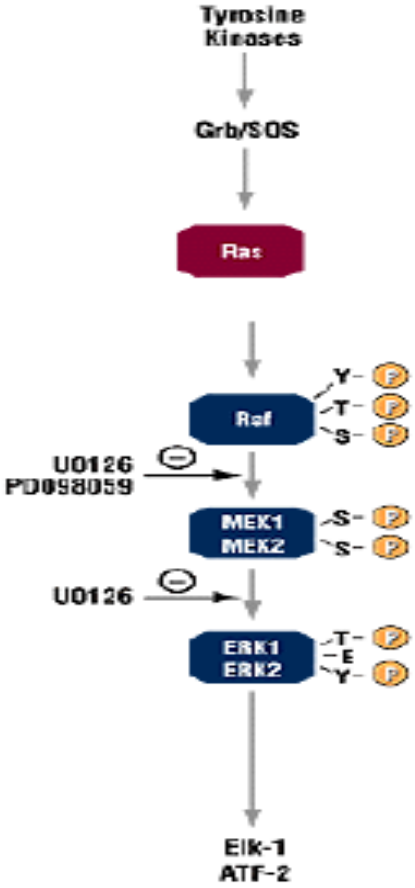


Figure D: A brief signalling pathway of MAP kinase pathway along with the functions of the inhibitors (www.cellsignal.com).

The differentiated cells are often considered to be normal healthy cells (Shelly, Petruzzelli et al. 1998, Ahmad, Muiwo et al. 2014). In our previous studies we have shown that miR-22 is a tumor suppressor and its downregulated in K562 or CML patient, but the expression of miR-22 is restored in PMA induced differentiated megakaryocytic ells. And we have shown that NET1 is the target of miR-22 (Ahmad, Muiwo et al. 2014). We have also shown that Fos B, a transcription factor whose expression is induced by PMA treatment, is responsible for up regulating of the expression of miR-22 in the same cell (Ahmad, Muiwo

et al. 2017). The other targets of miR-22 such as IPO7, MTHFR and YARS are also predicted in the studies. The relationship between isomiRs of miR-22 and the predicted targets gene have been studied and the results are presented in this chapter. Our previous studies has showed that PMA could stimulate the expression of microRNA (miR-22) (Ahmad, Muiwo et al. 2014), in order to further investigate we have carried out small RNA sequencing from C-camp (two datasets) of DMSO (control) and PMA treated k562 cells. If PMA is stimulating the expression of microRNA it will be a good system to understand the characteristic of isomiRs. Majority of the changes observed among isomiRs are template dependent. Therefore it is possible that these changes could be stochastic in nature. It is also not clear if the number of isomiRs observed for different miRNAs follow a pattern. We have attempted to answer these questions using a system where the levels of miRNAs are induced using a signalling molecule.

4.2.0 Results

4.2.1 Sequencing details

Illumina 1.9 Encoding was used to encode the quality score of the fastq files (fig 1.1). Distribution of sequence length showed that more than 30 million reads were between 18 and 28 nucleotides window. Considering the standard size of a miRNA, we added an additional layer of data quality filter and opted for length cut-off of sequences between 11 to 25 nucleotides. After counting the frequency of each unique sequence, we discarded those sequences which displayed frequencies less than 10. The mapping was performed with bowtie aligner tool. For PMA, 15410 (42.68%) of the reads mapped with the precursor 20, whereas for DMSO 20264 (34.40%) reads mapped to the reference database.

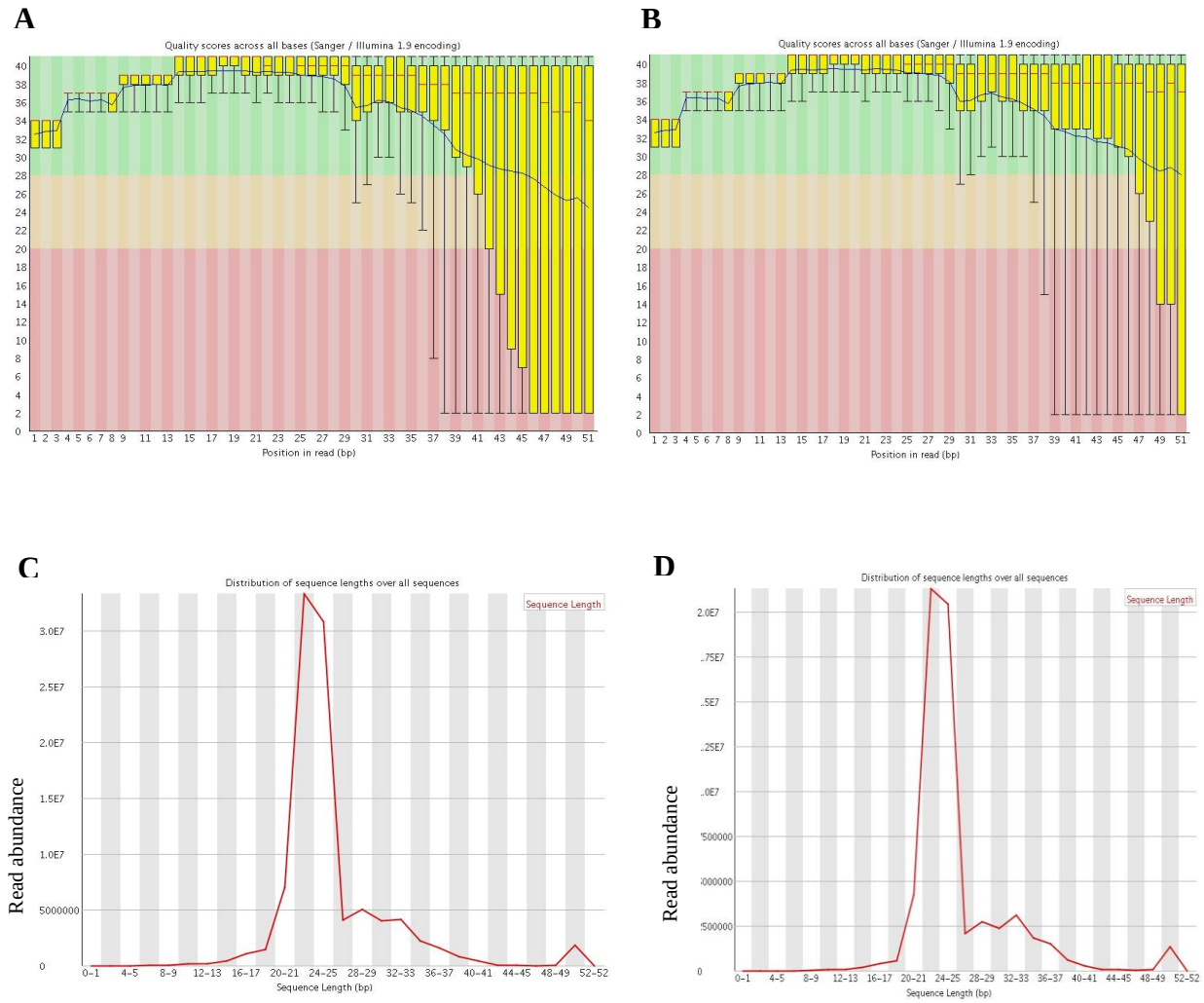


Figure 1.1: Phred quality score of the sequenced dataset DMSO (a) and PMA (b) samples. The length distribution of the miRNA in DMSO (c) and PMA (d) samples.

4.2.2 PMA stimulates miRNAs population

PMA induced differentiation of K562 cells caused a sizeable increase in some of the miRNA levels. We have carried out small RNA sequencing in order to analyse miRNA profiles. Levels of 24.4% (71 miRNAs out of 291) of microRNAs showed enhanced expression (more than 2 fold), whereas only few of the miRNAs showed decline (10%, 30 miRNAs out of 291) on PMA stimulation in both datasets (Appendix II). The expression profiles of the canonical miRNAs were found to be similar to that observed in studies including *in vitro* differentiation of CD34 (+) hematopoietic progenitors to megakaryocytes, where the expression miR-144, miR-126 and miR-20a are repressed in megakaryocytes but miR-126 and miR-135 go up (though some of it may be observed in only one dataset) (Garzon, Pichiorri et al. 2006, Zarif, Soleimani et al. 2013, Kohrs, Kolodziej et al. 2016). We have analysed the isomiR pattern of all miRNAs from these cells before and after PMA treatment.

4.2.3 Analysis of isomiRs before and after PMA treatment in K562 cells

IsomiRs are generated during the processing of pre-miRNAs. When there is an overall escalation in the level of a miRNA, it is expected that different isomiRs of that miRNA may also get upregulated. In order to comprehend the mechanism by which isomiRs are generated we computed relative proportions of different isomiRs before and after PMA treatment. The two replicates show high degrees of similarity shown in graphical form in Appendix III. In general, the relative proportions of different isomiRs remained unchanged independent of expression status of miRNAs after PMA treatment. One of the illustrative examples is miR-127-3p *(Table 1).* The overall increase in expression of this miRNA was more than 10 fold (38 vs 437 TPM) after PMA treatment. IsomiR1 made up the major fraction of miR-127-3p and there was no notable difference in relative proportions (61 vs 66%) after PMA treatment. Other three isomiRs also showed similar relative proportions before and after treatment. Out of 113 miRNAs analysed, 92.9% displayed similar isomiR relative pattern before and after induction of differentiation. This was also seen in samples from CML patients and normal individuals with majority of miRNAs maintaining relative isomiR levels *(Table 2).*

Table 1. Expression profile of miR-127-3p isomiRs in PMA treated K562 cells

DMSO TPM	%	hsa-mir-127-3p	PMA TPM	%
29.1726442	61.081	TCGGATCCGTCTGAGCTTGGCT (1)	359.9927	66.816
3.714331202	7.777	TCGGATCCGTCTGAGCTTGGC (2)	24.97113	4.6347
2.646261715	5.5407	TCGGATCCGTCTGAGCTTGGCTA (3)	31.75371	5.8936
2.327435002	4.8732	TCGGATCCGTCTGAGCTTGGCTAT (4)	21.09873	3.9160

Table 2: isomiRs profile of miR-378a-3p in CML patient

hsa-miR-378a-3p	N1 TPM	N1 %	N2 TPM	N2 %	P1 TPM	P1 %	P2 TPM	P2 %
ACTGGACTTGGAGTCAGAAGGC	1326	22	1520	26	1842	27	3450	32
ACTGGACTTGGAGTCAGAAGGCA	1184	20	1157	20	1275	18.7	1755	17
ACTGGACTTGGAGTCAGAAGGCAT	758	12.8	583	10	732	10.7	1029	10
ACTGGACTTGGAGTCAGAAGG	737.9	12.5	649	11	791	11.6	1033	10
ACTGGACTTGGAGTCAGAAGGA	359	6	293	5.	438	6.4	386.	3.8

N1, N2- *healthy volunteer:* P1, P2- *CML patient*

However, isomiR profiles of a few miRNAs (7%) were found to alter after PMA treatment *(Table. 3).* For instance, isomiR1 of miR-27b-3p is the most abundant in control cells but not in PMA treated cells where isomiR2 was found to be the most abundant *(Table 4).* Similarly, for miR-767-5p relative proportions of isomiR2 increased (15 to 30%) after PMA treatment (*Fig 1.2 and table 4).* The results are also presented as ratio (TPM) of specific isomiR before and after PMA treatment for better visualization of the two categories, that is, miRNAs where profile does not change and those that have different isomiR profiles after treatment *(Fig 1.2).*

Table 3. The list of miRNAs whose isomiRs profiles changed when PMA is added.

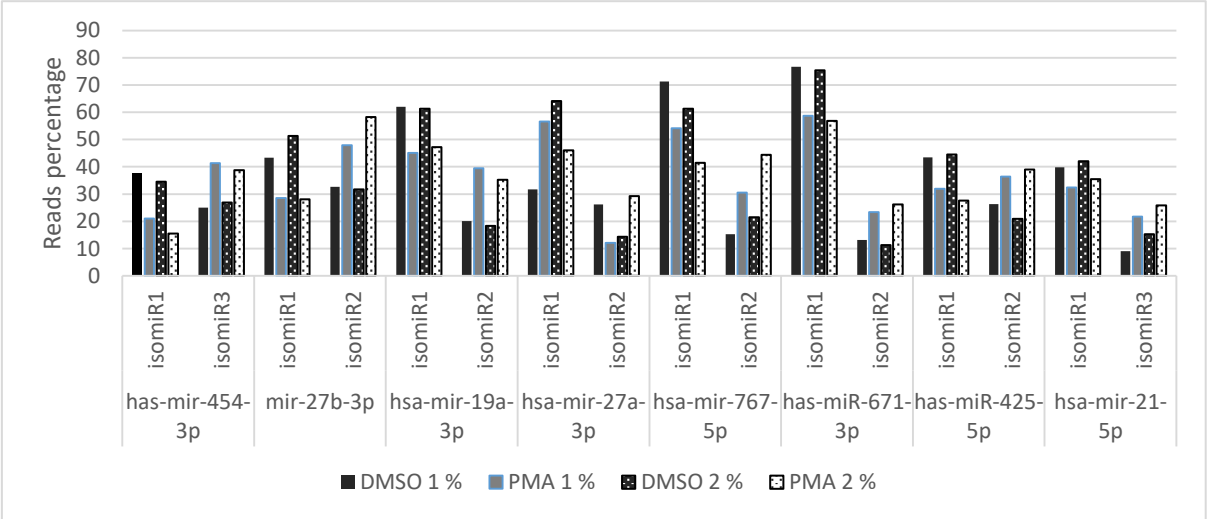
miR-19a-3p	miR-425-5p	miR-671-3p
miR-21-5p	miR-27a-3p	miR-454-3p
miR-27b-3p	miR-767-5p	

Table 4. Expression profile of mir-27b-3p and miR-767-5p isomiRs in PMA treated K562 cells

DMSO			PMA	
TPM	%	mir-27b-3p	TPM	%
1124.85252	43.307	TTCACAGTGGCTAAGTTCTGC (1)	711.7008	28.49157
848.49353	32.667	TTCACAGTGGCTAAGTTCTG (2)	1197.417	47.9363
387.262866	14.91	TTCACAGTGGCTAAGTTCTGCT (3)	358.8662	14.36651
77.6343045	2.9889	TTCACAGTGGCTAAGTTCTGCA (4)	72.66036	2.908818

DMSO	%	mir-767-5p	PMA	%
187.6454	71.301	TGCACCATGGTTGTCTGAGCATGC (1)	115.7497	54.1502
40.20404846	15.277	TGCACCATGGTTGTCTGAGCATG (2)	65.26759	30.5336
10.66475354	4.0523	TGCACCATGGTTGTCTGAGCATGA	9.129484	4.270971
6.918539663	2.6289	TGCACCATGGTTGTCTGAGCATGCA	4.341785	2.031181

A



B

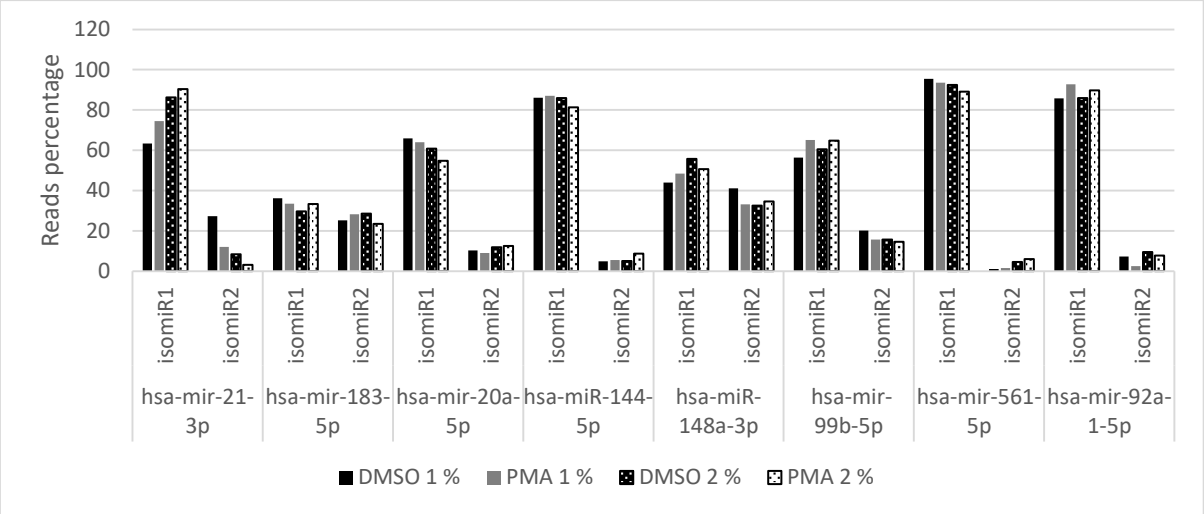


Figure 1.2: Patterns of different isomiRs of indicated miRNAs. (A) MiRNAs that display different isomer profiles before and after PMA treatment, (B) miRNAs that display similar pattern. The solid dark bar indicates the reads percentage of an isomiR in DMSO dataset 1, the solid light bar indicates the reads percentage of an isomiR in PMA dataset 1, the black bar with white dots indicates the reads percentage of an isomiR in DMSO dataset 2, the white bar with black dots indicates the reads percentage of an isomiR in PMA dataset 2.

We carried out functional analysis of miRNAs that displayed altered isomiR profiles after PMA treatment, based on literature search *(Table 5).* The results suggested that most of these were oncogenic, that is highly expressed in cancer cells as compared to normal tissues. Onco-miRs are generally expected to be upregulated in cancer cells as compared to that in healthy individuals, and its inhibition causes tumor regression (Si, Zhu et al. 2007, Voorhoeve, le Sage et al. 2007, Zhang, Pan et al. 2007).

Table 5. Analysis of miRNAs that displayed altered isomiR profile after PMA treatment.

miRNA	**Role of the miRNA**	**Type of cancer**	**Literature**
miR-19a-3p	Oncogenic	Lung cancer CML	(Yamamoto, Ito et al. 2015) (Venturini, Battmer et al. 2007)

miR-21-5p	Oncogenic	Breast cancer Many cancers	(Si, Zhu et al. 2007) (Buscaglia and Li 2011)
miR-27b-3p	Oncogenic	Breast cancer Breast cancer	(Takahashi, Miyazaki et al. 2015) (Wang, Rathinam et al. 2009)
miR-425-5p	Oncogenic Deregulation	Gastric cancer CML	(Ma, Liu et al. 2014) (Suresh, McCallum et al. 2011)
miR-27a-3p	Oncogenic	Lung cancer Breast cancer	(Li, Wang et al. 2014) (Mertens-Talcott, Chintharlapalli et al. 2007)
miR-767-5p	Oncogenic	Melanoma cell line Many cancer	(Loriot, Van Tongelen et al. 2014) (Plaisier, Pan et al. 2012)
miR-454-3p	Oncogenic	Breast cancer Lung cancer	(Cao, Li et al. 2016) (Zhu, Li et al. 2016)
miR-671-3p	No reports on oncogenicity		

4.2.4 Number of isomiRs generated during pre-miRNA processing

Number of isomiRs and their relative expression varied from miRNA to miRNA. For example, reference copy of miR-22-3p made up 95% of the total expression value (TPM =33,769) in PMA treated samples. IsomiRs of a few miRNAs showed equal expression. For example, two most abundant isomiRs of miR-21-5p and miR-411-displayed similar levels of expression. A few miRNAs showed multiple isomiRs with different relative levels *(Table 6a-c).* Total second dataset tables are presented in Appendix IV.

Table 6a: miRNAs that display a single dominant isomiR

Column1	isomiR 1 (%)	isomiR 2 (%)
miR-301a-3p	93	1.7
miR-185-5p	77.7	7.2
miR-16-1-5p	93.5	3.4
miR-22-3p	95.9	0.6
miR-192-5p	59	9
miR-18a-5p	72	7
miR-362-5p	82	5

Table 6b: miRNAs that display equal expression

Column1	isomiR 1 (%)	isomiR 2 (%)	isomiR 3(%)
miR-327b-3p	43	32	14
miR-186-5p	48.7	31.4	8.6
miR-660-5p	45	33	11
miR-142-5p	41.2	31.8	7
miR-27a-3p	31.7	26	18
miR-21-5p	39.8	36.9	9
miR-411-5p	51	48.6	

Table 6c: miRNAs that display multiple isomiRs

Column1	isomiR 1 (%)	isomiR 2 (%)	isomiR 3(%)
miR-140-3p	48.4	13.2	9.2
miR-125a	57	17.9	12
miR-222-3p	98	16	8
miR-19a-3p	62	20	1.9
miR-182-3p	57	22	11.2

4.2.5 Analysis of 5p and 3p

We have also analysed isomiR profiles of 3p and 5p isoforms of miRNAs. It is clear from our results that isomiR species abundance patterns of 5p and 3p isoforms of most miRNAs are similar. MiR-21 appears to be an exception. MiR-21-5p level is mainly due to expression of 2 template dependent isomiRs (39.8 and 39.9 %) and reference miRNA making up only 9% (DMSO)/ 21% (PMA) of total expression. On the other hand, expression of miR-21-3p reference miRNA was found to be the main species making up 65% (DMSO)/ 75% (PMA) of total reads Table 7. The other replicate shows the same result.

Table 7: The isomiR profile of miR-21 5p and miR-21-3p

TPM DMSO	%	hsa-mir-21-5p	PMA TPM	%
27717.35967	39.854	TAGCTTATCAGACTGATGTTGACT	32247.68	32.40312
25688.05953	36.936	TAGCTTATCAGACTGATGTTGAC	36099.93	36.27393
6327.881296	9.0987	TAGCTTATCAGACTGATGTTGA	21669.29	21.77373
5163.74932	7.4248	TAGCTTATCAGACTGATGTTGACC	2104.71	2.114854
1992.666954	2.8652	TAGCTTATCAGACTGATGTTGACA	3136.811	3.15193
246.0863981	0.3538	TAGCTTATCAGACTGATGTTGACG		

TPM DMSO	%	hsa-mir-21-3p	PMA TPM	%
3395.727668	63.328	CAACACCAGTCGATGGGCTGT	2091.426	74.47578
1466.873881	27.356	CAACACCAGTCGATGGGCTGTC	336.8287	11.99448
185.8281494	3.4656	CAACACCAGTCGATGGGCTGTCT	101.011	3.597008
23.30623269	0.4346	CAACACCAGTCGATGGGCTGTT		

(Dark shade highlighting the reference miRNA)

4.2.6 The relation between number of isomiRs and TPM value

The number of different isomiRs from a single miRNA was found to be independent of total number of reads or expression level of the miRNA (*Fig. 1.3).* In this analysis isomiRs with more than 5% of the total miRNA reads and TPM of 100 to 12000 reads were taken into consideration. There was no correlation between TPM and number of isomiRs.

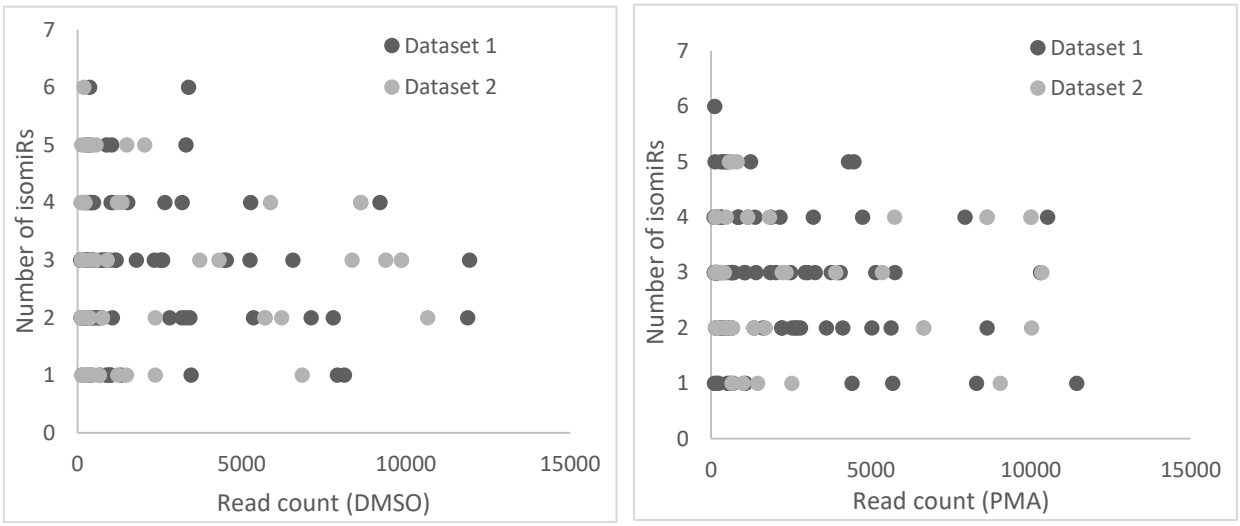
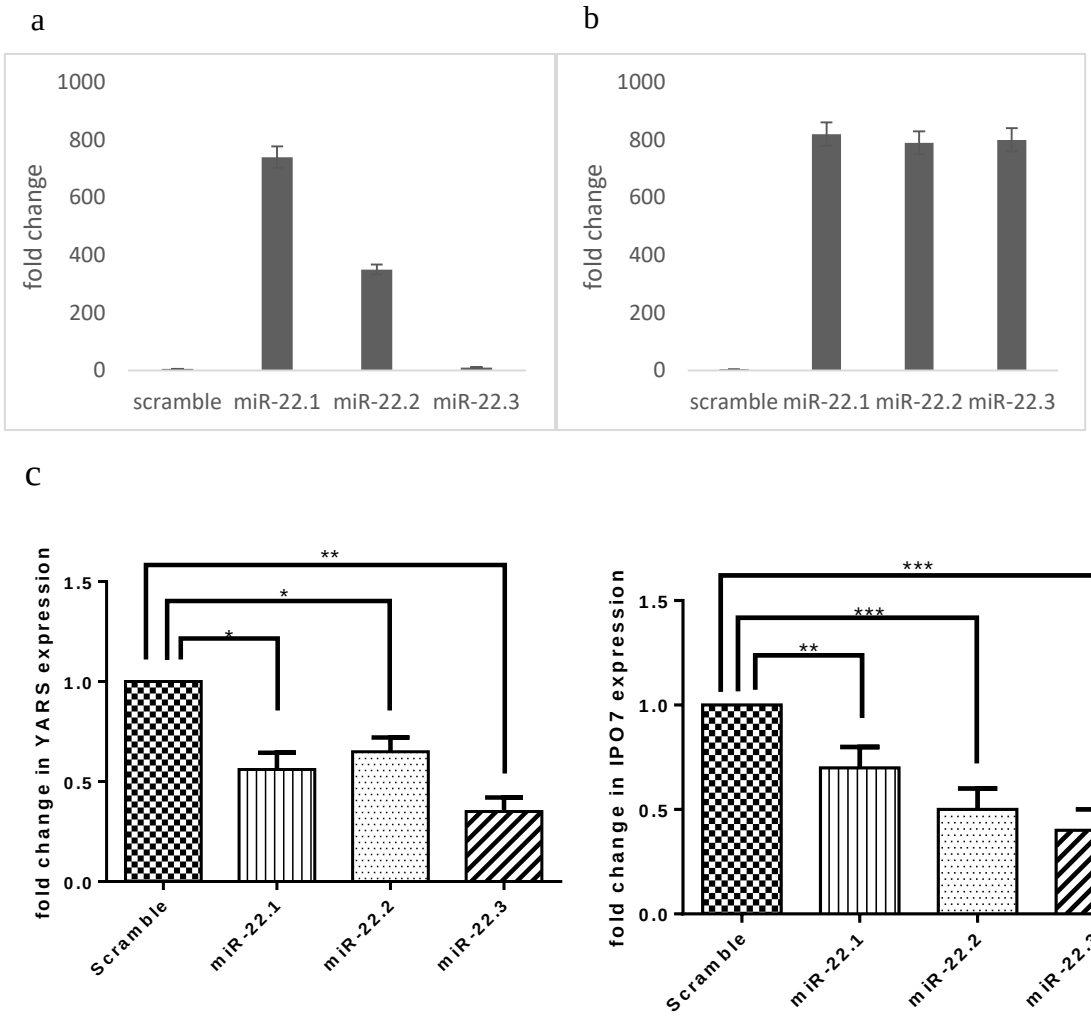


Figure 1.3: Scatter plot showing the relationship between number of isomiRs against experimental value in control and PMA treated K562 cells. The darker shade dots represents dataset 1 and the lighter shade dots represents dataset 2.

4.2.7 The functions of IsomiRs of miR-22

Mir-22 is one of the miRNAs induced by PMA treatment in K562 cells. It has been shown to be anti-tumorigenic in different types of cancers (Nagaraja, Creighton et al. 2010; Zang et al. 2010).We had previously shown that miR-22 down regulates NET1 expression in K562 cell (Ahmad et al 2015). A few other target genes were also identified and some of these were selected for testing functionality of three isomiRs of miR-22 by transfecting them in the K562 cell line. When the transfected RNA oligos were quantitated by the standard set of primers designed for canonical miR-22, we could not detect the isomiRs with equal efficiency compared to canonical miR-22 by qRT-PCR *(Fig 1.4a*). So, we designed a specific stem loop primer for each isomiRs *(Fig 1.4b).* Transcript levels of some of the potential target genes NET1, YARS, IPO7 and MTHFR were estimated using qRT-PCR system. The results showed that the levels of downregulation of the target genes were

different for each isomiR (*Fig 1.4c*). MiR-22.1 and miR-22.2 could efficiently downregulate NET1 (62% and 80% respectively), whereas mir-22.3 isomiR was not so efficient in downregulating NET1 (45%) comparatively, but very effective in case of YARS mRNA (64%) unlike MiR-22.1 and miR-22.2 (50% and 40% respectively). Moreover, in case of IPO7 and MTHFR, miR-22.3 is more efficient in downregulating the target gene compared to two other isomiRs. MiR-22.1 and miR-22.2 differed only in one nucleotide at the 3' end and the ability to target the cognate mRNA was found to be similar. However, miR-22.3 has a guanidine residue at the 3' end instead of adenine in canonical miRNA-22.1. This difference profoundly alters the functionality of the miR-22.



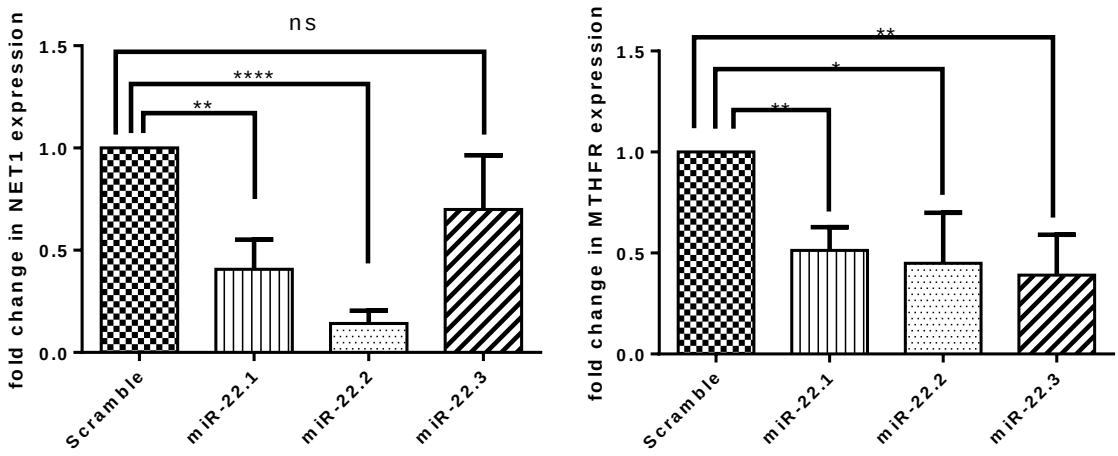


Figure 1.4: Quantitation of the isomiRs with (a) the canonical miR-22 primer set and (b) isomiR specific primer set by qRT-PCR. (c) Target gene (NET1, IPO7, YARS and MTHFR) quantitation in cells transfected with different isomiRs of miR-22. Graphical data points denote mean ± SEM. (t-test (*) $p< 0.05$; (**) $p<0.01$; (***) $p<0.001$; (****) $p<0.0001$; (ns) $p>0.05$.

4.3.0 Discussion

NGS analysis of small RNAs showed that miRNAs are made of a heterogenous population of sequences that mainly varied in the ends and occasionally even within the core region. Variable ends can be a result of imprecise cuts during processing resulting in generation of a family of sequences whose composition may be stochastic in origin. Sequence differences in the body of miRNAs may possibly be due to errors during transcription. If both these variations are stochastic in nature then we will likely see relative levels of different isomiRs vary within and between systems. PMA treatment greatly alters the production of miRNAs in K562 cells. Expression of many miRNAs (24.4%) are up regulated while that of a few are downregulated (10%). We believe that this inducible system of altering miRNA profiles is a useful way to understand if stochasticity drives the composition of different isomiRs. Our conclusion from the results is that there is no significant stochasticity in generating different isomiRs. The processing pathways are highly precise and for majority of miRNAs different isomiRs are generated in a precise ratio according to a certain plan independent of total number of reads or level of expression. However, for a few miRNAs PMA treatment led to altered processing plans and relative levels of different isomiRs. It is not clear by which mechanism the isomiR generation is regulated and the role played by the signalling pathway initiated by PMA. If stochastic processes were involved in generation of isomiRs, then one would expect a proportionality in generation of number of different isomiRs on increasing expression of miRNAs. However, our results did not show any correlation suggesting again a definitive processing plan for generation of isomiRs. It is not clear if the cause is the altered isomiR profile or effect of differentiation to megakaryocytes. At present it is difficult to comment due to paucity of data.

The isomiR profiles of a few miRNAs were found to be altered after PMA treatment and literature analysis suggested that most of these miRNAs were oncogenic. Since K562 cells undergo differentiation into megakaryocytes and these differentiated cells are considered equivalent to normal cells due to some of the displayed phenotypes (Shelly, Petruzzelli et al. 1998, Ahmad, Muiwo et al. 2014), it is likely that the oncogenic potential of these miRNAs arise due to altered isomiR profiles. However, it doesn't preclude that those miRNAs that do not show differential profile are likely to be non-oncogenic. There are many miRNAs, such as miR-127 and miR-18a-5p, that act as oncogene in lung cancer (Shi, Wang et al. 2017) (Liang, Zhang et al. 2017) . The expression profiles of the canonical miRNAs

are found to support their potential role in megakaryocytes. However, we cannot comment on the role of isomiRs as isomiR profiling has not been carried out in the same system. The profiling has been done on human platelets using NGS datasets, but we could find no similarity with respect to generation of different isomiR patterns (Ple, Landry et al. 2012).

The functional role of different isomiRs is not clear. It has been suggested that isomiRs diversify the target selection, enhance the stability and co-operatively or in some case show synergistic effect in downregulating the target gene (Cloonan, Wani et al. 2011, Llorens, Banez-Coronel et al. 2013). It is likely that isomiRs could target either different genes or the same gene but with different efficiency. Our results clearly show that PMA treatment alters overall miRNA and isomiR levels. However, it is not clear what the likely mechanism is for this change. There is a possibility that isomiRs can target some of the genes involved in synthesis, processing and stability of these molecules. In a study, relative abundance of miR-31 isomiRs was shown to vary in breast, lung and prostrate cell lines. Only one isomiR of miR-31 was found to effectively repress the expression of Dicer, a crucial enzyme in miRNA processing (Chan, Lin et al. 2013). There are a number of different possibilities, such as the abundance of the isomiRs, cellular localization of miRNAs, differential loading of isomiRs onto Ago complex which may be due to the preference of uridine and Adenine at the 5' or 3' end nucleotide by Ago 2 (Mi, Cai et al. 2008, Felice, Salzman et al. 2009, Frank, Sonenberg et al. 2010, Chan, Lin et al. 2013, Vickers, Sethupathy et al. 2013). Our results using isomiRs of miR-22 showed that isomiRs can regulate different target genes with different efficiency. A change in single nucleotide at the 3' end could alter the level of target gene regulation. Our results are consistent with other studies. A single nucleotide change in the seed sequence was able to completely change the target gene (Jones, Quinton et al. 2009, Karali, Persico et al. 2016). This shows that from a single precursor sequence different elements that can regulate target genes in different ways can be synthesized, suggesting an intricate and adaptive regulatory system for cellular control. Quantitation of the canonical microRNA by qRT-PCR is not absolute, rather it is a crude expression profile of the isomiRs. Only few techniques are available to quantitate the expression of isomiR, like small RNA sequencing (Vaz, Mer et al. 2011), NanoString Technologies' nCounter platform (Wyman, Knouf et al. 2011) and dumbbell PCR (Honda and Kirino 2015). So one has to be careful in analysing the expression of the canonical miRNAs especially in the case where the canonical is not the most abundant isomiR in the system. Our study shows that isomiRs could be good biomarkers for leukemia, though validation of this observation needs

further studies with cancer samples. The support for this conclusion comes from a recent study, where an isomiR was found to be of diagnostic value for identification of cancer cells with 90% sensitivity and false discovery rate of 3%. In this study it was observed that the discriminatory isomiR is differentially expressed in normal and cancerous cells (Telonis, Magee et al. 2017). Overall, we can conclude that maintaining isomiR pattern is an important regulatory feature during biogenesis of miRNAs.

4.4.0 Appendix-II

NGS of small RNAs shows that microRNA expression I enhavced by PMA treatment. Levels of 24.4% (71 miRNAs out of 291) of miRNAs displayed increase expression (more than 2 fold) and few of the miRNAs also showed decline (10%, 30 miRNAs out of 291) on PMA treatment in both datasets

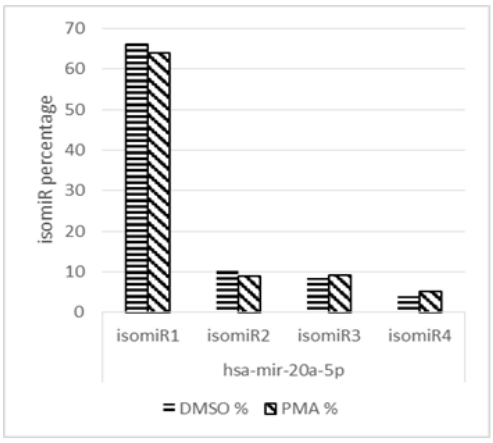
Fold change (PMA TPM/ DMSO TPM)					
	Upregulated miRNAs			Downregulated miRNAs	
microRNA list	Dataset 1	Dataset 2	microRNA list	Dataset 1	Dataset 2
hsa-let-7b-5p	2.3798244	5.9	hsa-miR-1285-3p	0.48227	0.293103
hsa-let-7d-5p	2.0939031	2.3	hsa-miR-16-2-3p	0.306235	0.190476
hsa-let-7e-5p	3.2060278	3.821519	hsa-miR-1296	0.274385	0.447368
hsa-let-7i-5p	2.026036	3.302326	hsa-miR-1303	0.283945	0.407407
hsa-miR-125a-5p	2.2788619	5.100391	hsa-miR-18a-3p	0.417521	0.408696
hsa-miR-126-3p	2.0249243	2.906159	hsa-miR-18a-5p	0.372746	0.384667
hsa-miR-127-3p	14.77826	13.14444	hsa-miR-19b-1-5p	0.245555	0.093023
hsa-miR-1286	11.449001	23	hsa-miR-191-3p	0.189086	0.066667
hsa-miR-132-3p	16.067009	32.72222	hsa-miR-193b-3p	0.39782	0.393701
hsa-miR-132-5p	8.0492505	27.875	hsa-miR-193b-5p	0.373991	0.444444
hsa-miR-134	37.642219	35	hsa-miR-20a-3p	0.319008	0.461538
hsa-miR-135b-5p	2.5401417	8.166667	hsa-miR-218-5p	0.298882	0.447368
hsa-miR-136-3p	12.863058	9.25	hsa-miR-219-1-3p	0.342593	
hsa-miR-143-3p	2.4399206	3.666667	hsa-miR-25-5p	0.154939	0.413793
hsa-miR-146a-5p	44.518315	652	hsa-miR-26b-3p	0.45994	0.278261
hsa-miR-146b-3p	2.9273027	3.661538	hsa-miR-27b-5p	0.149125	0.288136
hsa-miR-146b-5p	3.7330889	6.586257	hsa-miR-30b-3p	0.353488	0.206897
hsa-miR-153	67.111609	41.66667	hsa-miR-30c-1-3p	0.449515	0.3
hsa-miR-181a-2-3p	4.1521564	4.138889	hsa-miR-30d-3p	0.278918	0.241228
hsa-miR-181a-5p	6.6955591	6.516854	hsa-miR-3180	0.362592	0.205882

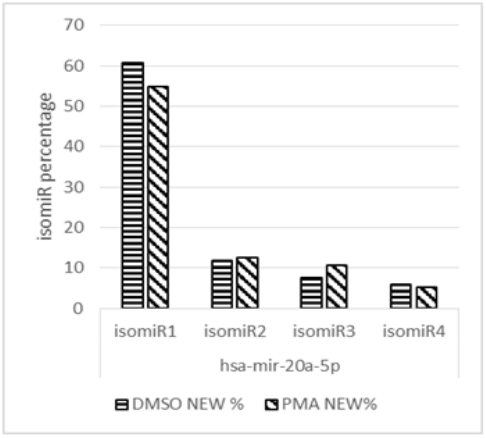
hsa-miR-181b-5p	6.2863691	5.764706	hsa-miR-345-5p	0.49088	0.493976
hsa-miR-181c-5p	5.420278	3.75	hsa-miR-4746-5p	0.423203	0.462366
hsa-miR-181d	5.5271836	12.125	hsa-miR-548b-3p	0.298172	0.076923
hsa-miR-188-3p	3.0811484	4.333333	hsa-miR-548b-5p	0.299209	0.381579
hsa-miR-194-5p	2.2568146	3.794643	hsa-miR-548j	0.447117	0.266667
hsa-miR-212-3p	24.313306	54.47368	hsa-miR-550a-5p	0.190437	0.221519
hsa-miR-212-5p	17.372784	32.39623	hsa-miR-641	0.324627	0.390909
hsa-miR-21-5p	4.1010185	7.36747	hsa-miR-7-1-3p	0.318466	0.088235
hsa-miR-23b-3p	2.2857602	2.888889	hsa-miR-92a-1-5p	0.204085	0.185185
hsa-miR-24-3p	3.7005759	5.857143	hsa-miR-93-3p	0.476514	0.25
hsa-miR-221-3p	13.644411	49.52273	hsa-miR-942	0.309581	0.1
hsa-miR-222-3p	12.429866	42			
hsa-miR-22-5p	2.2548032	3.75			
hsa-miR-23a-3p	5.5341645	9.871681			
hsa-miR-22-3p	2.4526029	4.440211			
hsa-miR-224-5p	5.118682	12.75			
hsa-miR-299-3p	42.062566	24			
hsa-miR-29a-3p	3.0486427	9.717647			
hsa-miR-29b-1-5p	4.4541514	3.333333			
hsa-miR-29b-3p	2.839383	12.72727			
hsa-miR-328	7.4931818	16			
hsa-miR-3605-3p	2.4139109	7.714286			
hsa-miR-361-3p	2.542935	6.5			
hsa-miR-362-5p	2.3657556	3.435233			
hsa-miR-370	15.178005	7			
hsa-miR-375	53.503364	63.27604			
hsa-miR-376c-3p	26.2391	25			

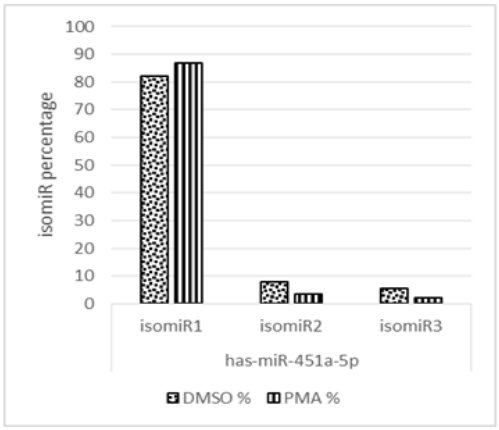
hsa-miR-409-3p	189.07023	123			
hsa-miR-410	119.34595	473			
hsa-miR-411-5p	65.061054	113.7			
hsa-miR-381-3p	65.777477	182.2143			
hsa-miR-431-5p	14.810053	4			
hsa-miR-452-5p	5.695226	4.47619			
hsa-miR-4662a-5p	35.262032	48			
hsa-miR-487b	41.500699	33			
hsa-miR-493-3p	11.884611	25			
hsa-miR-493-5p	13.96653	30			
hsa-miR-505-3p	2.1263332	2.714286			
hsa-miR-532-3p	2.4005867	2.066667			
hsa-miR-99b-5p	2.6098906	2.093796			
hsa-miR-539-3p	49.114973	37			
hsa-miR-574-3p	3.2837253	15.36364			
hsa-miR-574-5p	8.9472562	18.4			
hsa-miR-758-3p	25.036043	83			
hsa-miR-889	64.068	332			
hsa-miR-9-3p	2.9498046	18			
hsa-miR-9-5p	3.14892	5.595238			

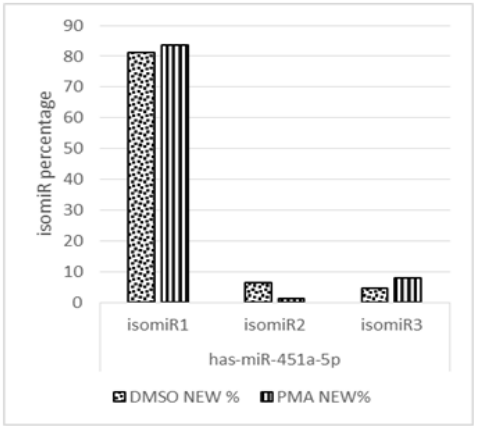
4.5.0. Appendix III

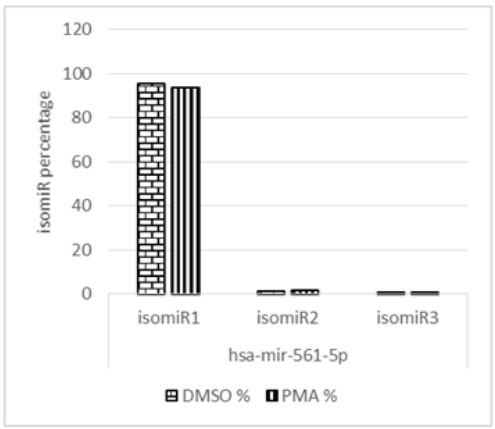
Graphical representation of isomiR profile of a few miRNAs showing that the two replicates essentially showed similar pattern

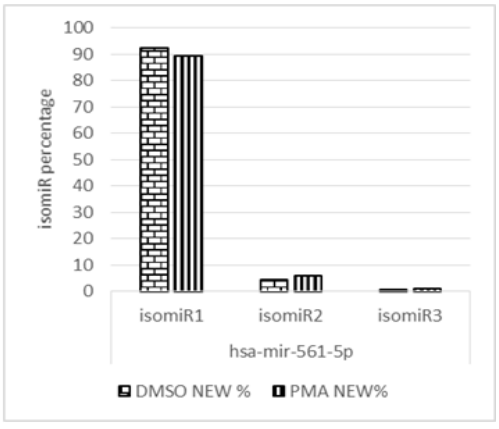


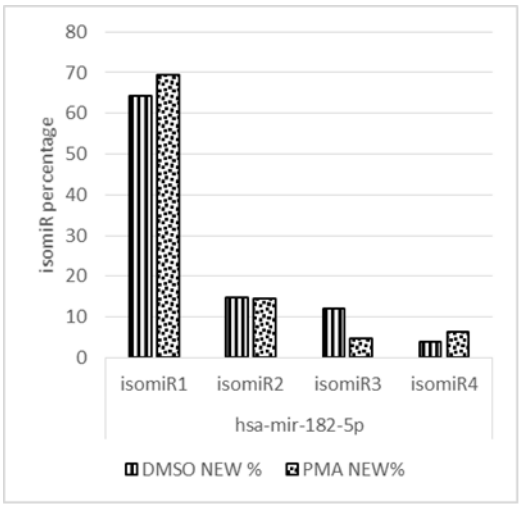


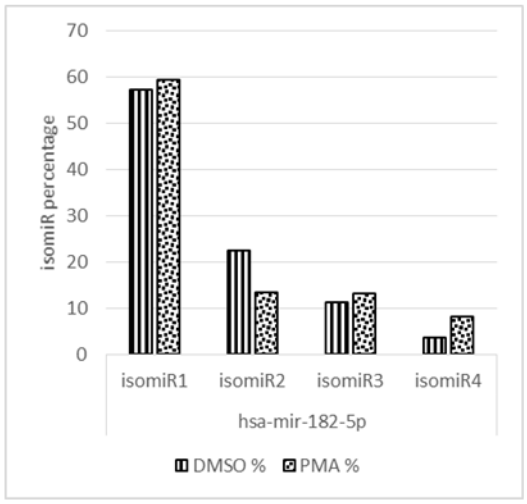


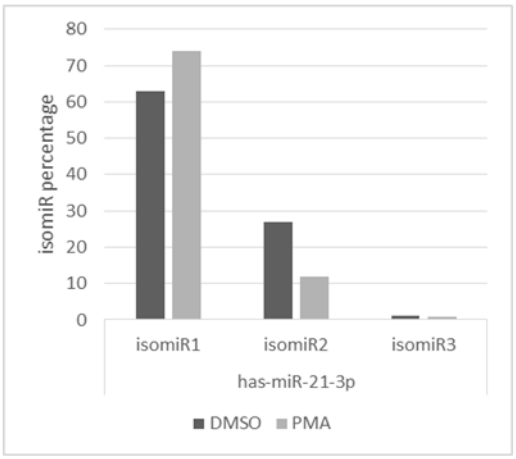


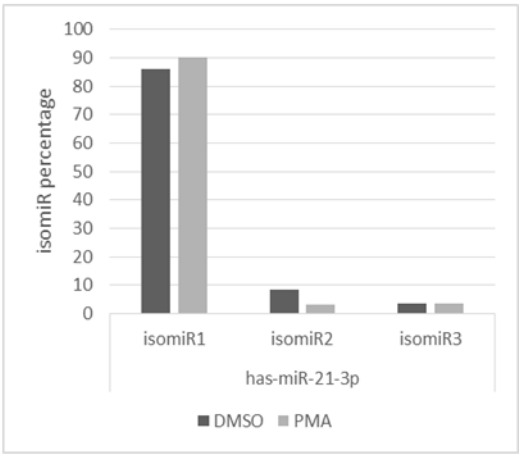


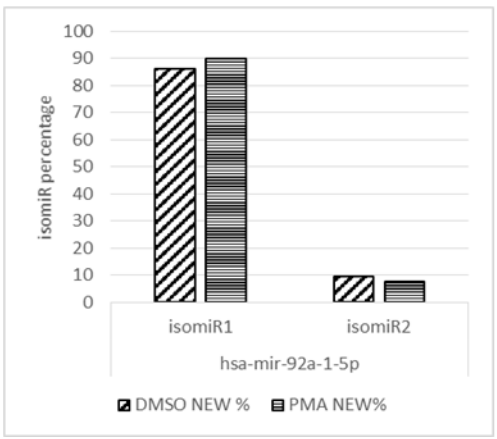


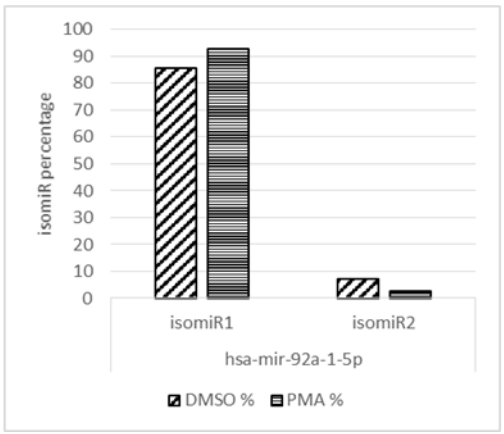

80
70
60
50
40
30
20
10
0
isomiR percentage
isomiR1
isomiR2
isomiR3
isomiR4
hsa-mir-182-5p
DMSO NEW %
PMA NEW%


70
60
50
40
30
20
10
0
isomiR percentage
isomiR1
isomiR2
isomiR3
isomiR4
hsa-mir-182-5p
DMSO %
PMA %


80
70
60
50
40
30
20
10
0
isomiR percentage
isomiR1
isomiR2
isomiR3
has-miR-21-3p
DMSO
PMA


100
90
80
70
60
50
40
30
20
10
0
isomiR percentage
isomiR1
isomiR2
isomiR3
has-miR-21-3p
DMSO
PMA


100
90
80
70
60
50
40
30
20
10
0
isomiR percentage
isomiR1
isomiR2
hsa-mir-92a-1-5p
DMSO NEW %
PMA NEW%


100
90
80
70
60
50
40
30
20
10
0
isomiR percentage
isomiR1
isomiR2
hsa-mir-92a-1-5p
DMSO %
PMA %

4.6.0 Appendix IV

The dataset 2 of the tables in chapter-1

Table 1. Expression profile of miR-127-3p isomiRs in PMA treated K562 cells

DMSO	%	mir-127-3p	PMA	%
17.47115	54.54545	TCGGATCCGTCTGAGCTTGGCT	96.67544	58.85572
4.076601	12.72727	TCGGATCCGTCTGAGCTTGGC	12.99357	7.910447
4.076601	12.72727	TCGGATCCGTCTGAGCTTGGCA	13.56562	8.258706

Table 4. Expression profile of mir-27b-3p and miR-767-5p isomiRs in PMA treated K562 cells

DMSO	%	miR-27b-3p	PMA	%
722.529	51.29548	TTCACAGTGGCTAAGTTCTGC	318.8737	28.04168
446.4849	31.6979	TTCACAGTGGCTAAGTTCTG	662.2635	58.23931
154.5226	10.97023	TTCACAGTGGCTAAGTTCTGCT	91.20016	8.020122
19.41239	1.37817	TTCACAGTGGCTAAGTTCTGCA	10.54195	0.927057

dmso	%	mir-767 5p	PMA	%
49.30746	61.35266	TGCACCATGGTTGTCTGAGCATGC	16.262394	41.45833
17.27702	21.49758	TGCACCATGGTTGTCTGAGCATG	17.406482	44.375
3.105982	3.864734	TGCACCATGGTTGTCTGAGCATGA	1.307529	3.333333
2.135363	2.657005	TGCACCATGGTTGTCTGAGCA	0.653765	1.666668

Table 6a: miRNAs that display a single dominant isomiR

miRNA	isomiR 1(%)	isomiR 2(%)
miR-301a-3p	93.41317	1.996008
miR-185-5p	64.82412	7.537688
miR-22-3p	95.62805	0.749358
miR-192-5p	60.42615	13.89671
miR-18a-5p	67.93478	67.93478
miR-362-5p	92.3445	4.30622

Table 6b: miRNAs that display equal expression

miRNA	isomiR 1(%)	isomiR 2(%)
miR-327b-3p		
miR-660-5p	37.45819	33.11037
miR-142-5p	50.88505	27.87363
miR-27a-3p	46.07246	29.22411
miR-21-5p	35.42067	30.50206
miR-411-5p	49.29547	46.3256

Table 6c: miRNAs that display multiple isomiRs

miRNA	isomiR 1(%)	isomiR 2(%)	isomiR 3(%)
miR-140-3p	44.56215	18.4322	12.64124
miR-125a	46.10858	20.21026	12.05084
miR-19a-3p	61.26943	18.34197	1.924422
miR-182-5p	64.2667	14.75683	11.97881

CHAPTER-2

REGULATION OF MIRNA BIOSYNTHETIC ENZYMES

5.1.0 Introduction

MicroRNA machinery are often altered in cancerous cells and they play an important role in carcinogenesis in many tumor (Horikawa, Wood et al. 2008, Ahmad, Muiwo et al. 2017). The global repression of the miRNA processing machinery (Drosha, DGCR8, DICER1, TRBP and XPO5) promotes transformation and tumorigenesis (Kumar, Lu et al. 2007, Melo and Esteller 2011). A study has shown that in liver cancer, ERK activation is responsible for global downregulation of miRNAs. ERK phosphorylates Exportin-5, thus hindering the translocation of pre-miRNAs from nucleus to cytoplasm (Sun, Cui et al. 2016). A mutation in XPO-5 gene showed defective pre-miRNA transport along with enhanced tumorigenicity. Restoration with the wild type protein showed tumor supressing features (Melo, Moutinho et al. 2010). Inheritable lung tumor Pleuropulmonary blastoma (PPB) patient are often found to be susceptible of malignant transformation in infant stage. Sequencing results showed that PPB families carry a germline mutations in the Dicer gene, however the member carrying only one mutated Dicer allele showed normal phenotype (Hill, Ivanovich et al. 2009). Loss of Dicer in cortex and hippocampus displayed abnormalities in cell number and functioning (Davis, Cuellar et al. 2008). Downregulation of Dicer in mouse lung adenocarcinoma cells causes enhanced cellular transformation and tumorigenesis (Kumar, Lu et al. 2007). Morover Dicer silencing in subcutaneous MCF-7 xenografts impair global miRNA population and enhanced proliferation and invasion (Han, Zhang et al. 2010). The detailed mechanism of altered miRNA population and tumorigenesis is not fully understood but likely due to the repression of the tumor suppressor gene that regulates the oncogenes. The dysregulation of miRNA synthesis has become one of the cancer specific mechanism to boost tumorigenesis. Therefore, understanding dysregulation of the miRNA machinery and their ultimate consequences in human cancer is a necessity to combat the disease and will also help in finding prognostic biomarkers of the disease.

5.1.1 Drosha ribonuclease III (DROSHA)

Drosha is a double-stranded RNA-specific ribonuclease III which catalyzes the initial processing step of miRNA synthesis. It cleaves >1kb long pri-miRNA to release a ~70 nucleotide pre-miRNA along with a partner protein DGCR8, they are known as microprocessor complex and found in the nucleus as mentioned in the introduction of chapter 1. Drosha has been known to contain two binding sites for DGCR3 in each of the RNase III domain (Kwon, Nguyen et al. 2016). A study has also shown that cytoplasmic Drosha can also process pre-miRNA (Dai, Chen et al. 2016, Zhang, Hou et al. 2016) and the

level of cytoplasmic Drosha could be accompanied by cancer progression in gastric cancer tissue (Zhang, Hou et al. 2016). A recurring mutation is found in Drosha in 12% of Wilms tumour, and this hampers the miRNA expression (Torrezan, Ferreira et al. 2014). It was also shown that Drosha displays copy number variation, and this poorly correlated with survival (Czubak, Lewandowska et al. 2015). Moreover, synthesis of canonical miRNA was abolished in Drosha deleted cells (Kim, Kim et al. 2016). Drosha showed no significant changes of expression in CLL (Zhu, Fan et al. 2012). In K562 cells the Drosha was upregulated as mentioned earlier.

5.1.2 Dicer ribonuclease III

Dicer (Dcr), also known as endoribonuclease, is an enzyme encoded by *DICER 1* gene in humans. Mammalian genomes encode one Dicer1 gene but have many transcript variants due to alternative promoter and splicing. The gene is located in chromosome 14, and has 26 exons. It is a 220kDa protein endonuclease belonging to RNase III class. It contains DExH-box RNA helicase-like domain, a PAZ domain, a double-stranded RNA-binding domain (dsRBD) and two RNase III domains (MacRae and Doudna 2007). Dicer processes the pre-miRNA to release a ~21 nucleotide mature miRNA with the help of partner protein like Ago2, TRBP and PACT (Koscianska, Starega-Roslan et al. 2011). Dicer is known to be deregulated in many cancers. There were some inconsistencies in the reports of the expression of Drosha and Dicer in some tumor. A study showed that high expression of *DICER1* was associated with longer survival in various cancers including lung cancer (Czubak, Lewandowska et al. 2015). Other studies showed that Dicer downregulation was correlated with decreased survival and poor prognosis in CLL, non-small cell lung cancer, hepatocarcinoma and breast cancer (Dedes, Natrajan et al. 2011, Zhu, Fan et al. 2012) . Moreover, loss of transcription factor Tap63 is known to downregulate Dicer protein which occurs frequently in cancer. It regulates Dicer by binding directly to it promoter region (Su, Chakravarti et al. 2010). High expression of Dicer was also reported in prostate adenocarcinoma (Chiosea, Jelezcova et al. 2006) and AML (Martin, Payton et al. 2009). Let-7 (Tokumaru, Suzuki et al. 2008) and miR-103/107 (Martello, Rosato et al. 2010) are known to regulate the expression of Dicer by binding to its 3'UTR region.

5.1.3 EIF4EBP1 eukaryotic translation initiation factor 4E binding protein 1

Eukaryotic translation initiation factor 4E binding protein1 or EIF4EBP1 or 4EBP1 belongs to a family of eIF4E binding protein (4EBP1, 2 and 3). It is a translation inhibitor protein. 4EBP1 and 2 show high similarity with 4EBP1, but the latter is known to be more

deregulated in cancer compared to the former two (Musa, Orth et al. 2016). 4EBP1 inhibits protein synthesis by binds the eukaryotic translation initiation 4E protein (eIF4E). And EIF4E is the limiting constituent of the multi-subunit complex that recruits 40S ribosomal subunits to the 5′-cap of mRNAs. When 4EBP1 interacts with eIF4E, it inhibits the binding with eIF4G and thus prevent initiation complex formation of cap dependent and not the cap independent translation (Gingras, Kennedy et al. 1998, Qin, Jiang et al. 2016) . The interaction of 4EBP1 and eIF4E is reversible. It depends on many growth factor signals, including serum and hormones. 4EBP1 has 6 phosphorylation sites that get phosphorylated by the nature of the signal received. The sites are Thr 37, Thr 46, Ser 65, Thr 70, Ser 83, and Ser 112. Depending on the site, phosphorylated 4EBP1 are designated as α (less phosphorylation), β (Thr^{37},Thr^{46}), γ (Thr^{37} ,Thr^{46},Thr^{70}) or δ (Thr^{37},Thr^{46} ,Thr^{70}, Ser^{65}). The affinity of 4EBP1 to eIF4E depends on their phosphorylation sate, hyperphosphorylation leads to weak binding with eIF4E whereas hypophosphorylated 4EBP1 have high affinity for eIF4E. The phosphorylation of 4EBP1 weakens their interaction, making 4E available to bind the 5' cap of mRNA, and initiates translation processes. The phosphorylation of these sites is controlled by AKT (PKB) and MAPK/ERK signalling pathway (Gingras, Kennedy et al. 1998, Herbert, Tee et al. 2002). eIF4E may generally control the protein synthesis of cap dependent protein synthesis, but contributes to tumor progression by selectively assisting limited pool of mRNAs (De Benedetti and Graff 2004). 4EBP1, considered a tumor suppressor, supresses the activity of eIF4E, which is otherwise often upregulated in cancer cells (Musa, Orth et al. 2016). 4E-BP1 can also regulate (enhance or supress) the translation of subset of mRNA by competing with the eIF4G to bind with eIF4E (Colina, Costa-Mattioli et al. 2008, Petroulakis, Parsyan et al. 2009). The inhibition of 4EBP1 may not affect the global protein synthesis but regulate the translation of selected set of proteins (Magagnin, van den Beucken et al. 2008, Petremand, Bulat et al. 2009). Moreover, loss of 4EBP1 leads to induced EMT (Epithelial-mesenchymal transition) and promoted cancer migration by specifically upregulating snail expression (Cai, Ye et al. 2014). 4EBP1 inhibition significantly increased protein synthesis of S100 calcium-binding protein A4 (S100A4) and transgelin 2 in HeLa cells (Magagnin, van den Beucken et al. 2008)

In our previous studies we had shown that miRNA constituted only 18% of the total small RNAs in K562 cells, whereas in normal PBMCs (Peripheral blood mononuclear cells) and HL60 cells, the majority of the small RNAs are made up by 61 % miRNA and 71 % miRNA respectively *(Fig. E*) (Vaz, Ahmad et al. 2010). Moreover, normal healthy individuals also

showed higher proportion of miRNA when compared to CML patients (Vaz, Ahmad et al. 2013). The analysis of miRNA expression showed that majority of the miRNAs were downregulated in K562 compared to PBMCs. Interestingly, Drosha, DGCR8 and XPO5 were upregulated but the cytoplasmic processing enzyme Dicer was decreased when compared to the control PBMCs.

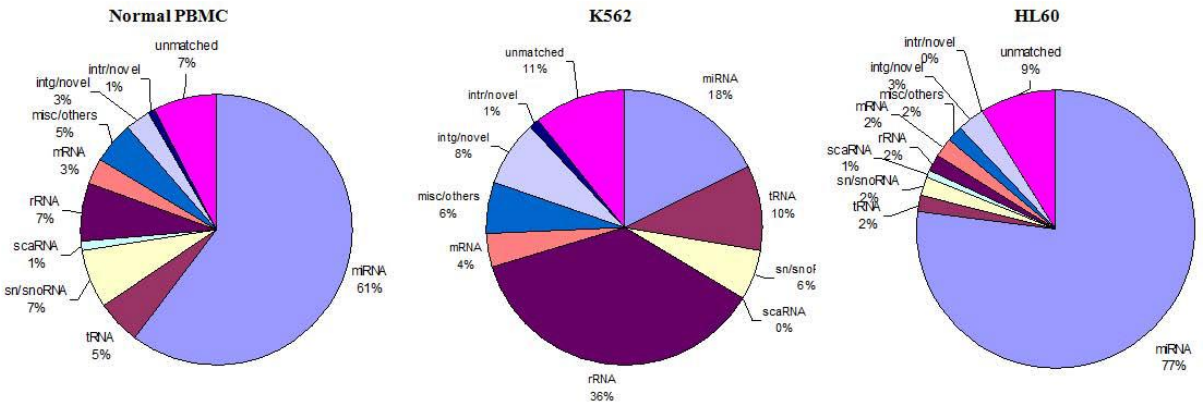


Figure E: Pie chart of different classes of small RNA species found in sequencing data (Vaz, Ahmad et al. 2010).

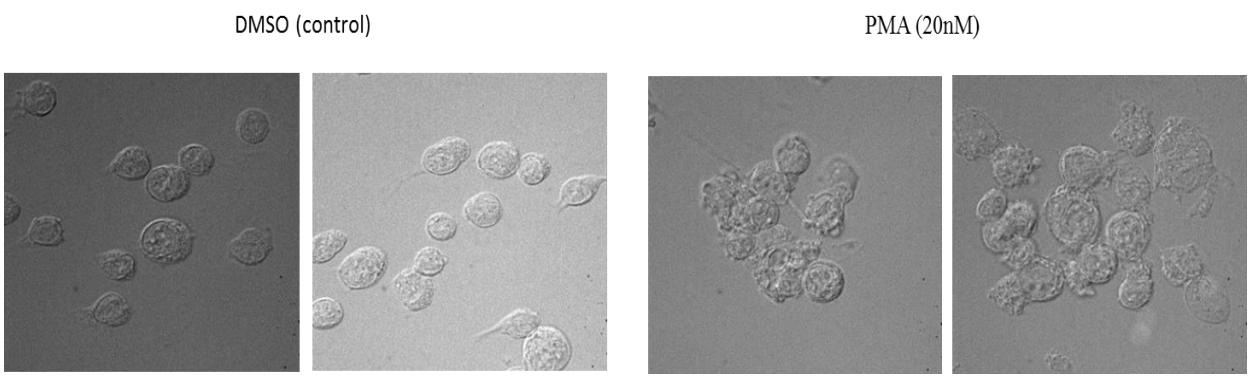
In the previous chapter we have seen that PMA enhances the expression of total miRNAs reads by 42.6% as compared to 18% in K562 cells. Levels of 24.4% of miRNAs showed enhanced expression whereas only 10% of the miRNA showed declined expression on PMA stimulation on both datasets (Appendix II). So, we wanted to understand if this upregulation can be correlated with the expression levels of the biosynthetic machinery. The dysregulation of the miRNA is likely to be a major cause of the tumorigenesis. Understanding the dysregulation of the miRNA machinery and their ultimate consequences in human cancer is a necessity to combat the disease and will also help in finding the prognostic biomarkers of the disease.

5.2.0. Results

5.2.1 PMA differentiate k562 into megakaryocytes

Total fraction of all small RNAs that make up miRNAs was found to be 18% for K562 cells. But when the cells were stimulated by PMA, 42.6 % of the small RNA reads mapped/aligned with the pre-miRNA. Moreover, 24.4% of the miRNAs were upregulated while only 10% were downregulated as a consequence of PMA treatment. We found that PMA treatment also altered the morphology and the cells tend to form aggregates due to increased expression of cell adhesion molecules *(Fig. 2.1a).* The markers for megakaryocytes CD41 and CD61 (Matsumura-Takeda, Sogo et al. 2007) were highly expressed in PMA treated cells (CD41 p value .001 and CD61 p value .0003). Thus PMA differentiated the immature progenitor cell K562 into mature megakaryocytes *(Fig. 2.1b)*

a



b

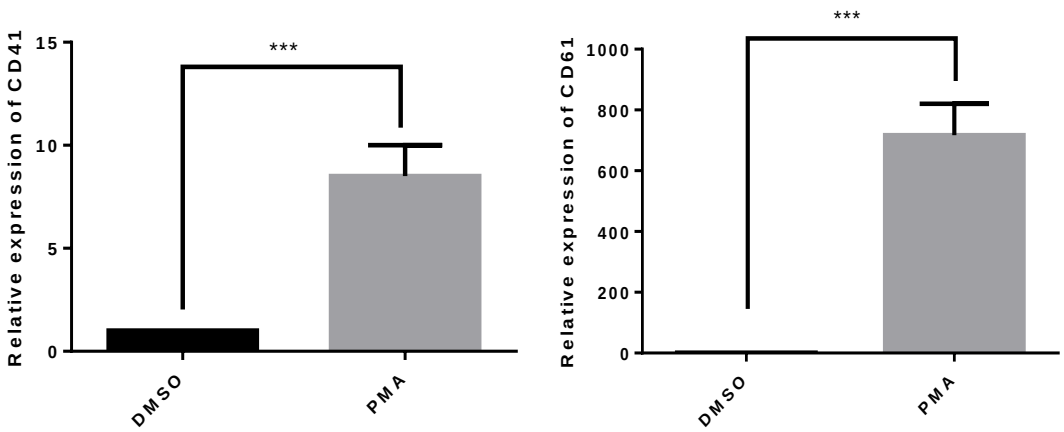
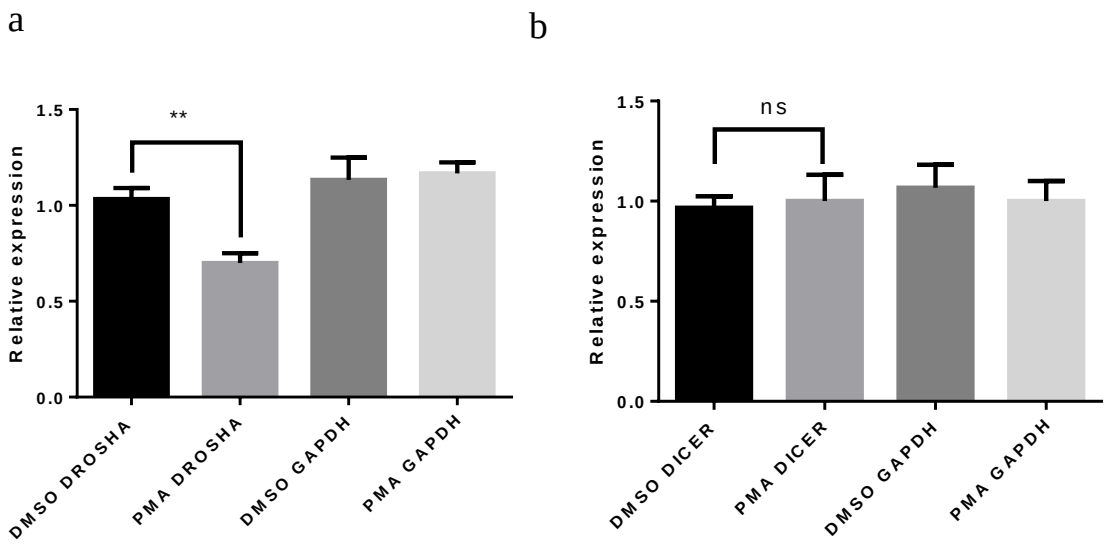


Figure: 2.1. (a) The DIC (Differential interference contrast) image of treated and untreated cell K562 cells. The aggregation were seen consistently in 70% of the PMA treated K562 cells (b) Quantification of the megakaryocyte marker CD41 (p value .001) and CD61 (pvalue .0003) by qRT-PCR. Fold change was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns- p>0.05.

5.2.2 Analysis of the expression of miRNA biosynthetic enzyme

The expression of miRNA biosynthetic emzymes Drosha and Dicer were quantitated by qRT-PCR and western blots in absence or presence of PMA treatment for 48h (*Fig. 2.2)*. In case of Drosha, the transcript was downregulated by 30% (p value .0036) in PMA treated cell when compared with DMSO control (*Fig 2.2a).* The Drosha protein levels were also repressed by the treatment compared to the untreated cells *(Fig 2.2c).* The transcript of Dicer was not altered by PMA (not significant) *(Fig 2.2b)*, but at the protein level the expression was stimulated. The analysis of the Dicer expression showed that Dicer is post-transcriptional regulated in PMA treated K562 cell. Literature survey showed that the transcript and the protein level of Dicer are often poorly regulated (Wiesen and Tomasi 2009, He, Wang et al. 2014). A study also had shown that Dicer was induced during melanocyte differentiation (Levy, Khaled et al. 2010). We found no significant changes in the expression of Ago 2, so we did not include Ago 2 in further studies.



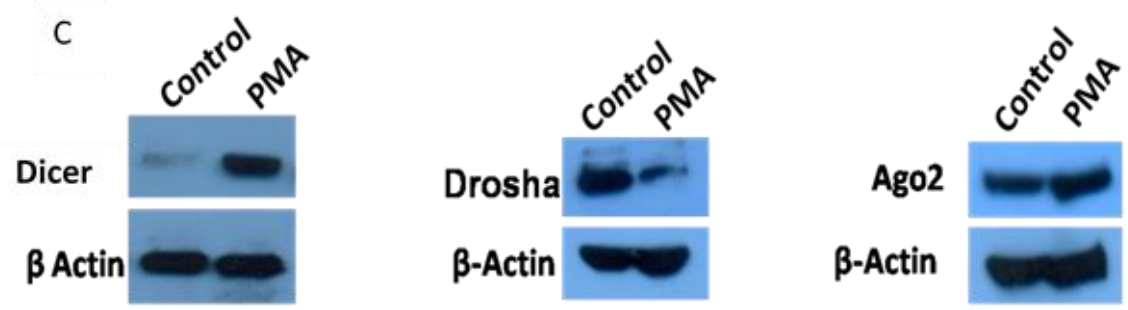


Figure 2.2: The expression of microRNA biosynthetic machinery in PMA treated cells: (a) qRT-PCR of the transcript level of Dicer and Drosha in presence or absence of PMA; (b) Western blots of Dicer, Drosha and Ago 2 in DMSO (control) and PMA treated K562 cells for 48h. Fold change for qRT-PCR was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns-p>0.05.

5.2.3 Analysis of factors that regulates expression post transcriptionally

From the above experiment it is clear that Dicer is regulated post transcriptionally in PMA-treated k562 cells. Small RNA molecules are not the only post transcriptional regulator of the gene expression, many RNA binding proteins (RBPs) does influence gene expression. RBPs mechanism involves modulating the stability and degradation of the mRNA, regulating the assembly of ribosomal protein for translation, assisting binding of other molecules and regulator of translational terminator (Rouhana, Shibata et al. 2010, Van Assche, Van Puyvelde et al. 2015). In our case, RBPs that involved in mRNA stability and translational regulator seems to be the potential regulator of Dicer protein level in our system. From our lab generated microarray expression data (PMA treated and untreated K562 cells) a few differentially expressed RBPs were selected (*Table 2.1).* EIF4EBP1, EDC4 and ANAPC5 were chosen for further experiments because inhibition of these RBPs is known to induce gene expression or restore the transcript level (Eulalio, Rehwinkel et al. 2007, Magagnin, van den Beucken et al. 2008, Ho, Garg et al. 2013, Cai, Ye et al. 2014, Chang, Bercovich et al. 2014). These 3 genes were transiently knocked down by siRNAs, and the Dicer expression was checked by Western blots *(Fig 2.3).* Compared to negative control only EIF4EBP1 (eukaryotic initiation factor 4E binding protein1) knockdown could elevate the expression of Dicer and not the others *(fig 2.3*). Therefore, 4EBP1 was investigated.

Table 2.1: List of differentially expressed trans acting factor in PMA-treated k562 cells

Differentially expressed trans acting factor	Functions	Expression fold change in PMA (fold change expressed in log_2)
EIF4EBP1	Inhibitor of Translation Initiation	-1.62
EDC4	Enhancer of Decapping/Localizes to P Body	-1.04
ANAPC5	Interact with PolyA. Block IRES mediated translation	-1.10
RBX1	May Influence protein turn over	-1.03
GSPT2	mRNA Stability/Translation Termination	1.34
EIF4G3	Translation Initiation	1.27

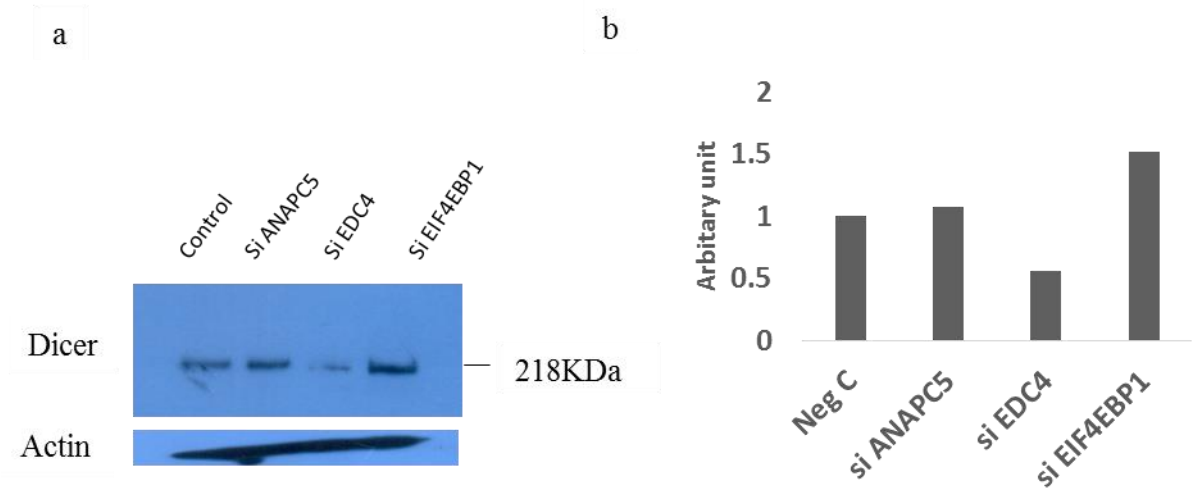


Figure 2.3: Analysis of post-transcriptional acting factors (a) Western blots of transient knockdown of EDC4, ANAPC5 and EIF4EBP1 and (b) quantitating the expression of DCR1 by densitometry analysis. Scramble siRNA is used as controls. For densitometry analysis AplhaErase FC was used. The density of the blot was normalized with that of the loading control. Using the control sample as reference the change in density of other experimental samples was measured. Neg C= negative control.

5.2.4 EIF4EBP1 expression in PMA treated k562 cells

Eukaryotic Initiation factor 4E Binding Protein, also called 4EBP1, inhibited the translation of a mRNA by binding EIF4E, thus preventing initiation complex formation. It is a18kDa molecular weight protein and has 6 phosphorylation sites. We checked the expression of 4EBP1 in PMA treated K562 at 12, 24 and 48h was checked and it was found that both the transcript and the protein levels were downregulated compare to DMSO sample *(fig 2.4).* In our K562 cells microarray data we found that this protein was upregulated when compared with the normal PBMCs *(Table 2.2).* Since the expression of Dicer and 4EBP1 are always inversely correlated we speculated that 4EBP1 could be the modulator of Dicer in PMA treated k562 cells.

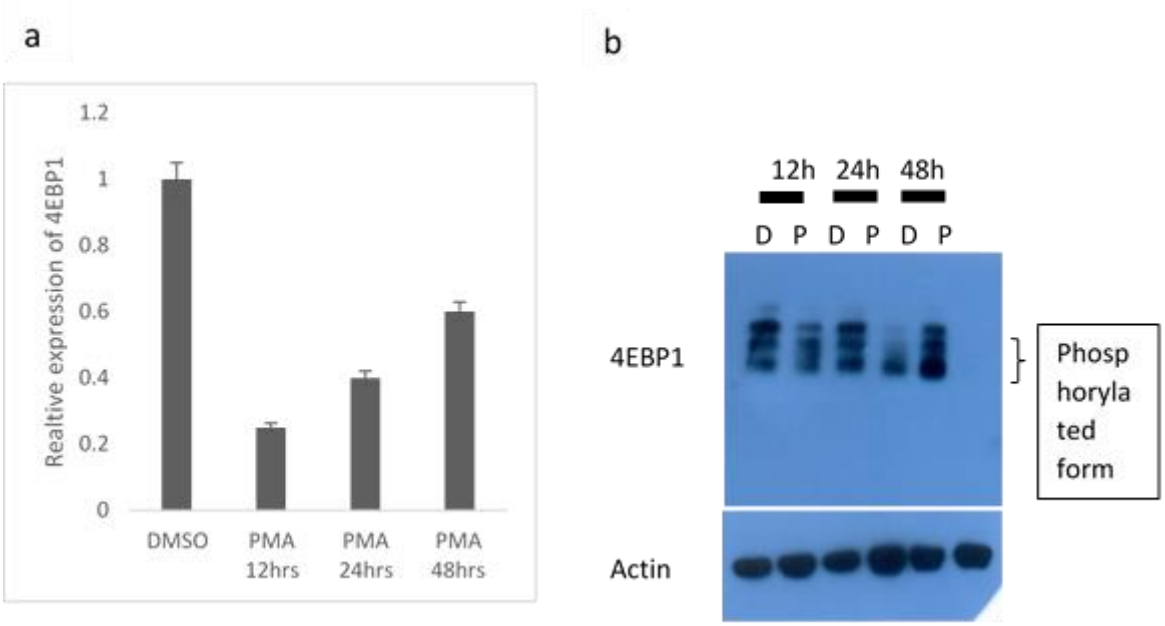


Figure 2.4: The expression of 4EBP1 at different time point (12, 24 and 48h) of PMA treatment by (a) qRT-PCR (b) western blots of 4EBP1. Fold change for qRT-PCR was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** D= DMSO, P=PMA**.**

Table 2.2: Microarray expression data of 4EBP1 in k562 cells

Gene	Fold change (K562/normal PBMC) (fold change expressed in log_2)
EIF4EBP1	1.77

5.2.5 Is 4EBP1 regulation specific for Dicer?

The specificity of regulation of Dicer by 4EBP1 was then investigated by checking expression of Dicer, Drosha and Ago2 in 4EBP1 knockdown cells *(Fig 2.5).* The downregulation of 4EBP1 by 50% could elevate the Dicer expression by 1.5 fold (p value .0012). But the change was not significant in case of Drosha and Ago2. Thus, 4EBP1 translation regulation for dicer is specific in PMA treated K562 cells. To further confirm the regulation of 4EBP1 on Dicer protein 4eBP1 was over-expressed *(Fig 2.5c).* It was found that Dicer protein is 0.5 fold (p value .073) downregulated compared to that with control vector *(Fig 2.5c&d).* In both the downregulation and overexpression of 4EBP1 the transcript levels of Dicer was found to be unchanged *(Fig 2.5e&f).*

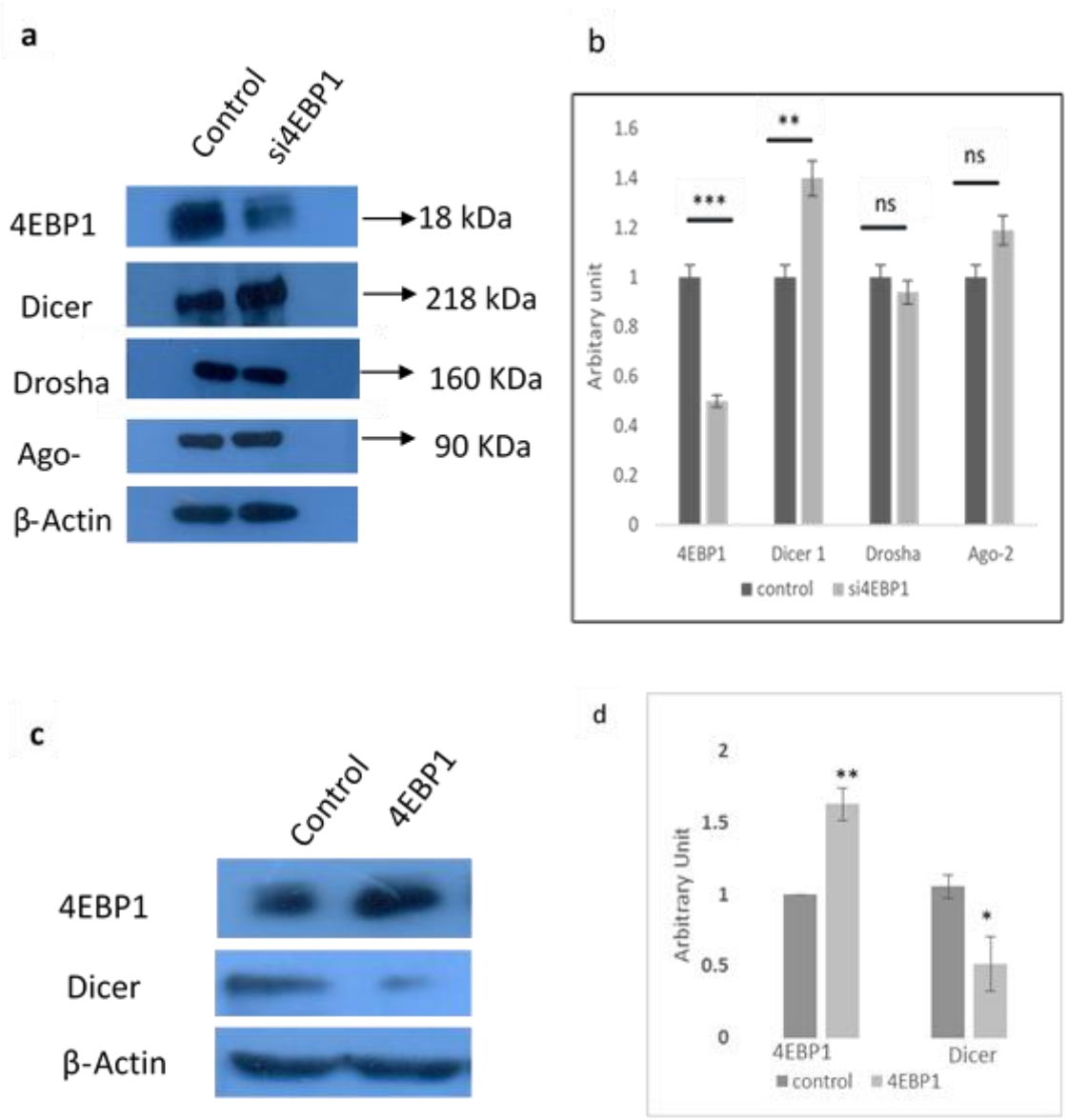

a
Control
si4EBP1
4EBP1
18 kDa
Dicer
218 kDa
Drosha
160 KDa
Ago-
90 KDa
β-Actin
b
Arbitary unit
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
**
ns
ns
4EBP1
Dicer 1
Drosha
Ago-2
control
si4EBP1
c
Control
4EBP1
4EBP1
Dicer
β-Actin
d
Arbitrary Unit
2
1.5
1
0.5
0
**
*
4EBP1
Dicer
control
4EBP1
e
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Dicer
Drosha
Ago
control
si 4EBP1
f
Relative fold of expression
3.5
3
2.5
2
1.5
1
0.5
0
4EBP1
Dicer
control
4EBP1 overexpression

Figure 2.5: 4EBP1 regulation is specific for Dicer protein; (a) Western blot showing the effects of 4EBP1 downregulation on Dicer, Drosha and Ago 2 protein; (b) Densitometry analysis of the western blot (a) using AplhaEaseFC software (for details see *materials and methods*); (c) Overexpression of 4EBP1 showing increased Dicer expression; (d) Densitometry analysis of the western blot (c) using AplhaEaseFC software; (e) Quantification of the transcript level of Dicer, Drosha And Ago 2 in 4EBP1 downregulated cells by qRT-PCR; (f) Quantification of the transcript level of 4EBP1 and Dicer in 4EBP1 overexpressed cells by qRT-PCR. Fold change for qRT-PCR was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** For densitometry analysis AplhaErase FC was used. The density of the blot was normalized with that of the loading control. Using the control sample as reference the change in density of other experimental samples was measured. (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns- p>0.05. *(All experiments were carried out three times. Scrambled siRNA and empty vector were used as controls.)*.

5.2.6 4EBP1 and MAP kinase signalling

It is known that 4EBP1 has 6 phosphorylation sites and that MAP kinase is one of the signalling pathways that regulate the phosphorylation of these sites. PMA is known to activate MAP kinase by activating Protein Kinase C (PKC). In order to find out if the regulator of the Dicer, 4EBP1, is under the modulation of the MAP kinase signalling in PMA treated K562 cells, MAP kinase inhibitor PD98059 was used. PD98059 selectively inhibited the MEK1 and MEK2 activity, thus preventing activation of ERK protein. The ERK phosphorylation increased upon PMA stimulation, but this did not alter the levels of unphosphorylated ERK. Upon the addition of inhibitor PD98059 before PMA stimulation it was observed that the ERK phosphorylation had been inhibited. It showed that PMA did function via MAP kinase signalling pathway *(Fig 2.6).* 4EBP1 expression was also altered at the transcription level since both the transcript and protein levels were down regulated when treated with PMA. PMA treatment inhibited the 4EBP1 transcript level by 40% (p value .0011) *(Fig 2.6c).* Inhibiting the MAP kinase pathway using PD98059 alone elevated the expression of 4EBP1 when compared to DMSO (p value 0.025), showing that the endogenous MAP kinase signalling also inhibits the 4EBP1. Treatment of K562 cells by PD98059 before PMA stimulation showed no significant changes in PMA expression (p value .369). In the same way the densitometry analysis of the western blot showed almost the pattern *(Fig 2.6b).* The blot showed 70% (p value .0172) inhibition of 4EBP1 when PMA is added but the inhibition is relived when PMA signalling is blocked by PD98059 (DMSO

vs PD+PMA p value .16). Thus 4EBP1 was regulated by PMA at transcript and protein level by MAP kinase signalling.

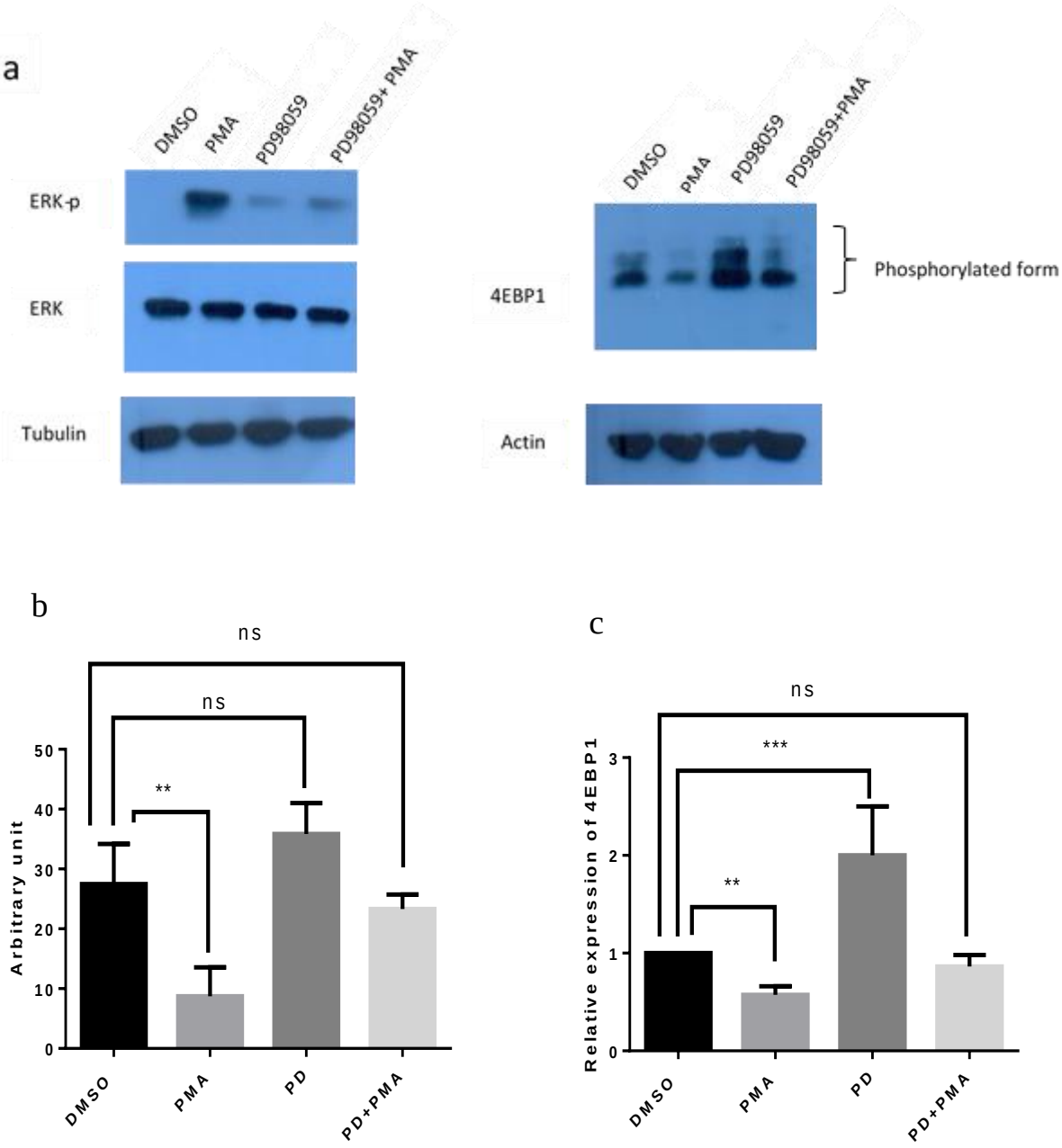


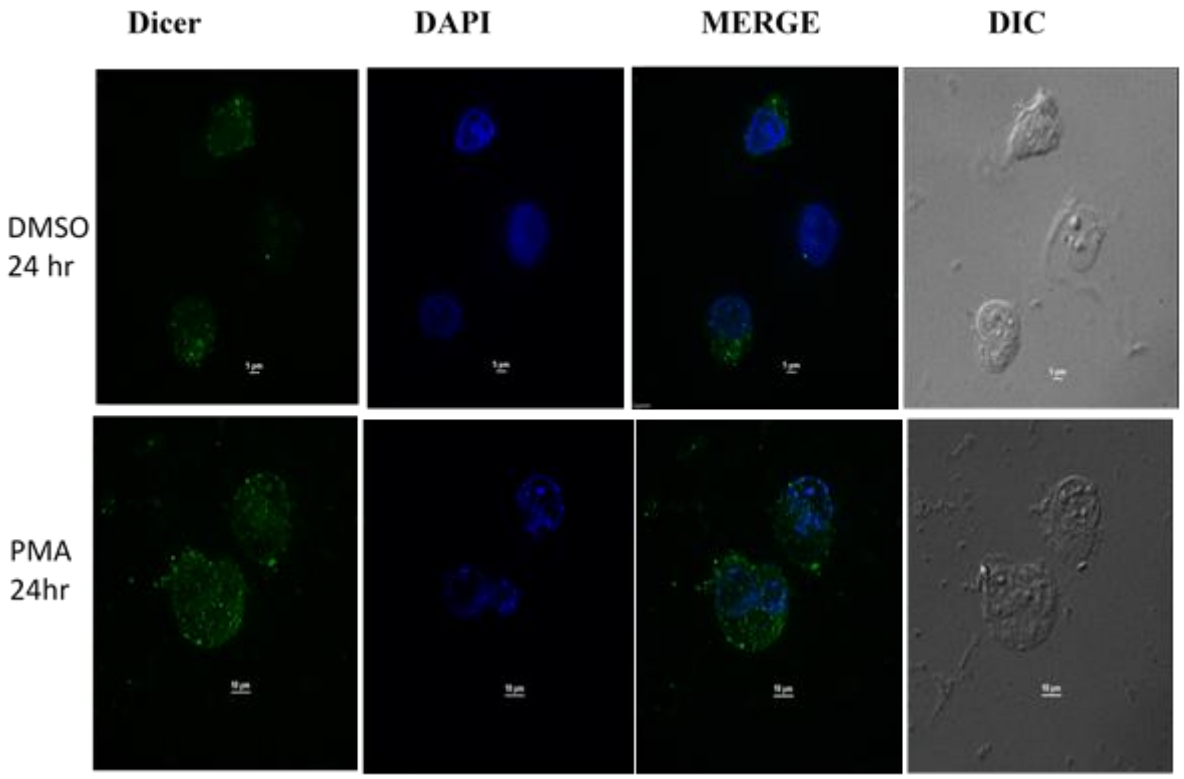
Figure 2.6: 4EBP1 is regulated by MAP kinase signalling pathway; (a) The western blot of ERK-P, ERK and 4EBP1 in presence or absence of PMA and MAP kinase inhibitor PD98059; (b) Densitometry analysis of the western blot (a) using AplhaEaseFC software

(for details see *materials and methods*); (c) qRT-PCR of 4EBP1 in presence or absence of PMA and MAP kinase inhibitor PD98059. Fold change for qRT-PCR was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** For densitometry analysis AplhaErase FC was used. The density of the blot was normalized with that of the loading control. Using the control sample as reference the change in density of other experimental samples was measured. (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns- p>0.05). (*All experiments were carried out three times); PD= PD98059.*

5.2.7 Localization of Dicer in PMA treated cells

In mouse, Dicer is exclusively found in the cytoplasm (Much, Auchynnikava et al. 2016). On the other hand, human Dicer protein is known observed both in cytoplasm and nucleus (White, Schlackow et al. 2014). Though the function of nucleus dicer is not well defined some studies have suggested that it helps in processing of double stranded RNAs that arise during convergent transcription which otherwise is deleterious for cells and also helps in formation of heterochromatin structure in the nuclei (Fukagawa, Nogami et al. 2004, White, Schlackow et al. 2014). We checked the localization of Dicer in PMA treated K562 cells and found that in control sample (DMSO) and 24h of PMA treatment, Dicer was localized both in cytoplasm and nucleus *(Fig 2.7a)* but after 48h of PMA treatment Dicer was translocated into the nucleus *(Fig 2.7b).* We observed this phenomena in about 60% of the cells. A study showed that ERK phosphorylated Dicer on two conserved residues, and this phosphorylation triggered nuclear translocation (Drake, Furuta et al. 2014). We can conclude that PMA treatment leads to nuclear translocation of Dicer via MAP kinase signalling.

a


Dicer
DAPI
MERGE
DIC
DMSO
24 hr
PMA
24hr

b

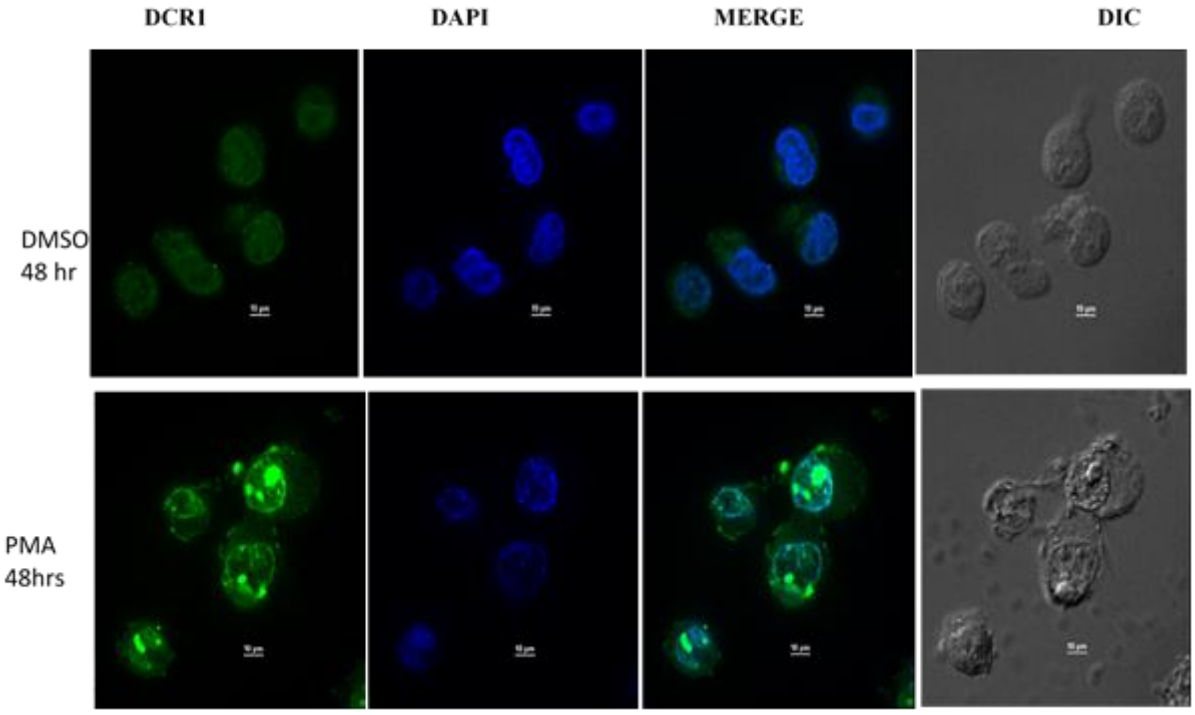

DCR1
DAPI
MERGE
DIC
DMSO
48 hr
PMA
48hrs

Figure 2.7: Imaging of Dicer in PMA treated K562 cells. Cells cultured with or without PMA for 24h and 48h were fixed on the coverslip. Then incubated the fixed cells with Dicer primary antibody. To detect the primary antibody we used Alexa 488 conjugated secondary antibody. For nucleus detection, we used DAPI stain. Cells were viewed under a confocal microscope (Olympus fluoview FV1000). Around 60% of the cells shows the translocation of Dicer in the nucleus in 48h treated PMA.

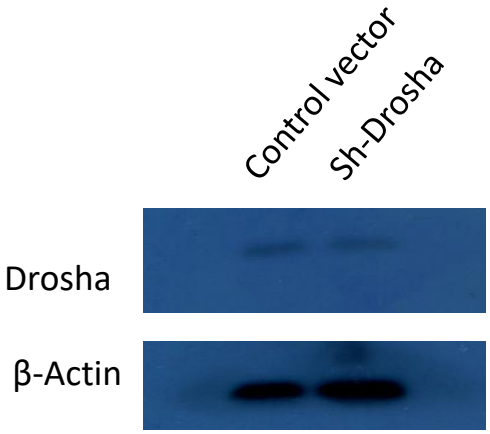
5.2.8 Drosha expression in PMA treated K562

Drosha is a endoribonuclease that cleaves the pri-miRNA into ~70 nt pre-miRNA in the nucleus. There are miRNAs that by pass the Drosha processing like the mirtons. These are the miRNAs that stem from short hairpin introns. Mirtrons are generated from the mRNA splicing and lariat debranching introns that mimic the pre-miRNA to enter the Dicer processing, and they are proven to be functional miRNA (Yang and Lai 2011). Other Drosha independent miRNAs are miRNAs which are generated from snoRNA, tRNA, and endo-shRNAs. In K562 cells, miRNA constitute only 18% of the total small RNA. But when treated with PMA, 46.8 % of the small RNA reads mapped with the pre-miRNA. And as mentioned earlier 24.4% of the miRNAs were upregulated while only 10% were downregulated. Despite this huge stimulation of miRNA population the Drosha level was downregulated in PMA treated K562 cells. So it is likely that Drosha may not always be involved in miRNA processing. Drosha knockdown stable cell line was generated *(Fig 2.8).* The knockdown showed 50% downregulation of Drosha expression (qRT-PCR- p value .0016) *(Fig 2.8a).* After PMA treatment was given to the knockdown cell line, Drosha level all showed 80% inhibition (qRT-PCR p value .0018) compared to the control (empty vector with only DMSO) *(Fig 2.9b&c).* At this level of Drosha it was we expected that if the miRNAs were independent of Drosha processing the miRNA expression would be unaffected when compared with controls (control vector with DMSO treatment). In order to test this three miRNAs (that Let-7, miR-23a and miR-22) were randomly chose. The PMA treatment could significantly (p value 0.021) enhanced the expression of the three miRNAs compared to that of the control vector *(Fig 2.8d).* The knockdown of Drosha does affect the expression of these miRNAs (p value 0.0078) in the only DMSO added condition. In the Drosha knockdown cell line the PMA could no longer upregulate (not significant) the expression of these three miRNAs. This showed that Let-7, miR-23a and miR-22 are indeed

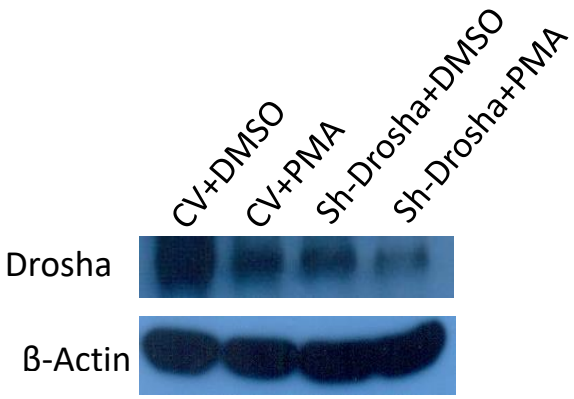
dependent on Drosha processing and there may be no alternative protein for pri-miRNA processing in PMA treated K562 cells.

We conclude that the inverse correlation of Dicer and Drosha is a regulatory mechanism to balance the synthesis of miRNA populations in PMA treated cells.

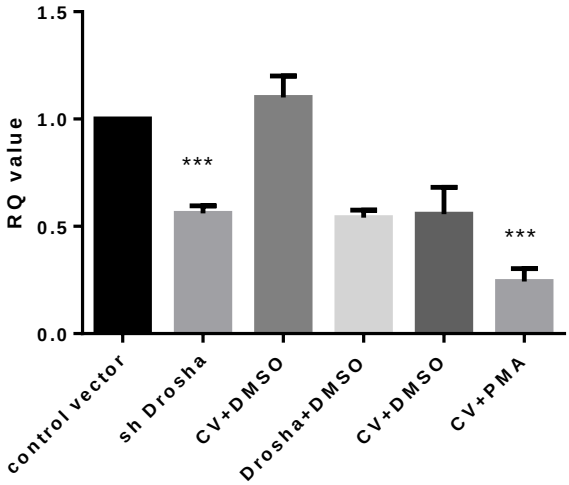
a



b



c



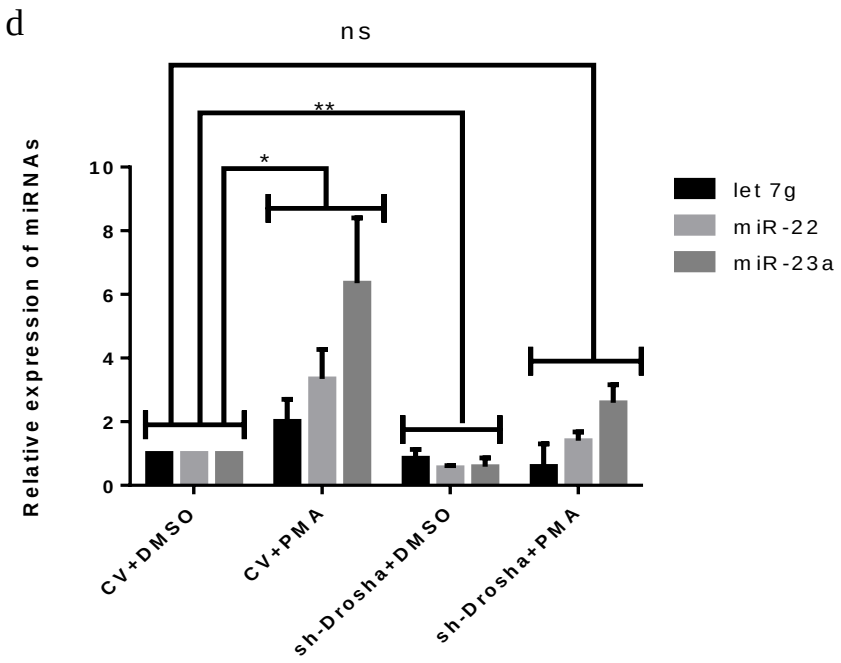


Figure 2.8: Effects of PMA on knockdown of Drosha cell line; (a) Western blots of Drosha knockdown (b) Western blots showing the effects of PMA treament on Drosha expression in the cell line; (c) qRT-PCR of Drosha knockdown cell line in presence or absence of PMA quantified; (d) Expression of Let-7, miR-23a, miR-22 in presence or absence of PMA quantited by qRT-PCR in Drosha knowdown cell line. Fold change for qRT-PCR was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** For densitometry analysis AplhaErase FC was used. The density of the blot was normalized with that of the loading control. Using the control sample as reference the change in density of other experimental samples was measured. (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns- p>0.05. CV=control vector, ns= non-significant.

5.3. Discussion

The expressions of microRNA are regulated depending on the type of cells, tissues, developmental stages and diseases. Generally the regulation is at trancription and post trancription for a particlular set of miRNAs (Winter, Jung et al. 2009). A study showed that only 25% of the differentail miRNA expression could be attributed to change in transcription in B cells (Kuchen, Resch et al. 2010). Also, the alteration of miRNA level is also linked to impairment of biosynthetic machinary (Winter, Jung et al. 2009). Downregulating the three different components of miRNA processing machinary – Dicer, Drosha and Ago 2, could substantially decrease steady-state miRNA levels, and loss of miRNA could enhance tumorogenesis by deregulating oncogenic target gene (Kumar, Lu et al. 2007). In our study the stimulation of miRNA synthesis could be correlated to Dicer protein expression. In K562 cells, Dicer was expressed at lower level and miRNA constituted only 18% of the total small RNA population. When K562 cells were treated with PMA, the miRNA population rose to 42.6% with an increase in Dicer protein expresion. So we could concluded that the escalation in miRNA synthesis was due to Dicer processing. However, the Drosha expression was repressed in PMA sample. This suggested a alternative pri-miRNA processing in this cell system. When the Drosha knockdown cell line was treated with PMA, the expression of Drosha reduced to 80% compare to the control. But, the expression of few miRNAs were reduced in the Drosha downregulated cell. This excluded the posiblity of an alaternative pathway as the miRNAs levels were dependent on the expression of Drosha. A study has also reported an inverse expression of Drosha (upregulated) and Dicer (downregulated) in Triple negative breast cancer patient (Avery-Kiejda, Braye et al. 2014). Thus, PMA inhibiting the Drosha protein seems to be one of the way for regulation of miRNA biogenesis.

It was interesting to find that Dicer is post trancriptionally regulated in our system. The trancript level was unchanged but the protein level was upregulated. It is clear from the result presented that 4EBP1, a translation inhibitor protein, whose expression is always inversely corrleated with Dicer in K562 cells and PMA treated K562 cells, regulated Dicer expression. 4EBP1 may be a general translation inhibitor but it can modulate the translation of a specific set of trancript. This depend on factor like the ratio of 4EBP1 and eIF4E, the 5’ UTR structure, the GC content and length of the 5’ UTR (Musa, Orth et al. 2016). A study showed that an active 4EBP1 mutant could enhance the translation of the reporter contruct that had a shorter and less structured 5’ UTR (Zid, Rogers et al. 2009). Phosphorylated 4EBP1 were reported in colorectal, breast lung and leukemia and these were corrleated with poor prognosis, metatasis and tumor progression. Phosphorylated 4EBP1 can not bind eIF4E. Thus there was no translational inhibition for proto oncogenic eIF4E-sensitive transcripts (Musa, Orth et al. 2016). We believed that the inhibition of 4EBP1 expression and the phosphorylation by MAP kinase signalling in PMA treated cell gave Dicer mRNA transcipt an easy access to bind eIF4E and eIF4G freely leading to smooth translation.

Based on the result presented, it is hypothesized that PMA activates MAP kinase signaling via PKC protein. The MAP kinase pathway leads to downregulaion of the transcript level and protein level. The inhibition relived Dicer transcript from translation inhibition and thus more Dicer transcipt are translated. Increase levels of Dicer protein help in generating more mature miRNAs. From literature survey it has been found that ERK phosphorylated Dicer, and this phosphorylation was needed for tanslocation of Dicer to nucleus. Dicer showed enrichement in nucleus at 48h of PMA treatment as mentioned above. In the nucleus, Dicer processed the endogenous shRNA from a facultative heterochromatin along with RNA pol II to generate RNAi response through Ago protein, and this process was necessary for viabiltiy of cells (White, Schlackow et al. 2014). In other studies, nulcear Dicer was associated with methylation of a promoter and increased tumor size (Cheng, Qi et al. 2017).

A number of studies have correlated miRNA abundance with cancer progression that is, more miRNAs more tumorigenesis. Jun Lu et al 2005 had analysed 217 mammalian miRNAs from 334 samples, including multiple human cancers, and came to the conclusion that tumors showed general downregulation of miRNAs compared to healthy tissues (Lu, Getz et al. 2005). Another group has also shown the donwregulation of miR-379/miR-656 cluster in multiple cancer (Laddha, Nayak et al. 2013). Similarly, in our previous studies we had shown that miRNA population were lesser in K562 cells and CML patients compared to normal PBMCs (Vaz, Ahmad et al. 2010, Vaz, Ahmad et al. 2013). The mechanism is not clear yet but it is likey due to relieving the inhibition of the proto-oncogenes in the cell.

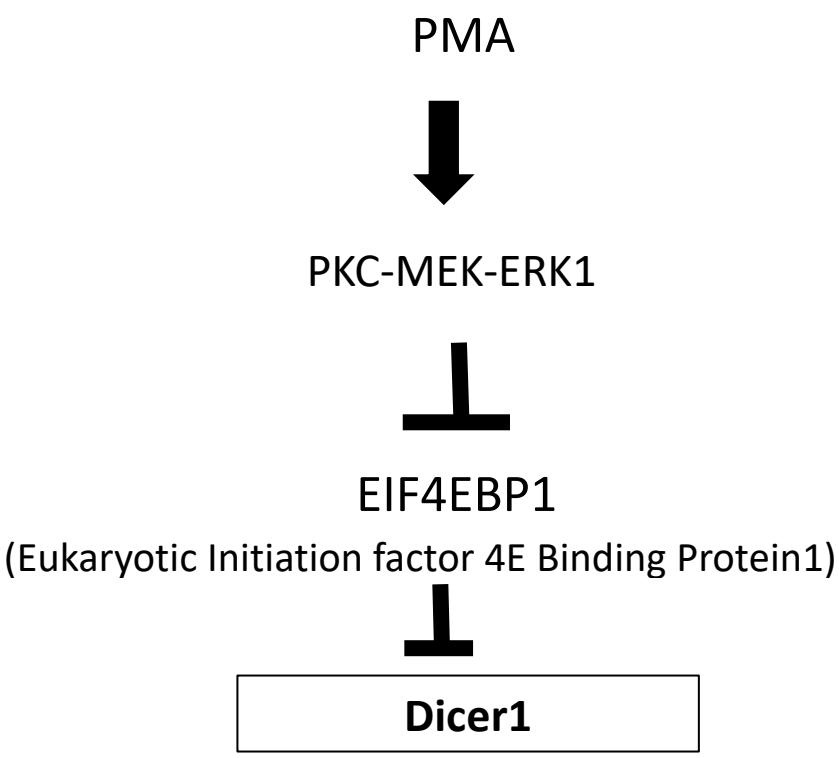


Figure 2.9: Model showing the PMA treatment activating the PKC-MEK-ERK1 pathway, thereby, inhibiting the translation inhibitor 4EBP1 to relieve the Dicer transcript from translational repression.

CHAPTER-3

REGULATION OF MIRNA BIOSYNTHETIC ENZYMES

6.1.0 Introduction

Chronic Myeloid Leukaemia (CML) is a myeloproliferative disorder resulting from a clonal expansion of a transformed multipotent hematopoietic stem cell. The discovery of Philadelphia (Ph) chromosome in 1960 led to better understanding of the pathogenesis of the disease (Nowell 1962). Ph chromosome is a reciprocal translocation between chromosomes 9 and 22. The translocation was found to involve the head to tail fusion of BCR gene on chromosome 22q11 with ABL1 gene located on chromosome 9q34 that creates the fusion gene *bcr-abl1* (Bartram, de Klein et al. 1983, Groffen, Stephenson et al. 1984). This chromosomal abnormality occurs in more than 95% of CML patients (Kumar 2006) and in some patients with acute lymphoblastic leukaemia (ALL) or acute myelogenous leukaemia (AML). The BCR-ABL encodes a tyrosine kinase which is constantly in active form and activates the downstream pro-survival, growth and anti-apoptosis signalling pathways including JAK/STAT, RAS/RAF/MEK/ERK, PI3K/AKT, and BAD/BCL-X_L (Eide and O'Hare 2015).

CML is a diphasic disease. Usually, the patient is diagnosed at chronic phase (CP) and later progresses to a terminal acute leukaemia-like phase, called blast crisis (BC), sometimes preceded by an accelerated phase (AP) (Bennour, Saad et al. 2016). CP is characterised by the presence of less than 10% blast cells (immature white blood cells) which can last for several years. About 90% of CML are diagnosed at this stage. Acceleration phase have 10-19% blasts in blood and bone marrow, and tend to have other cytogenetic changes in addition to Ph chromosome. Blast crisis have more than 20% blasts in their blood and bone marrow. The symptoms of CML includes anaemia, fatique, abdominal discomfort, enlarged spleen, fever and weight loss (www.cancer.net).. Moreover, about 40% of CML patients are asymptomatic and are diagnosed as a result of atypical blood counts and cytogenetically by presence of Ph chromosome (Goldman and Melo 2003, Chereda and Melo 2015).

In India, 60-70% of the leukemia cases reported in adult are CML (Bansal, Prabhash et al. 2013) but in US only 10-15% of leukemia are CML according to Surveillance, Epidemiology & End Results program data (SEERP). The median age at diagnosis of disease is 65 in US and 38-40 in India though this disease affects people of all age groups, including children (Au, Caguioa et al. 2009). The incidence of CML is higher in male compared to

female (male:female ratio is 1.3-1.35: 1) and the only proven risk is high exposure to ionizing radiation (Corso, Lazzarino et al. 1995).

6.1.1 Molecular basis for CML

ABL gene has non-receptor tyrosine kinase activity. It has a role in cell growth, apoptosis, DNA repair and cellular signalling. On the other hand BCR gene function is not well known. BCR-ABL fusion protein formed could be of different molecular weight depending on the of the break point of BCR gene. However, the breakpoint in the ABL region could occur anywhere within 300 KB areas at its 5' end in chromosome 9q34 (Deininger, Goldman et al. 2000). Majority of the breakpoints of BCR gene are either between exon 13 and 14, or 14 and 15. In ABL gene the patients have breakpoints in intron 1 or 2. This fusion transcript generates a 210 kDa protein and is responsible for malignant transformation and phenotypic abnormalities of chronic phase CML (*Fig F*) (Salesse and Verfaillie 2002). Normally ABL protein is under negative regulation through its SH3 domain, but after the fusion process this negative regulation is lost, and so BCR-ABL is constitutively active (Mayer and Baltimore 1994). This leads to activation of signalling pathways like JAK/STAT, RAS/RAF/MEK/ERK, PI3K/AKT, and BAD/BCL-X_L that ultimately alter adhesion to marrow stroma and extracellular matrix, reduction of apoptosis, enhanced cell proliferation and transformation, and reduction of proteasome mediated degradation of ABL inhibitory proteins. All these contribute to neoplastic transformation of the cell (Salesse and Verfaillie 2002, Eide and O'Hare 2015). The progression of the disease from CP to BC has additional chromosomal abnormalities like double Ph chromosomes, trisomy 8, isochromosome 17q, trisomy 19, translocations t (3,21) and t(7,11) with molecular abnormalities p53, p16ARF, Rb and RAS mutations (Calabretta and Perrotti 2004).

It is still not clear whether BCR-ABL fusion alone is sufficient to induce CML in humans. Some studies have mentioned the requirement of additional genetic abnormalities for induction of CML as 30% of the healthy individuals have BCR-ABL protein in their blood but only a small percentage develops CML. An older person may have higher t(9:22) translocation in their hematopoietic stem cells. A mathematical modelling has suggested the requirement of at least three mutations in the stem cell for a chronic disease to develop and one more mutation at the stem cell or committed cell induce progression to blast stage (Ren 2005).

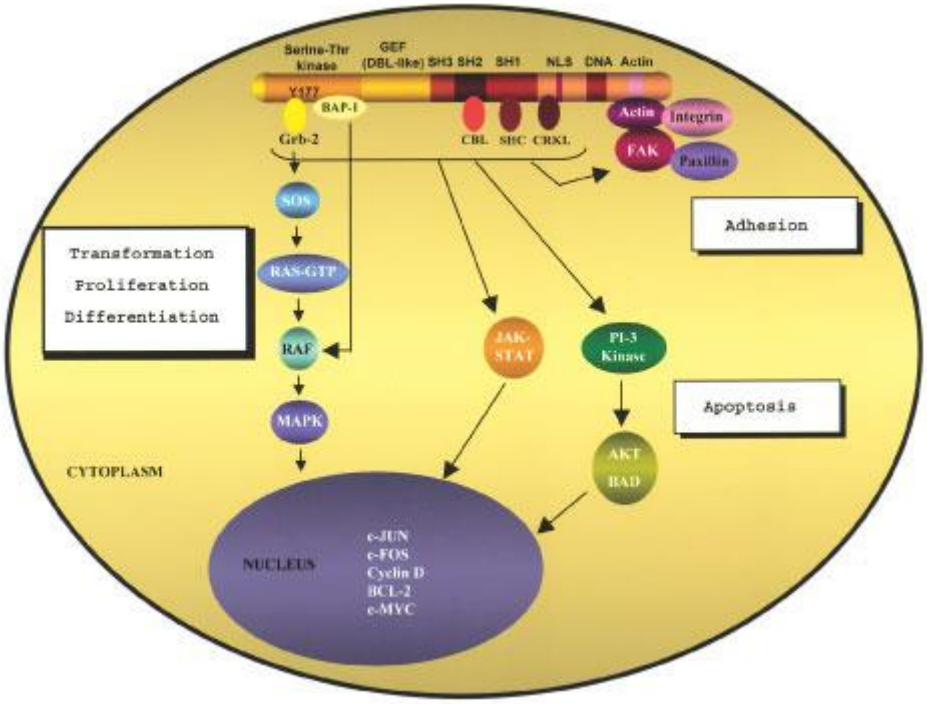


Figure F: Signalling pathway of BCR-ABL protein. Activation of JAK/STAT, RAS/RAF/MEK/ERK, PI3K/AKT, and BAD/BCL-X_L and ultimately leading to reduced apoptosis, increased cell adhesion and cell proliferation and transformation (Salesse and Verfaillie 2002).

6.1.2 Tyrosine Kinase Inhibitors (TKI)

Until early 1990's hydroxyurea, a ribonucleotide reductase inhibitor, busulfan, Interferon alpha and allogeneic stem cell transplantation were used to treat CML. But in late 1990's, the discovery that BCR-ABL is central to disease pathogenesis and progression led to discovery of a tyrosine kinase inhibitor, Imatinib. It revolutionized the treatment of CML; increasing overall survival rate profoundly (Huang, Cortes et al. 2012, Bennour, Saad et al. 2016). Imatinib specifically binds the tyrosine kinase active sites and inhibits its activity. New generation of TK inhibitors, Nilotinib (TasignaR), Dasatinib (SprycelR), Bosutinib (BosulifTM), Ponatinib (IclusigTM) and Omacetaxine (SynriboTM) are also available.

A complete cytogenetic response (CCyR) is defined as the absence of the Ph chromosome in bone marrow cells, and major molecular response (MMR) is a condition where the BCR-ABL: ABL ratio is less than 0.1%. Five years of Imatinib treatment showed a significant CCyR and MMR of 77% and 58% respectively (Druker, Guilhot et al. 2006).

It appears that 20-25% of the CML are resistant to Imatinib treatment (Apperley 2007). The patient may fail to show response to the drug or the patient may achieve and subsequently lose its response. Imatinib cannot eradicate all neoplastic stem cells (subclones) in CML which can lead to overpopulation of the subclones carrying mutation that leads to imatinib resistance (Branford, Rudzki et al. 2002, Kantarjian, Giles et al. 2006). Moreover the most common mechanism of Imatinib resistance stems from BCR/ABL mutation. Around 50%-90% of cases with clinical resistance to Imatinib were reported to have 40 different mutations at the kinase domain. The mutation restrains the conformational changes of the kinase domain to which imatinib binds. Mutation can be at the ATP binding loop (P-loop), activation loop (A-loop), catalytic domain and others (Apperley 2007, Experts in Chronic Myeloid 2013).

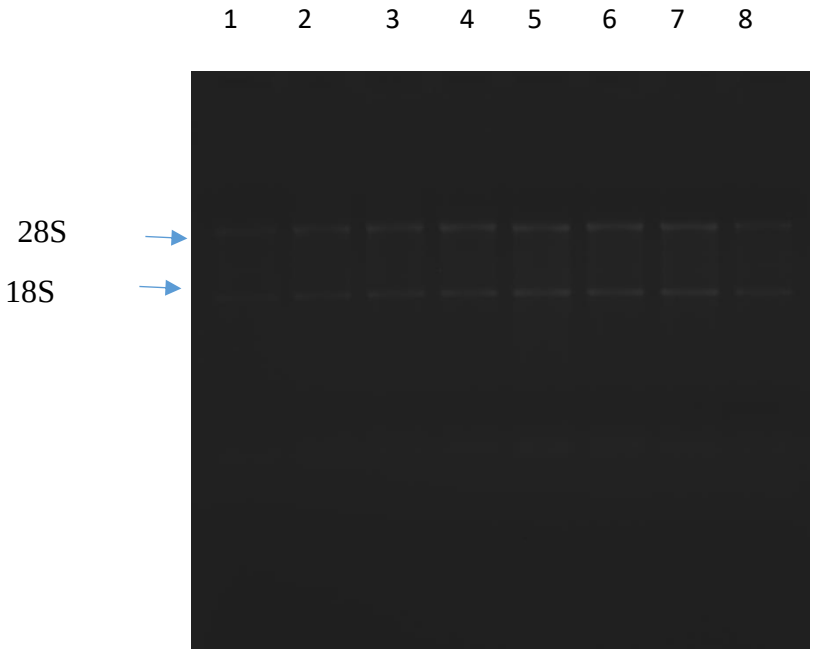
In this chapter we investigate to correlate expression levels of differentially expressed miRNAs in CML with respect to imatinib responders. About 25% of the patients are known to be resistant to Imatinib, and prolonged usage of the drug renders mutation in the drug binding site of BCR-ABL protein that leads to resistance (Apperley 2007, Experts in Chronic Myeloid 2013, Kagita, Uppalapati et al. 2014). Moreover, a case of increased efflux gylycoprotein and reduced influx transporter are reported (Apperley 2007). For these issues, there is a need of a reliable biomarker that can predict the ineffectiveness of drug therapy. This study has been carried out in collaboration with All India Institute of Medical Sciences (AIIMS), Department of Pharmacology and Oncology. Patient handling protocol was carried out at AIIMS, but all the molecular analysis was performed at SLS, JNU. Ethical clearance for this work was taken from JNU. The patients were assessed for their response to therapy based on white cell counts, platelet count and bone marrow biopsy. A complete hematologic remission (CHR) is the condition of absence of immature cells in peripheral blood and a white blood cell count less than 10×10^9/L. A complete cytogenetic response is the condition where there is no visible BCR-ABL protein in the bone marrow. In this study, a patient who achieved a CHR within 3 months of Imatinib mesylate at a dose of 400 mg/day is defined as a responder. Primary resistant patients are defined as those who failed to achieve the expected response (3 month CHR, 6 month CCyR, 12 month molecular response). Secondary resistant patients are those that eventually lose response to the therapy.

6.2.0 Result

6.2.1 Sample collection

For this study we recruited 10 healthy individuals, and 20 patients who were newly diagnosed with CML and were naïve to imatinib. Out of the 20 CML patients, 19 were found to be in blast crisis and one of them in acceleration stage. We followed up these patients for 3 months during their imatinib treatment. Also 10 Imatinib resistant patient were recruited, who were already on. Out of 10 resistant patient, 2 were found to be primary and rest 8 were secondary resistant patient. The patients were recruited irrespective of their age and sex. We found that majority of the patient were males (65% in newly diagnosed patients and 70% in resistant patient).

PBMCs (peripheral blood mononuclear cells) was isolated from the volunteering individuals by careful layering of the blood sample on Histopaque 1077 and then centrifuged it. The total RNA was isolated using Ambion kit. The RNA quality is shown in the figure 3.1a.and the flowchart of the study is shown in figure 3.1b.



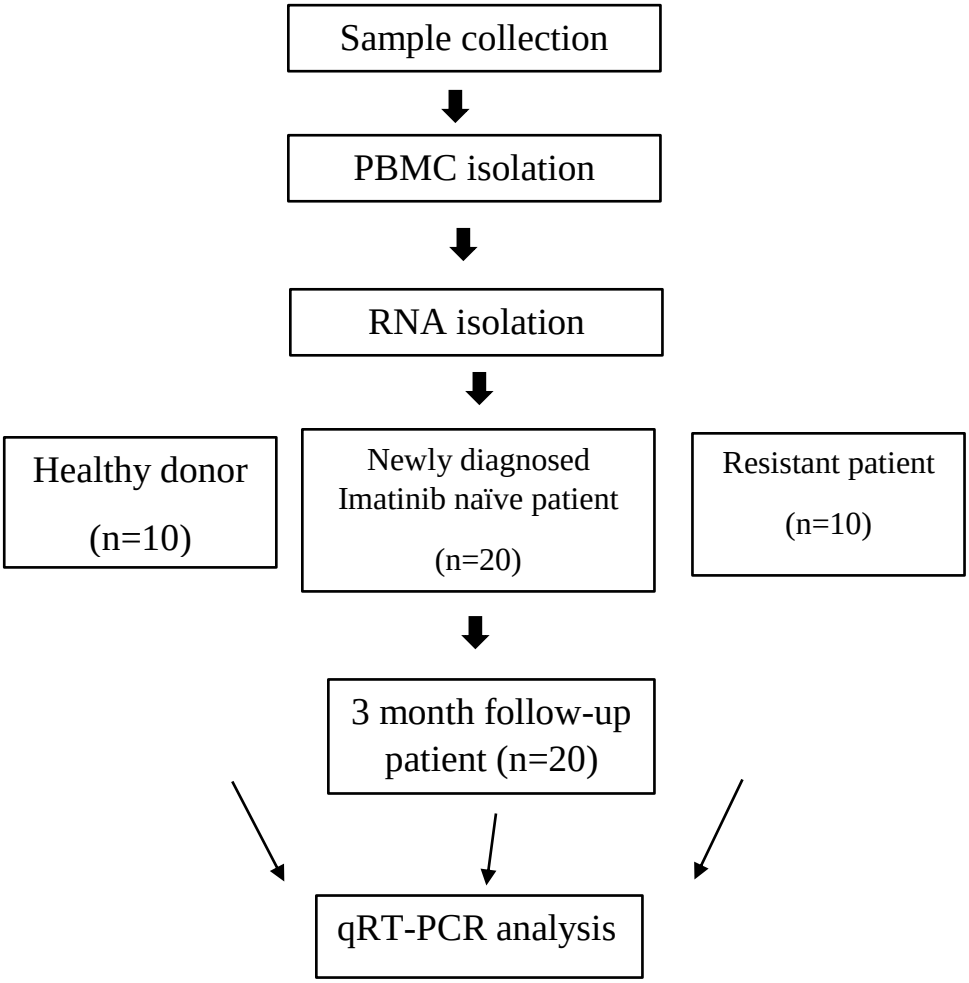


Figure 3.1 (a) A representative denaturing agarose gel picture of total RNA of the recruited individuals. Lane 1-3= healthy donor, lane 7-8 newly diagnosed imatinib naïve patient. Total 1ug of RNA was loaded in 0.8% denaturing agarose gel. (b) A flowchart of study outline.

6.2.2 Screening of miRNAs in CML patient

Out of the twenty newly diagnosed CML patients, 18 were found to be imatinib responders. They achieved complete cytogenetic response after 3 months of imatinib treatment. Based on the literature survey and our previous lab data (Vaz, Ahmad et al. 2010, Machova Polakova, Lopotova et al. 2011) we chose 8 differentially expressed miRNAs (miR-18a-5p, miR-20a-3p, miR-19a-3p, miR-19b-3p, mir-92a-3p, miR-193b-5p, miR-193b-3p and miR-505-5p) to check their expression in healthy donors (n=5) and newly diagnosed patients

naïve to imatinib (n=7). We found that 50% of the selected miRNAs were significantly differentially expressed in CML *(Fig 3.2).* MiR-505-5p, miR-20a-3p ,miR-18a-5p, and miR-193b-3p were significantly downregulated in CML 5-, 2-, 4- and 2.5-fold respectively (p value .005, .048, .004,.03 respectively) in 7 newly diagnosed patients when compared to 5 healthy donors individuals by qRT-PCR (*fig 3.2).*

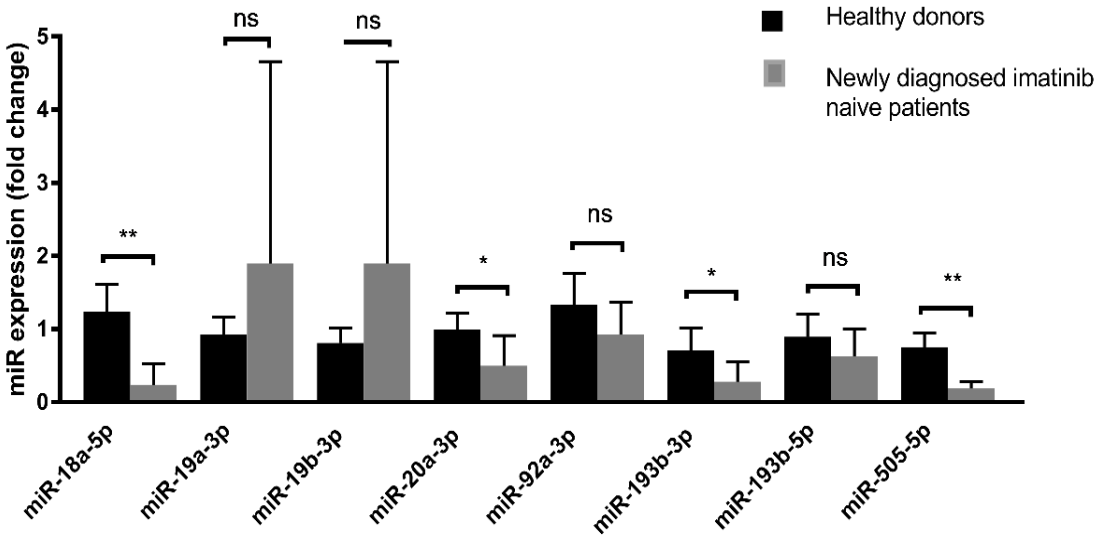


Figure 3.2: Screening of miRNA in CML patient by qRT-PCR; the relative expression of miR-18a-5p, miR-20a-3p, miR-19a-3p, miR-19b-3p, mir-92a-3p, miR-193b-5p, miR-193b-3p and miR-505-5p in heathy donors (n=5) and newly diagnosed CML patients (n=7). Fold change was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns- p>0.05.

6.2.3 Expression of miR-193b-3p in imatinib responder and resistant patient

MiR-193b-3p acts as a tumor suppressor in Acute lymphoblastic leukemia (ALL). It is known to supress the expression of oncogenic transcription factor MYB in T-ALL (Mets, Van der Meulen et al. 2015). MiR-193b gene is often hypermethylated and it targets cyclin D1 in prostate cancer supressing cell growth (Kaukoniemi, Rauhala et al. 2015). The epitopic over-expression of miR-193b and miR-365a inhibit proliferation and migration of epidermal squamous cell carcinoma (Gastaldi, Bertero et al. 2014). In CML also, miR-193b-3p is significantly downregulated suggesting the possible tumor suppressing role in the disease. Next, we checked the expression of miR-193b-3p in separate cohorts of healthy

donors (n=5), newly diagnosed patients (n=19), imatinib responders (n=18) and resistant patients (n=10). In the study of new cohorts, miR-193b-3p showed about 5 fold downregulation in newly diagnosed patients (p value <.0001) *(Fig 3.3).* After 3 months of imatinib therapy the patients' miR-193b-3p level shot up to 7.5 fold (p value <.0001) compared to imatinib naïve condition. But in the imatinib resistant patients the change of miR-193b-3p level was not significantly different from that of the newly diagnosed patients. This showed the dependence of miR-193b expression on BCR-ABL signalling pathway.

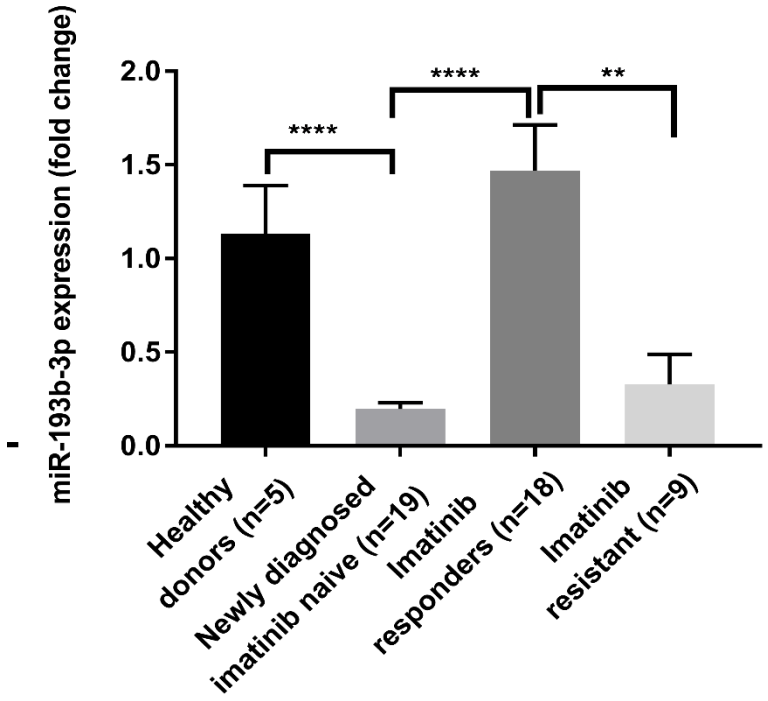


Figure 3.3: The relative expression of miR-193b-3p in healthy donor (n=5), newly diagnosed patient (n=19), Imatinib responder (n=18) and imatinib resistant (n=9) by qRT-PCR. Fold change was calculated using comparative CT method ($2^{-\Delta\Delta CT}$**).** (Graphical data points denote mean±SE). *p< 0.05; **p<0.01;***p<0.001; ns- p>0.05.

6.3 Discussion

CML is one of the commonest adult leukemia in India, accounting for 40-60% of all adult leukemia. The median age varied from 38 to 40 years in India (Bansal, Prabhash et al. 2013). A molecular targeted therapy of Imatinib against the BCR-ABL protein has revolutionised the therapy of the CML since 2001. It has largely increased the survival rate when compared to other chronic diseases like diabetics and hypertension (Huang, Cortes et al. 2012). The use of tyrosine kinase inhibitors has increase the overall survival rate of CML to more than 80%. Despite this, about 25% resistant cases have been reported against Imatinib. Of these the most common mechanism is mutation in fusion protein, which are detected in 20-30% of blast crisis patient and 70-80% of the chronic phase patient (Soverini, Hochhaus et al. 2011). And the resistant patient relayed on second generations of TKIs such as dasatinib, nilotinib, and bosutinib. Besides these, there are also other limitations – a monetary burden to the patient for life long therapy and refractoriness to imatinib therapy (Apperley 2007, Experts in Chronic Myeloid 2013). The prolonged treatment also leads to point mutations in BCR-ABL binding domain leading to more resistance (Kagita, Uppalapati et al. 2014) and over expression of the fusion gene. Also a case of increased efflux gylycoprotein and reduced influx transporter are reported (Apperley 2007). So, there is a need for a biomarker to predict the response to a therapy to overcome the drug resistance. In this study an attempt has been made to identify a miRNA whose expression reflects Imatinib resistance.

For this study we have selected a few miRNAs that are known to be deregulated in CML based on our previous studies and the literature. The deregulated miRNAs are likely to be the important modulators or the traits of that reflect of the cancer cell signalling, and so a good target for biomarkers in cancer studies. The selected miRNAs were found to be deregulated in the CML patients. When we looked deeper into the literature, miR-193b-3p was found to be often associated with cancer. It was found to play a tumor suppressor role in T-cell ALL. It is expressed at lower levels in ovarian cancer and often associated with poor prognosis. It also has a low expression in AML (acute myeloid leukimia) compared to that of the normal individual (Fan, Huang et al. 2013). In hematopoietic stem cell miR-193b provides a negative feedback to prevent uncontrolled expansion by limiting excessive signalling (Haetscher, Feuermann et al. 2015). On the other hand a study has shown that miR-193b enhances tumor progression by targeting neurofibromin 1 in HNSCC cell line (Lenarduzzi, Hui et al. 2013). In the same way our study also suggested the role of tumor suppressor in CML.

We also found that miR-193b-3p expression is suppressed in CML compared to that in healthy donors (5 fold lower p value<.001), and the suppression is relieved by Imatinib. Therefore, miR-193b expression inversely correlates with Imatinib responder. It also shows that miR-193b-3p expression is dependent on BCR-ABL signalling. Interestingly, we found that the miR-193b level does not change in resistant patient. To our best knowledge, this is the first study to show the involvement of miR-193b-3p in Imatinib resistance. However, a larger clinical trials is needed for longer duration in order for miR-193b-3p to be use as a biomarker for predicting Imatinib resistance.

SUMMARY AND CONCLUSIONS

Summary and conclusions

It has been estimated that 75% of the cells' energy is spent on translational processes, while only ~2% of the energy is spent in transcription. Cells have devised a beautiful mechanism to regulate the gene expression network by using minimum energy, a small regulatory RNA molecules. MicroRNAs can regulate the gene expression by binding mostly at the 3' UTR of the transcript, thus inhibiting and destabilizing the translational machinery. MiRNAs are predicted to regulate 60% of all the protein coding genes in mammalian cells. Their roles have been implicated in almost all physiological processes such as cell cycle, apoptosis, signalling, cell division, proliferation and cell death. Dysregulation of miRNA profile has been reported in many diseases. In many cancer cells total downregulation of miRNA population has been observed. Dyregulation of miRNA genes is due to genetic and epigenetic alteration, impairment of biosynthetic machinery and transcription factor regulation.

MicroRNAs can successfully classify the stages of tumor, thus making them good candidates for diagnostic marker. They are highly stable in preserved tissues and blood samples for a long time, probably due to their small size (Melo and Esteller 2011). Biomarkers suggested for different cancers are: miR-203 for colorectal cancer (Fu, Zhang et al. 2016), miR-505 and miR-193b for CML (Ramachandran, Muiwo et al. 2017), miR-22 and miR-20a for gastric cancer (Jafarzadeh-Samani, Sohrabi et al. 2017), and miR-196a is in pancreatic ductal adenocarcinoma (PDAC) (Melo and Esteller 2011). MiRNAs can function as oncogenes and tumour suppressor. This can be exploited for therapeutic tools by overexpression or inhibition of miRNA. For this purpose synthetic siRNA oligos and chemically modified oligo ribonucleotide has been developed. A targeted delivery of miRNAs using nano particles have also been developed recently. Synthetic siRNA oligos are effective in *in vivo* for almost a couple of weeks in mouse model. To overcome this a plasmid vector containing hairpin RNA cloned in tissue specific promoter is used. A sponge vector is also used to eliminate the endogenous miRNA from inhibiting their natural target gene.

IsomiRs are not widely studied because it they are thought not to behave differently from the reference miRNA and functionally considered to be complementing or assisting the canonical miRNA. But with time this myth has been disproved as discussed earlier. Recent

study has shown that the differentially expressed isomiRs could be a potential biomarker to differentiate normal individuals from cancerous patients.

In this study we have analysed the patterns and characterised a few of the isomiRs produced in PMA differentiated K562 cells, and also checked the function of miR-22 isomiRs in K562 cells. In the second chapter, we have focussed on the regulation of miRNA biosynthetic enzymes, mainly Dicer. And in the last chapter we have studied expression of a few miRNAs in Imatinib responders and resistant CML patients for a biomarker study. The results are summarised as below:

1. PMA stimulates microRNA production in K562 cells. 24.4% (71 miRNAs out of 291) of the miRNA goes up while only 10% (30 out of 291) miRNA are downregulated (list in appendix II).

2. Out of 113 miRNAs analysed, 92.9% displayed similar isomiR-relative pattern before and after induction of differentiation. Irrespective of upregulation or downregulation of the miRNA, there was no notable difference in relative proportions of isomiRs before and after treatment.

3. The isomiR profile of only 7% miRNAs were found to alter after PMA treatment. The ratios of the isomiRs were found to be notably different before and after treatment.

4. Literature search suggested that most of the miRNAs that displayed altered isomiR profiles were found to be oncogenic and are highly expressed in cancer cells.

5. Number of isomiRs and their relative expression varied among the miRNAs. In some case a single isomiR dominated the expression (eg: one isomiR made up 95% of the total TPM). Also two or more isomiRs of a miRNA displayed similar expression. A few miRNAs showed multiple isomiRs with different relative levels.

6. In most cases the 5p and 3p of a miRNA were processed similarly, except for miR-21. The isomiRs generated from 3p of miR-21 are dominated by one isomiR (75%) whereas two almost equal ratios of isomiRs were generated from 5p of miR-21 (39% and 36%).

7. We found that the number of isomiRs produced from the pre-miRNA was independent of the TPM of the miRNA.

8. The real time PCR of a miRNA using stem loop primer gave the quantification/levels of a pool of isomiRs. So,one has to be careful while designing the primer sets, especially when the reference miRNA is not the most abundant isomiR.

9. Change in single nucleotide at the 3' end (from U to G) could significantly alter the inhibition level of the target genes. We found that the level of inhibition was different for the different isomiRs.

10. PMA treatment upregulates the Dicer protein but not the transcript level. Drosha expression was repressed both at transcript and protein level.

11. 4EBP1 is always negatively correlated with Dicer (K562 or PMA treated K562 cells). The downregulation or upregulation of 4EBP1 affected the levels of Dicer protein. 4EBP1 expression is regulated by MAP kinase signalling at both transcript and protein level as shown by using MAP kinase inhibitor.

12. We propose that PMA treatment activates MAP kinase signalling via PKC and ultimately leads to downregulation of 4EBP1. This inhibition relived Dicer transcript from translation inhibition by 4EBP1, and thus more Dicer are translated. Hence, increased levels of Dicer helps in processing more mature miRNA.

13. Drosha knockdown does affect the expression of miRNAs hinting that probably Drosha is the rate limiting substrate in the biosynthesis of miRNA in PMA treated K562 cells.

14. Dicer are found to be localised in nucleus and cytoplasm in PMA treated K562 cells.

15. Expression of miR-18a-5p, miR-19a-3p, miR-19b-3p, miR-20a-3p, miR-92a-3p, miR-193b-3p, miR-193b-5p and miR-505-5p were checked in CML patients. When compared with the normal individuals, miR-18a-5p, miR-20a-3p, miR-193b-3p and miR-505-5p were found to be significantly downregulated in CML patients.

16. MicroRNA 193b-3p expression is supressed in CML patients. But the Imatinib responder patients showed increased miR-193b-3p expression after 3 months of Imitinib treatment. Interestingly, in Imatinib resistant patients the level of miR-193b-3p did not change.

ABBREVIATIONS AND SYMBOLS

mM	Milli molar
nm	Nanometer
ºC	Degree centigrade
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
RNA	Ribonucleic Acid
rpm	Revolutions per minute
Sec	Second
TEMED	N,N,N′,N′, Tetramethylethylenediamine
T_m	Annealing temperature
Tris	Tris (hyroxymethyl) amino ethane
v/v	volume/volume
w/v	weight/volume
γ	gamma
λ	lambda
µg	Microgram
µl	Microliter
µM	Micromolar
ATCC	American Type Culture Collection
bp	base pair
$CHCl_3$	Chloroform
Cl	Chloride
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetate
h	Hour/hours
min	Minute
s	second/seconds

kb	Kilobase pair
kDa	Kilo Dalton
M	Marker
mA	Milli ampere
Mg	Magnesium
mg	Milligram
ml	Milliliter
CML	Chronic myeloid leukemia
AML	Acute myeloid leukemia
miR	microRNA
PMA	Phorbol 12-Myristate 13-Acetate
DMSO	Dimethyl sulfoxide
4EBP1/EIF4EBP1	Eukaryotic initiation factor 4E binding protein
TPM	Trimmed per million
Ago2	Argonaute 2
DGCR8	DiGeorge syndrome criticle region gene 8
RISC	RNA-inducing silencing complex
CID-miRNA	computational identification of miRNA
TRBP	TAR-RNA binding protein
NGS	Next generation sequencing
DAPI	4′,6-diamidino-2-phenylindole
DIC	Differential interference contrast
Pri-miRNA	primary miRNA
Pre-miRNA	precursor miRNA
cDNA	complementary DNA

REFERENCES

References

Agarwal, V., G. W. Bell, J. W. Nam and D. P. Bartel (2015). "Predicting effective microRNA target sites in mammalian mRNAs." Elife **4**.

Ahmad, H. M., P. Muiwo, R. Muthuswami and A. Bhattacharya (2017). "FosB regulates expression of miR-22 during PMA induced differentiation of K562 cells to megakaryocytes." Biochimie **133**: 1-6.

Ahmad, H. M., P. Muiwo, S. S. Ramachandran, P. Pandey, Y. K. Gupta, L. Kumar, R. Kulshreshtha and A. Bhattacharya (2014). "miR-22 regulates expression of oncogenic neuro-epithelial transforming gene 1, NET1." FEBS J **281**(17): 3904-3919.

Ahn, K., J. A. Gim, H. S. Ha, K. Han and H. S. Kim (2013). "The novel MER transposon-derived miRNAs in human genome." Gene **512**(2): 422-428.

Apperley, J. F. (2007). "Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia." Lancet Oncol **8**(11): 1018-1029.

Askou, A. L., L. Aagaard, C. Kostic, Y. Arsenijevic, A. K. Hollensen, T. Bek, T. G. Jensen, J. G. Mikkelsen and T. J. Corydon (2015). "Multigenic lentiviral vectors for combined and tissue-specific expression of miRNA- and protein-based antiangiogenic factors." Mol Ther Methods Clin Dev **2**: 14064.

Au, W. Y., P. B. Caguioa, C. Chuah, S. C. Hsu, S. Jootar, D. W. Kim, I. Y. Kweon, W. M. O'Neil, T. K. Saikia and J. Wang (2009). "Chronic myeloid leukemia in Asia." Int J Hematol **89**(1): 14-23.

Avery-Kiejda, K. A., S. G. Braye, J. F. Forbes and R. J. Scott (2014). "The expression of Dicer and Drosha in matched normal tissues, tumours and lymph node metastases in triple negative breast cancer." BMC Cancer **14**: 253.

Avruch, J., A. Khokhlatchev, J. M. Kyriakis, Z. Luo, G. Tzivion, D. Vavvas and X. F. Zhang (2001). "Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade." Recent Prog Horm Res **56**: 127-155.

Bansal, S., K. Prabhash and P. Parikh (2013). "Chronic myeloid leukemia data from India." Indian J Med Paediatr Oncol **34**(3): 154-158.

Barshack, I., G. Lithwick-Yanai, A. Afek, K. Rosenblatt, H. Tabibian-Keissar, M. Zepeniuk, L. Cohen, H. Dan, O. Zion, Y. Strenov, S. Polak-Charcon and M. Perelman (2010). "MicroRNA expression differentiates between primary lung tumors and metastases to the lung." Pathol Res Pract **206**(8): 578-584.

Bartel, D. P. (2009). "MicroRNAs: target recognition and regulatory functions." Cell **136**(2): 215-233.

Bartram, C. R., A. de Klein, A. Hagemeijer, T. van Agthoven, A. Geurts van Kessel, D. Bootsma, G. Grosveld, M. A. Ferguson-Smith, T. Davies, M. Stone and et al. (1983). "Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia." Nature **306**(5940): 277-280.

Bennour, A., A. Saad and H. Sennana (2016). "Chronic myeloid leukemia: Relevance of cytogenetic and molecular assays." Crit Rev Oncol Hematol **97**: 263-274.

Betel, D., M. Wilson, A. Gabow, D. S. Marks and C. Sander (2008). "The microRNA.org resource: targets and expression." Nucleic Acids Res **36**(Database issue): D149-153.

Boele, J., H. Persson, J. W. Shin, Y. Ishizu, I. S. Newie, R. Sokilde, S. M. Hawkins, C. Coarfa, K. Ikeda, K. Takayama, K. Horie-Inoue, Y. Ando, A. M. Burroughs, C. Sasaki, C. Suzuki, M. Sakai, S. Aoki, A. Ogawa, A. Hasegawa, M. Lizio, K. Kaida, B. Teusink, P. Carninci, H. Suzuki, S. Inoue, P. H. Gunaratne, C. Rovira, Y. Hayashizaki and M. J. de Hoon (2014). "PAPD5-mediated 3' adenylation and subsequent degradation of miR-21 is disrupted in proliferative disease." Proc Natl Acad Sci U S A **111**(31): 11467-11472.

Branford, S., Z. Rudzki, S. Walsh, A. Grigg, C. Arthur, K. Taylor, R. Herrmann, K. P. Lynch and T. P. Hughes (2002). "High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance." Blood **99**(9): 3472-3475.

Brennecke, J., A. Stark, R. B. Russell and S. M. Cohen (2005). "Principles of microRNA-target recognition." PLoS Biol **3**(3): e85.

Bueno, M. J., I. Perez de Castro, M. Gomez de Cedron, J. Santos, G. A. Calin, J. C. Cigudosa, C. M. Croce, J. Fernandez-Piqueras and M. Malumbres (2008). "Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression." Cancer Cell **13**(6): 496-506.

Burke, J. M., D. P. Kelenis, R. P. Kincaid and C. S. Sullivan (2014). "A central role for the primary microRNA stem in guiding the position and efficiency of Drosha processing of a viral pri-miRNA." RNA **20**(7): 1068-1077.

Buscaglia, L. E. and Y. Li (2011). "Apoptosis and the target genes of microRNA-21." Chin J Cancer **30**(6): 371-380.

Cai, W., Q. Ye and Q. B. She (2014). "Loss of 4E-BP1 function induces EMT and promotes cancer cell migration and invasion via cap-dependent translational activation of snail." Oncotarget **5**(15): 6015-6027.

Calabretta, B. and D. Perrotti (2004). "The biology of CML blast crisis." Blood **103**(11): 4010-4022.

Calin, G. A., C. Sevignani, C. D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini and C. M. Croce (2004). "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers." Proc Natl Acad Sci U S A **101**(9): 2999-3004.

Cao, Z. G., J. J. Li, L. Yao, Y. N. Huang, Y. R. Liu, X. Hu, C. G. Song and Z. M. Shao (2016). "High expression of microRNA-454 is associated with poor prognosis in triple-negative breast cancer." Oncotarget **7**(40): 64900-64909.

Chan, Y. T., Y. C. Lin, R. J. Lin, H. H. Kuo, W. C. Thang, K. P. Chiu and A. L. Yu (2013). "Concordant and discordant regulation of target genes by miR-31 and its isoforms." PLoS One **8**(3): e58169.

Chang, C. T., N. Bercovich, B. Loh, S. Jonas and E. Izaurralde (2014). "The activation of the decapping enzyme DCP2 by DCP1 occurs on the EDC4 scaffold and involves a conserved loop in DCP1." Nucleic Acids Res **42**(8): 5217-5233.

Chendrimada, T. P., R. I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura and R. Shiekhattar (2005). "TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing." Nature **436**(7051): 740-744.

Cheng, W., Y. Qi, L. Tian, B. Wang, W. Huang and Y. Chen (2017). "Dicer promotes tumorigenesis by translocating to nucleus to promote SFRP1 promoter methylation in cholangiocarcinoma cells." Cell Death Dis **8**(2): e2628.

Chereda, B. and J. V. Melo (2015). "Natural course and biology of CML." Ann Hematol **94 Suppl 2**: S107-121.

Chiosea, S., E. Jelezcova, U. Chandran, M. Acquafondata, T. McHale, R. W. Sobol and R. Dhir (2006). "Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma." Am J Pathol **169**(5): 1812-1820.

Chou, C. H., N. W. Chang, S. Shrestha, S. D. Hsu, Y. L. Lin, W. H. Lee, C. D. Yang, H. C. Hong, T. Y. Wei, S. J. Tu, T. R. Tsai, S. Y. Ho, T. Y. Jian, H. Y. Wu, P. R. Chen, N. C. Lin, H. T. Huang, T. L. Yang, C. Y. Pai, C. S. Tai, W. L. Chen, C. Y. Huang, C. C. Liu, S. L. Weng, K. W. Liao, W. L. Hsu and H. D. Huang (2016). "miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database." Nucleic Acids Res **44**(D1): D239-247.

Choudhury, Y., F. C. Tay, D. H. Lam, E. Sandanaraj, C. Tang, B. T. Ang and S. Wang (2012). "Attenuated adenosine-to-inosine editing of microRNA-376a* promotes invasiveness of glioblastoma cells." J Clin Invest **122**(11): 4059-4076.

Christopher, A. F., R. P. Kaur, G. Kaur, A. Kaur, V. Gupta and P. Bansal (2016). "MicroRNA therapeutics: Discovering novel targets and developing specific therapy." Perspect Clin Res **7**(2): 68-74.

Cifuentes, D., H. Xue, D. W. Taylor, H. Patnode, Y. Mishima, S. Cheloufi, E. Ma, S. Mane, G. J. Hannon, N. D. Lawson, S. A. Wolfe and A. J. Giraldez (2010). "A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity." Science **328**(5986): 1694-1698.

Cloonan, N., S. Wani, Q. Xu, J. Gu, K. Lea, S. Heater, C. Barbacioru, A. L. Steptoe, H. C. Martin, E. Nourbakhsh, K. Krishnan, B. Gardiner, X. Wang, K. Nones, J. A. Steen, N. A. Matigian, D. L. Wood, K. S. Kassahn, N. Waddell, J. Shepherd, C. Lee, J. Ichikawa, K. McKernan, K. Bramlett, S. Kuersten and S. M. Grimmond (2011). "MicroRNAs and their isomiRs function cooperatively to target common biological pathways." Genome Biol **12**(12): R126.

Colina, R., M. Costa-Mattioli, R. J. Dowling, M. Jaramillo, L. H. Tai, C. J. Breitbach, Y. Martineau, O. Larsson, L. Rong, Y. V. Svitkin, A. P. Makrigiannis, J. C. Bell and N. Sonenberg (2008). "Translational control of the innate immune response through IRF-7." Nature **452**(7185): 323-328.

Coronnello, C. and P. V. Benos (2013). "ComiR: Combinatorial microRNA target prediction tool." Nucleic Acids Res **41**(Web Server issue): W159-164.

Corso, A., M. Lazzarino, E. Morra, S. Merante, C. Astori, P. Bernasconi, M. Boni and C. Bernasconi (1995). "Chronic myelogenous leukemia and exposure to ionizing radiation--a retrospective study of 443 patients." Ann Hematol **70**(2): 79-82.

Czubak, K., M. A. Lewandowska, K. Klonowska, K. Roszkowski, J. Kowalewski, M. Figlerowicz and P. Kozlowski (2015). "High copy number variation of cancer-related microRNA genes and frequent amplification of DICER1 and DROSHA in lung cancer." Oncotarget **6**(27): 23399-23416.

Dai, L., K. Chen, B. Youngren, J. Kulina, A. Yang, Z. Guo, J. Li, P. Yu and S. Gu (2016). "Cytoplasmic Drosha activity generated by alternative splicing." Nucleic Acids Res **44**(21): 10454-10466.

Davis, T. H., T. L. Cuellar, S. M. Koch, A. J. Barker, B. D. Harfe, M. T. McManus and E. M. Ullian (2008). "Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus." J Neurosci **28**(17): 4322-4330.

De Benedetti, A. and J. R. Graff (2004). "eIF-4E expression and its role in malignancies and metastases." Oncogene **23**(18): 3189-3199.

Dedes, K. J., R. Natrajan, M. B. Lambros, F. C. Geyer, M. A. Lopez-Garcia, K. Savage, R. L. Jones and J. S. Reis-Filho (2011). "Down-regulation of the miRNA master regulators Drosha and Dicer is associated with specific subgroups of breast cancer." Eur J Cancer **47**(1): 138-150.

Deininger, M. W., J. M. Goldman and J. V. Melo (2000). "The molecular biology of chronic myeloid leukemia." Blood **96**(10): 3343-3356.

Didiano, D. and O. Hobert (2006). "Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions." Nat Struct Mol Biol **13**(9): 849-851.

Drake, M., T. Furuta, K. M. Suen, G. Gonzalez, B. Liu, A. Kalia, J. E. Ladbury, A. Z. Fire, J. B. Skeath and S. Arur (2014). "A requirement for ERK-dependent Dicer phosphorylation in coordinating oocyte-to-embryo transition in C. elegans." Dev Cell **31**(5): 614-628.

Druker, B. J., F. Guilhot, S. G. O'Brien, I. Gathmann, H. Kantarjian, N. Gattermann, M. W. Deininger, R. T. Silver, J. M. Goldman, R. M. Stone, F. Cervantes, A. Hochhaus, B. L. Powell, J. L. Gabrilove, P. Rousselot, J. Reiffers, J. J. Cornelissen, T. Hughes, H. Agis, T. Fischer, G. Verhoef, J. Shepherd, G. Saglio, A. Gratwohl, J. L. Nielsen, J. P. Radich, B. Simonsson, K. Taylor, M. Baccarani, C. So, L. Letvak, R. A. Larson and I. Investigators (2006). "Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia." N Engl J Med **355**(23): 2408-2417.

Duncan, M. T., T. A. DeLuca, H. Y. Kuo, M. Yi, M. Mrksich and W. M. Miller (2016). "SIRT1 is a critical regulator of K562 cell growth, survival, and differentiation." Exp Cell Res **344**(1): 40-52.

Ebert, M. S. and P. A. Sharp (2010). "MicroRNA sponges: progress and possibilities." RNA **16**(11): 2043-2050.

Eide, C. A. and T. O'Hare (2015). "Chronic myeloid leukemia: advances in understanding disease biology and mechanisms of resistance to tyrosine kinase inhibitors." Curr Hematol Malig Rep **10**(2): 158-166.

Enright, A. J., B. John, U. Gaul, T. Tuschl, C. Sander and D. S. Marks (2003). "MicroRNA targets in Drosophila." Genome Biol **5**(1): R1.

Eulalio, A., E. Huntzinger and E. Izaurralde (2008). "GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay." Nat Struct Mol Biol **15**(4): 346-353.

Eulalio, A., J. Rehwinkel, M. Stricker, E. Huntzinger, S. F. Yang, T. Doerks, S. Dorner, P. Bork, M. Boutros and E. Izaurralde (2007). "Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing." Genes Dev **21**(20): 2558-2570.

Experts in Chronic Myeloid, L. (2013). "The price of drugs for chronic myeloid leukemia (CML) is a reflection of the unsustainable prices of cancer drugs: from the perspective of a large group of CML experts." Blood **121**(22): 4439-4442.

Fallah, P., N. Amirizadeh, B. Poopak, G. Toogeh, E. Arefian, F. Kohram, S. M. Hosseini Rad, M. Kohram, H. Teimori Naghadeh and M. Soleimani (2015). "Expression pattern of key microRNAs in patients with newly diagnosed chronic myeloid leukemia in chronic phase." Int J Lab Hematol **37**(4): 560-568.

Fan, L. P., H. B. Huang, J. Z. Shen, H. Y. Fu and H. R. Zhou (2013). "[MicroRNA-193b expression in newly diagnosed leukemia patients and its significance]." Zhongguo Shi Yan Xue Ye Xue Za Zhi **21**(4): 843-846.

Felice, K. M., D. W. Salzman, J. Shubert-Coleman, K. P. Jensen and H. M. Furneaux (2009). "The 5' terminal uracil of let-7a is critical for the recruitment of mRNA to Argonaute2." Biochem J **422**(2): 329-341.

Fernandez-Valverde, S. L., R. J. Taft and J. S. Mattick (2010). "Dynamic isomiR regulation in Drosophila development." RNA **16**(10): 1881-1888.

Frank, F., N. Sonenberg and B. Nagar (2010). "Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2." Nature **465**(7299): 818-822.

Friedman, R. C., K. K. Farh, C. B. Burge and D. P. Bartel (2009). "Most mammalian mRNAs are conserved targets of microRNAs." Genome Res **19**(1): 92-105.

Fu, Q., J. Zhang, X. Xu, F. Qian, K. Feng and J. Ma (2016). "miR-203 is a predictive biomarker for colorectal cancer and its expression is associated with BIRC5." Tumour Biol.

Fukagawa, T., M. Nogami, M. Yoshikawa, M. Ikeno, T. Okazaki, Y. Takami, T. Nakayama and M. Oshimura (2004). "Dicer is essential for formation of the heterochromatin structure in vertebrate cells." Nat Cell Biol **6**(8): 784-791.

Fukunaga, R., B. W. Han, J. H. Hung, J. Xu, Z. Weng and P. D. Zamore (2012). "Dicer partner proteins tune the length of mature miRNAs in flies and mammals." Cell **151**(3): 533-546.

Garzon, R., F. Pichiorri, T. Palumbo, R. Iuliano, A. Cimmino, R. Aqeilan, S. Volinia, D. Bhatt, H. Alder, G. Marcucci, G. A. Calin, C. G. Liu, C. D. Bloomfield, M. Andreeff and C. M. Croce (2006). "MicroRNA fingerprints during human megakaryocytopoiesis." Proc Natl Acad Sci U S A **103**(13): 5078-5083.

Gastaldi, C., T. Bertero, N. Xu, I. Bourget-Ponzio, K. Lebrigand, S. Fourre, A. Popa, N. Cardot-Leccia, G. Meneguzzi, E. Sonkoly, A. Pivarcsi, B. Mari, P. Barbry, G. Ponzio and R. Rezzonico (2014). "miR-193b/365a cluster controls progression of epidermal squamous cell carcinoma." Carcinogenesis **35**(5): 1110-1120.

Ghoshal, A., R. Shankar, S. Bagchi, A. Grama and S. Chaterji (2015). "MicroRNA target prediction using thermodynamic and sequence curves." BMC Genomics **16**: 999.

Gingras, A. C., S. G. Kennedy, M. A. O'Leary, N. Sonenberg and N. Hay (1998). "4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway." Genes Dev **12**(4): 502-513.

Goldman, J. M. and J. V. Melo (2003). "Chronic myeloid leukemia--advances in biology and new approaches to treatment." N Engl J Med **349**(15): 1451-1464.

Griffiths-Jones, S. (2004). "The microRNA Registry." Nucleic Acids Res **32**(Database issue): D109-111.

Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman and A. J. Enright (2006). "miRBase: microRNA sequences, targets and gene nomenclature." Nucleic Acids Res **34**(Database issue): D140-144.

Grimson, A., K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim and D. P. Bartel (2007). "MicroRNA targeting specificity in mammals: determinants beyond seed pairing." Mol Cell **27**(1): 91-105.

Groffen, J., J. R. Stephenson, N. Heisterkamp, A. de Klein, C. R. Bartram and G. Grosveld (1984). "Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22." Cell **36**(1): 93-99.

Ha, M. and V. N. Kim (2014). "Regulation of microRNA biogenesis." Nat Rev Mol Cell Biol **15**(8): 509-524.

Haetscher, N., Y. Feuermann, S. Wingert, M. Rehage, F. B. Thalheimer, C. Weiser, H. Bohnenberger, K. Jung, T. Schroeder, H. Serve, T. Oellerich, L. Hennighausen and M. A. Rieger (2015). "STAT5-regulated microRNA-193b controls haematopoietic stem and progenitor cell expansion by modulating cytokine receptor signalling." Nat Commun **6**: 8928.

Han, J., Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin and V. N. Kim (2004). "The Drosha-DGCR8 complex in primary microRNA processing." Genes Dev **18**(24): 3016-3027.

Han, J., Y. Lee, K. H. Yeom, J. W. Nam, I. Heo, J. K. Rhee, S. Y. Sohn, Y. Cho, B. T. Zhang and V. N. Kim (2006). "Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex." Cell **125**(5): 887-901.

Han, L., A. Zhang, X. Zhou, P. Xu, G. X. Wang, P. Y. Pu and C. S. Kang (2010). "Downregulation of Dicer enhances tumor cell proliferation and invasion." Int J Oncol **37**(2): 299-305.

He, L., H. Y. Wang, L. Zhang, L. Huang, J. D. Li, Y. Xiong, M. Y. Zhang, W. H. Jia, J. P. Yun, R. Z. Luo and M. Zheng (2014). "Prognostic significance of low DICER expression regulated by miR-130a in cervical cancer." Cell Death Dis **5**: e1205.

Helwak, A., G. Kudla, T. Dudnakova and D. Tollervey (2013). "Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding." Cell **153**(3): 654-665.

Herbert, T. P., A. R. Tee and C. G. Proud (2002). "The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E-BP1 at multiple sites." J Biol Chem **277**(13): 11591-11596.

Herrera, R., S. Hubbell, S. Decker and L. Petruzzelli (1998). "A role for the MEK/MAPK pathway in PMA-induced cell cycle arrest: modulation of megakaryocytic differentiation of K562 cells." Exp Cell Res **238**(2): 407-414.

Hill, D. A., J. Ivanovich, J. R. Priest, C. A. Gurnett, L. P. Dehner, D. Desruisseau, J. A. Jarzembowski, K. A. Wikenheiser-Brokamp, B. K. Suarez, A. J. Whelan, G. Williams, D. Bracamontes, Y. Messinger and P. J. Goodfellow (2009). "DICER1 mutations in familial pleuropulmonary blastoma." Science **325**(5943): 965.

Ho, A. W., A. V. Garg, L. Monin, M. R. Simpson-Abelson, L. Kinner and S. L. Gaffen (2013). "The anaphase-promoting complex protein 5 (AnapC5) associates with A20 and inhibits IL-17-mediated signal transduction." PLoS One **8**(7): e70168.

Hofacker, I. L. (2007). "How microRNAs choose their targets." Nat Genet **39**(10): 1191-1192.

Honda, S. and Y. Kirino (2015). "Dumbbell-PCR: a method to quantify specific small RNA variants with a single nucleotide resolution at terminal sequences." Nucleic Acids Res **43**(12): e77.

Horikawa, Y., C. G. Wood, H. Yang, H. Zhao, Y. Ye, J. Gu, J. Lin, T. Habuchi and X. Wu (2008). "Single nucleotide polymorphisms of microRNA machinery genes modify the risk of renal cell carcinoma." Clin Cancer Res **14**(23): 7956-7962.

Huang, J. C., T. Babak, T. W. Corson, G. Chua, S. Khan, B. L. Gallie, T. R. Hughes, B. J. Blencowe, B. J. Frey and Q. D. Morris (2007). "Using expression profiling data to identify human microRNA targets." Nat Methods **4**(12): 1045-1049.

Huang, X., J. Cortes and H. Kantarjian (2012). "Estimations of the increasing prevalence and plateau prevalence of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy." Cancer **118**(12): 3123-3127.

Humphreys, D. T., C. J. Hynes, H. R. Patel, G. H. Wei, L. Cannon, D. Fatkin, C. M. Suter, J. L. Clancy and T. Preiss (2012). "Complexity of murine cardiomyocyte miRNA biogenesis, sequence variant expression and function." PLoS One **7**(2): e30933.

Jafarzadeh-Samani, Z., S. Sohrabi, K. Shirmohammadi, H. Effatpanah, R. Yadegarazari and M. Saidijam (2017). "Evaluation of miR-22 and miR-20a as diagnostic biomarkers for gastric cancer." Chin Clin Oncol **6**(2): 16.

Jones, M. R., L. J. Quinton, M. T. Blahna, J. R. Neilson, S. Fu, A. R. Ivanov, D. A. Wolf and J. P. Mizgerd (2009). "Zcchc11-dependent uridylation of microRNA directs cytokine expression." Nat Cell Biol **11**(9): 1157-1163.

Kagita, S., S. Uppalapati, S. Jiwatani, V. G. Linga, S. Gundeti, N. Nagesh and R. Digumarti (2014). "Incidence of Bcr-Abl kinase domain mutations in imatinib refractory chronic myeloid leukemia patients from South India." Tumour Biol **35**(7): 7187-7193.

Kantarjian, H., F. Giles, L. Wunderle, K. Bhalla, S. O'Brien, B. Wassmann, C. Tanaka, P. Manley, P. Rae, W. Mietlowski, K. Bochinski, A. Hochhaus, J. D. Griffin, D. Hoelzer, M. Albitar, M. Dugan, J. Cortes, L. Alland and O. G. Ottmann (2006). "Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL." N Engl J Med **354**(24): 2542-2551.

Karali, M., M. Persico, M. Mutarelli, A. Carissimo, M. Pizzo, V. Singh Marwah, C. Ambrosio, M. Pinelli, D. Carrella, S. Ferrari, D. Ponzin, V. Nigro, D. di Bernardo and S. Banfi (2016). "High-resolution analysis of the human retina miRNome reveals isomiR variations and novel microRNAs." Nucleic Acids Res **44**(4): 1525-1540.

Karimiani, E. G., F. Marriage, A. J. Merritt, J. Burthem, R. J. Byers and P. J. Day (2014). "Single-cell analysis of K562 cells: an imatinib-resistant subpopulation is adherent and has upregulated expression of BCR-ABL mRNA and protein." Exp Hematol **42**(3): 183-191 e185.

Katoh, T., Y. Sakaguchi, K. Miyauchi, T. Suzuki, S. Kashiwabara, T. Baba and T. Suzuki (2009). "Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2." Genes Dev **23**(4): 433-438.

Kaukoniemi, K. M., H. E. Rauhala, M. Scaravilli, L. Latonen, M. Annala, R. L. Vessella, M. Nykter, T. L. Tammela and T. Visakorpi (2015). "Epigenetically altered miR-193b targets cyclin D1 in prostate cancer." Cancer Med **4**(9): 1417-1425.

Kawahara, Y., B. Zinshteyn, P. Sethupathy, H. Iizasa, A. G. Hatzigeorgiou and K. Nishikura (2007). "Redirection of silencing targets by adenosine-to-inosine editing of miRNAs." Science **315**(5815): 1137-1140.

Kertesz, M., N. Iovino, U. Unnerstall, U. Gaul and E. Segal (2007). "The role of site accessibility in microRNA target recognition." Nat Genet **39**(10): 1278-1284.

Khorshid, M., J. Hausser, M. Zavolan and E. van Nimwegen (2013). "A biophysical miRNA-mRNA interaction model infers canonical and noncanonical targets." Nat Methods **10**(3): 253-255.

Kim, Y. K., B. Kim and V. N. Kim (2016). "Re-evaluation of the roles of DROSHA, Export in 5, and DICER in microRNA biogenesis." Proc Natl Acad Sci U S A **113**(13): E1881-1889.

Klein, E., H. Ben-Bassat, H. Neumann, P. Ralph, J. Zeuthen, A. Polliack and F. Vanky (1976). "Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia." Int J Cancer **18**(4): 421-431.

Kohrs, N., S. Kolodziej, O. N. Kuvardina, J. Herglotz, J. Yillah, S. Herkt, A. Piechatzek, G. Salinas Riester, T. Lingner, C. Wichmann, H. Bonig, E. Seifried, U. Platzbecker, H. Medyouf, M. Grez and J. Lausen (2016). "MiR144/451 Expression Is Repressed by RUNX1 During Megakaryopoiesis and Disturbed by RUNX1/ETO." PLoS Genet **12**(3): e1005946.

Kong, Y. W., I. G. Cannell, C. H. de Moor, K. Hill, P. G. Garside, T. L. Hamilton, H. A. Meijer, H. C. Dobbyn, M. Stoneley, K. A. Spriggs, A. E. Willis and M. Bushell (2008). "The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene." Proc Natl Acad Sci U S A **105**(26): 8866-8871.

Koscianska, E., J. Starega-Roslan and W. J. Krzyzosiak (2011). "The role of Dicer protein partners in the processing of microRNA precursors." PLoS One **6**(12): e28548.

Krek, A., D. Grun, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel and N. Rajewsky (2005). "Combinatorial microRNA target predictions." Nat Genet **37**(5): 495-500.

Kuchen, S., W. Resch, A. Yamane, N. Kuo, Z. Li, T. Chakraborty, L. Wei, A. Laurence, T. Yasuda, S. Peng, J. Hu-Li, K. Lu, W. Dubois, Y. Kitamura, N. Charles, H. W. Sun, S. Muljo, P. L. Schwartzberg, W. E. Paul, J. O'Shea, K. Rajewsky and R. Casellas (2010). "Regulation of microRNA expression and abundance during lymphopoiesis." Immunity **32**(6): 828-839.

Kumar, L. (2006). "Chronic myelogenous leukaemia (CML): an update." Natl Med J India **19**(5): 255-263.

Kumar, M. S., J. Lu, K. L. Mercer, T. R. Golub and T. Jacks (2007). "Impaired microRNA processing enhances cellular transformation and tumorigenesis." Nat Genet **39**(5): 673-677.

Kunej, T., I. Godnic, J. Ferdin, S. Horvat, P. Dovc and G. A. Calin (2011). "Epigenetic regulation of microRNAs in cancer: an integrated review of literature." Mutat Res **717**(1-2): 77-84.

Kurzynska-Kokorniak, A., N. Koralewska, M. Pokornowska, A. Urbanowicz, A. Tworak, A. Mickiewicz and M. Figlerowicz (2015). "The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities." Nucleic Acids Res **43**(9): 4365-4380.

Kwon, S. C., T. A. Nguyen, Y. G. Choi, M. H. Jo, S. Hohng, V. N. Kim and J. S. Woo (2016). "Structure of Human DROSHA." Cell **164**(1-2): 81-90.

Laddha, S. V., S. Nayak, D. Paul, R. Reddy, C. Sharma, P. Jha, M. Hariharan, A. Agrawal, S. Chowdhury, C. Sarkar and A. Mukhopadhyay (2013). "Genome-wide analysis reveals downregulation of miR-379/miR-656 cluster in human cancers." Biol Direct **8**: 10.

Lagana, A., F. Russo, C. Sismeiro, R. Giugno, A. Pulvirenti and A. Ferro (2010). "Variability in the incidence of miRNAs and genes in fragile sites and the role of repeats and CpG islands in the distribution of genetic material." PLoS One **5**(6): e11166.

Lagos-Quintana, M., R. Rauhut, J. Meyer, A. Borkhardt and T. Tuschl (2003). "New microRNAs from mouse and human." RNA **9**(2): 175-179.

Lai, E. C., P. Tomancak, R. W. Williams and G. M. Rubin (2003). "Computational identification of Drosophila microRNA genes." Genome Biol **4**(7): R42.

Lawrie, C. H., S. Soneji, T. Marafioti, C. D. Cooper, S. Palazzo, J. C. Paterson, H. Cattan, T. Enver, R. Mager, J. Boultwood, J. S. Wainscoat and C. S. Hatton (2007). "MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma." Int J Cancer **121**(5): 1156-1161.

Lee, H. Y. and J. A. Doudna (2012). "TRBP alters human precursor microRNA processing in vitro." RNA **18**(11): 2012-2019.

Lee, R. C. and V. Ambros (2001). "An extensive class of small RNAs in Caenorhabditis elegans." Science **294**(5543): 862-864.

Lee, R. C., R. L. Feinbaum and V. Ambros (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-854.

Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim (2003). "The nuclear RNase III Drosha initiates microRNA processing." Nature **425**(6956): 415-419.

Lee, Y., I. Hur, S. Y. Park, Y. K. Kim, M. R. Suh and V. N. Kim (2006). "The role of PACT in the RNA silencing pathway." EMBO J **25**(3): 522-532.

Lenarduzzi, M., A. B. Hui, N. M. Alajez, W. Shi, J. Williams, S. Yue, B. O'Sullivan and F. F. Liu (2013). "MicroRNA-193b enhances tumor progression via down regulation of neurofibromin 1." PLoS One **8**(1): e53765.

Leoni, G. and A. Tramontano (2016). "A structural view of microRNA-target recognition." Nucleic Acids Res **44**(9): e82.

Levy, C., M. Khaled, K. C. Robinson, R. A. Veguilla, P. H. Chen, S. Yokoyama, E. Makino, J. Lu, L. Larue, F. Beermann, L. Chin, M. Bosenberg, J. S. Song and D. E. Fisher (2010). "Lineage-specific transcriptional regulation of DICER by MITF in melanocytes." Cell **141**(6): 994-1005.

Lewis, B. P., C. B. Burge and D. P. Bartel (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell **120**(1): 15-20.

Lewis, B. P., I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel and C. B. Burge (2003). "Prediction of mammalian microRNA targets." Cell **115**(7): 787-798.

Li, J., Y. Wang, Y. Song, Z. Fu and W. Yu (2014). "miR-27a regulates cisplatin resistance and metastasis by targeting RKIP in human lung adenocarcinoma cells." Mol Cancer **13**: 193.

Li, S. and D. J. Patel (2016). "Drosha and Dicer: Slicers cut from the same cloth." Cell Res **26**(5): 511-512.

Li, Y., A. Goldenberg, K. C. Wong and Z. Zhang (2014). "A probabilistic approach to explore human miRNA targetome by integrating miRNA-overexpression data and sequence information." Bioinformatics **30**(5): 621-628.

Liang, C., X. Zhang, H. M. Wang, X. M. Liu, X. J. Zhang, B. Zheng, G. R. Qian and Z. L. Ma (2017). "MicroRNA-18a-5p functions as an oncogene by directly targeting IRF2 in lung cancer." Cell Death Dis **8**(5): e2764.

Lin, S. and R. I. Gregory (2015). "MicroRNA biogenesis pathways in cancer." Nat Rev Cancer **15**(6): 321-333.

Liu, N., M. Abe, L. R. Sabin, G. J. Hendriks, A. S. Naqvi, Z. Yu, S. Cherry and N. M. Bonini (2011). "The exoribonuclease Nibbler controls 3' end processing of microRNAs in Drosophila." Curr Biol **21**(22): 1888-1893.

Llorens, F., M. Banez-Coronel, L. Pantano, J. A. del Rio, I. Ferrer, X. Estivill and E. Marti (2013). "A highly expressed miR-101 isomiR is a functional silencing small RNA." BMC Genomics **14**: 104.

Loher, P., E. R. Londin and I. Rigoutsos (2014). "IsomiR expression profiles in human lymphoblastoid cell lines exhibit population and gender dependencies." Oncotarget **5**(18): 8790-8802.

Loriot, A., A. Van Tongelen, J. Blanco, S. Klaessens, J. Cannuyer, N. van Baren, A. Decottignies and C. De Smet (2014). "A novel cancer-germline transcript carrying pro-metastatic miR-105 and TET-targeting miR-767 induced by DNA hypomethylation in tumors." Epigenetics **9**(8): 1163-1171.

Lozzio, B. B., C. B. Lozzio, E. G. Bamberger and A. S. Feliu (1981). "A multipotential leukemia cell line (K-562) of human origin." Proc Soc Exp Biol Med **166**(4): 546-550.

Lozzio, C. B. and B. B. Lozzio (1975). "Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome." Blood **45**(3): 321-334.

Lu, J., G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz and T. R. Golub (2005). "MicroRNA expression profiles classify human cancers." Nature **435**(7043): 834-838.

Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg and U. Kutay (2004). "Nuclear export of microRNA precursors." Science **303**(5654): 95-98.

Ma, H., Y. Wu, Q. Niu, J. Zhang, G. Jia, N. Manjunath and H. Wu (2016). "A sliding-bulge structure at the Dicer processing site of pre-miRNAs regulates alternative Dicer processing to generate 5'-isomiRs." Heliyon **2**(9): e00148.

Ma, J., J. Liu, Z. Wang, X. Gu, Y. Fan, W. Zhang, L. Xu, J. Zhang and D. Cai (2014). "NF-kappaB-dependent microRNA-425 upregulation promotes gastric cancer cell growth by targeting PTEN upon IL-1beta induction." Mol Cancer **13**: 40.

Macfarlane, L. A. and P. R. Murphy (2010). "MicroRNA: Biogenesis, Function and Role in Cancer." Curr Genomics **11**(7): 537-561.

Machova Polakova, K., T. Lopotova, H. Klamova, P. Burda, M. Trneny, T. Stopka and J. Moravcova (2011). "Expression patterns of microRNAs associated with CML phases and their disease related targets." Mol Cancer **10**: 41.

MacRae, I. J. and J. A. Doudna (2007). "Ribonuclease revisited: structural insights into ribonuclease III family enzymes." Curr Opin Struct Biol **17**(1): 138-145.

Macrae, I. J., K. Zhou, F. Li, A. Repic, A. N. Brooks, W. Z. Cande, P. D. Adams and J. A. Doudna (2006). "Structural basis for double-stranded RNA processing by Dicer." Science **311**(5758): 195-198.

Magagnin, M. G., T. van den Beucken, K. Sergeant, P. Lambin, M. Koritzinsky, B. Devreese and B. G. Wouters (2008). "The mTOR target 4E-BP1 contributes to differential protein expression during normoxia and hypoxia through changes in mRNA translation efficiency." Proteomics **8**(5): 1019-1028.

Manzano, M., E. Forte, A. N. Raja, M. J. Schipma and E. Gottwein (2015). "Divergent target recognition by coexpressed 5'-isomiRs of miR-142-3p and selective viral mimicry." RNA **21**(9): 1606-1620.

Marquardt, B., D. Frith and S. Stabel (1994). "Signalling from TPA to MAP kinase requires protein kinase C, raf and MEK: reconstitution of the signalling pathway in vitro." Oncogene **9**(11): 3213-3218.

Martello, G., A. Rosato, F. Ferrari, A. Manfrin, M. Cordenonsi, S. Dupont, E. Enzo, V. Guzzardo, M. Rondina, T. Spruce, A. R. Parenti, M. G. Daidone, S. Bicciato and S. Piccolo (2010). "A MicroRNA targeting dicer for metastasis control." Cell **141**(7): 1195-1207.

Martin, H. C., S. Wani, A. L. Steptoe, K. Krishnan, K. Nones, E. Nourbakhsh, A. Vlassov, S. M. Grimmond and N. Cloonan (2014). "Imperfect centered miRNA binding sites are common and can mediate repression of target mRNAs." Genome Biol **15**(3): R51.

Martin, M. G., J. E. Payton and D. C. Link (2009). "Dicer and outcomes in patients with acute myeloid leukemia (AML)." Leuk Res **33**(8): e127.

Mathonnet, G., M. R. Fabian, Y. V. Svitkin, A. Parsyan, L. Huck, T. Murata, S. Biffo, W. C. Merrick, E. Darzynkiewicz, R. S. Pillai, W. Filipowicz, T. F. Duchaine and N. Sonenberg (2007). "MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F." Science **317**(5845): 1764-1767.

Matsumura-Takeda, K., S. Sogo, Y. Isakari, Y. Harada, K. Nishioka, T. Kawakami, T. Ono and T. Taki (2007). "CD41+/CD45+ cells without acetylcholinesterase activity are immature and a major megakaryocytic population in murine bone marrow." Stem Cells **25**(4): 862-870.

Mayer, B. J. and D. Baltimore (1994). "Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase." Mol Cell Biol **14**(5): 2883-2894.

Melo, S. A. and M. Esteller (2011). "Dysregulation of microRNAs in cancer: playing with fire." FEBS Lett **585**(13): 2087-2099.

Melo, S. A. and M. Esteller (2011). "A precursor microRNA in a cancer cell nucleus: get me out of here!" Cell Cycle **10**(6): 922-925.

Melo, S. A., C. Moutinho, S. Ropero, G. A. Calin, S. Rossi, R. Spizzo, A. F. Fernandez, V. Davalos, A. Villanueva, G. Montoya, H. Yamamoto, S. Schwartz, Jr. and M. Esteller (2010). "A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells." Cancer Cell **18**(4): 303-315.

Mercey, O., A. Popa, A. Cavard, A. Paquet, B. Chevalier, N. Pons, V. Magnone, J. Zangari, P. Brest, L. E. Zaragosi, G. Ponzio, K. Lebrigand, P. Barbry and B. Marcet (2017). "Characterizing isomiR variants within the microRNA-34/449 family." FEBS Lett **591**(5): 693-705.

Mertens-Talcott, S. U., S. Chintharlapalli, X. Li and S. Safe (2007). "The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells." Cancer Res **67**(22): 11001-11011.

Mets, E., J. Van der Meulen, G. Van Peer, M. Boice, P. Mestdagh, I. Van de Walle, T. Lammens, S. Goossens, B. De Moerloose, Y. Benoit, N. Van Roy, E. Clappier, B. Poppe, J. Vandesompele, H. G. Wendel, T. Taghon, P. Rondou, J. Soulier, P. Van Vlierberghe and F. Speleman (2015). "MicroRNA-193b-3p acts as a tumor suppressor by targeting the MYB oncogene in T-cell acute lymphoblastic leukemia." Leukemia **29**(4): 798-806.

Mi, S., T. Cai, Y. Hu, Y. Chen, E. Hodges, F. Ni, L. Wu, S. Li, H. Zhou, C. Long, S. Chen, G. J. Hannon and Y. Qi (2008). "Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide." Cell **133**(1): 116-127.

Miranda, K. C., T. Huynh, Y. Tay, Y. S. Ang, W. L. Tam, A. M. Thomson, B. Lim and I. Rigoutsos (2006). "A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes." Cell **126**(6): 1203-1217.

Mitchell, P. S., R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin and M. Tewari (2008). "Circulating microRNAs as stable blood-based markers for cancer detection." Proc Natl Acad Sci U S A **105**(30): 10513-10518.

Morin, R. D., M. D. O'Connor, M. Griffith, F. Kuchenbauer, A. Delaney, A. L. Prabhu, Y. Zhao, H. McDonald, T. Zeng, M. Hirst, C. J. Eaves and M. A. Marra (2008). "Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells." Genome Res **18**(4): 610-621.

Mosakhani, N., S. Mustjoki and S. Knuutila (2013). "Down-regulation of miR-181c in imatinib-resistant chronic myeloid leukemia." Mol Cytogenet **6**(1): 27.

Much, C., T. Auchynnikava, D. Pavlinic, A. Buness, J. Rappsilber, V. Benes, R. Allshire and D. O'Carroll (2016). "Endogenous Mouse Dicer Is an Exclusively Cytoplasmic Protein." PLoS Genet **12**(6): e1006095.

Musa, J., M. F. Orth, M. Dallmayer, M. Baldauf, C. Pardo, B. Rotblat, T. Kirchner, G. Leprivier and T. G. Grunewald (2016). "Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1): a master regulator of mRNA translation involved in tumorigenesis." Oncogene **35**(36): 4675-4688.

Neilsen, C. T., G. J. Goodall and C. P. Bracken (2012). "IsomiRs--the overlooked repertoire in the dynamic microRNAome." Trends Genet **28**(11): 544-549.

Nishikura, K. (2016). "A-to-I editing of coding and non-coding RNAs by ADARs." Nat Rev Mol Cell Biol **17**(2): 83-96.

Nowell, P. C. (1962). "The minute chromosome (Phl) in chronic granulocytic leukemia." Blut **8**: 65-66.

Okada, C., E. Yamashita, S. J. Lee, S. Shibata, J. Katahira, A. Nakagawa, Y. Yoneda and T. Tsukihara (2009). "A high-resolution structure of the pre-microRNA nuclear export machinery." Science **326**(5957): 1275-1279.

Ovando-Vazquez, C., D. Lepe-Soltero and C. Abreu-Goodger (2016). "Improving microRNA target prediction with gene expression profiles." BMC Genomics **17**: 364.

Paraskevopoulou, M. D., G. Georgakilas, N. Kostoulas, I. S. Vlachos, T. Vergoulis, M. Reczko, C. Filippidis, T. Dalamagas and A. G. Hatzigeorgiou (2013). "DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows." Nucleic Acids Res **41**(Web Server issue): W169-173.

Park, J. E., I. Heo, Y. Tian, D. K. Simanshu, H. Chang, D. Jee, D. J. Patel and V. N. Kim (2011). "Dicer recognizes the 5' end of RNA for efficient and accurate processing." Nature **475**(7355): 201-205.

Parker, J. S., E. A. Parizotto, M. Wang, S. M. Roe and D. Barford (2009). "Enhancement of the seed-target recognition step in RNA silencing by a PIWI/MID domain protein." Mol Cell **33**(2): 204-214.

Pasquinelli, A. E., B. J. Reinhart, F. Slack, M. Q. Martindale, M. I. Kuroda, B. Maller, D. C. Hayward, E. E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson and G. Ruvkun (2000). "Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA." Nature **408**(6808): 86-89.

Pende, M., S. H. Um, V. Mieulet, M. Sticker, V. L. Goss, J. Mestan, M. Mueller, S. Fumagalli, S. C. Kozma and G. Thomas (2004). "S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway." Mol Cell Biol **24**(8): 3112-3124.

Peng, Z., Y. Cheng, B. C. Tan, L. Kang, Z. Tian, Y. Zhu, W. Zhang, Y. Liang, X. Hu, X. Tan, J. Guo, Z. Dong, Y. Liang, L. Bao and J. Wang (2012). "Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome." Nat Biotechnol **30**(3): 253-260.

Petremand, J., N. Bulat, A. C. Butty, C. Poussin, S. Rutti, K. Au, S. Ghosh, V. Mooser, B. Thorens, J. Y. Yang, C. Widmann and G. Waeber (2009). "Involvement of 4E-BP1 in the protection induced by HDLs on pancreatic beta-cells." Mol Endocrinol **23**(10): 1572-1586.

Petroulakis, E., A. Parsyan, R. J. Dowling, O. LeBacquer, Y. Martineau, M. Bidinosti, O. Larsson, T. Alain, L. Rong, Y. Mamane, M. Paquet, L. Furic, I. Topisirovic, D. Shahbazian, M. Livingstone, M. Costa-Mattioli, J. G. Teodoro and N. Sonenberg (2009). "p53-dependent translational control of senescence and transformation via 4E-BPs." Cancer Cell **16**(5): 439-446.

Pillai, R. S., S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand and W. Filipowicz (2005). "Inhibition of translational initiation by Let-7 MicroRNA in human cells." Science **309**(5740): 1573-1576.

Plaisier, C. L., M. Pan and N. S. Baliga (2012). "A miRNA-regulatory network explains how dysregulated miRNAs perturb oncogenic processes across diverse cancers." Genome Res **22**(11): 2302-2314.

Ple, H., P. Landry, A. Benham, C. Coarfa, P. H. Gunaratne and P. Provost (2012). "The repertoire and features of human platelet microRNAs." PLoS One **7**(12): e50746.

Profumo, V. and P. Gandellini (2013). "MicroRNAs: cobblestones on the road to cancer metastasis." Crit Rev Oncog **18**(4): 341-355.

Qin, X., B. Jiang and Y. Zhang (2016). "4E-BP1, a multifactor regulated multifunctional protein." Cell Cycle **15**(6): 781-786.

Ramachandran, S. S., P. Muiwo, H. M. Ahmad, R. M. Pandey, S. Singh, S. Bakhshi, L. Kumar, A. Bhattacharya and Y. K. Gupta (2017). "miR-505-5p and miR-193b-3p: potential biomarkers of imatinib response in patients with chronic myeloid leukemia." Leuk Lymphoma **58**(8): 1981-1984.

Rehmsmeier, M., P. Steffen, M. Hochsmann and R. Giegerich (2004). "Fast and effective prediction of microRNA/target duplexes." RNA **10**(10): 1507-1517.

Ren, R. (2005). "Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia." Nat Rev Cancer **5**(3): 172-183.

Ro, S., C. Park, D. Young, K. M. Sanders and W. Yan (2007). "Tissue-dependent paired expression of miRNAs." Nucleic Acids Res **35**(17): 5944-5953.

Robins, H., Y. Li and R. W. Padgett (2005). "Incorporating structure to predict microRNA targets." Proc Natl Acad Sci U S A **102**(11): 4006-4009.

Rodriguez, A., S. Griffiths-Jones, J. L. Ashurst and A. Bradley (2004). "Identification of mammalian microRNA host genes and transcription units." Genome Res **14**(10A): 1902-1910.

Rouhana, L., N. Shibata, O. Nishimura and K. Agata (2010). "Different requirements for conserved post-transcriptional regulators in planarian regeneration and stem cell maintenance." Dev Biol **341**(2): 429-443.

Rupaimoole, R. and F. J. Slack (2017). "MicroRNA therapeutics: towards a new era for the management of cancer and other diseases." Nat Rev Drug Discov **16**(3): 203-222.

Saetrom, P., B. S. Heale, O. Snove, Jr., L. Aagaard, J. Alluin and J. J. Rossi (2007). "Distance constraints between microRNA target sites dictate efficacy and cooperativity." Nucleic Acids Res **35**(7): 2333-2342.

Salem, O., N. Erdem, J. Jung, E. Munstermann, A. Worner, H. Wilhelm, S. Wiemann and C. Korner (2016). "The highly expressed 5'isomiR of hsa-miR-140-3p contributes to the tumor-suppressive effects of miR-140 by reducing breast cancer proliferation and migration." BMC Genomics **17**(1): 566.

Salesse, S. and C. M. Verfaillie (2002). "BCR/ABL: from molecular mechanisms of leukemia induction to treatment of chronic myelogenous leukemia." Oncogene **21**(56): 8547-8559.

Saunders, M. A., H. Liang and W. H. Li (2007). "Human polymorphism at microRNAs and microRNA target sites." Proc Natl Acad Sci U S A **104**(9): 3300-3305.

Seggerson, K., L. Tang and E. G. Moss (2002). "Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation." Dev Biol **243**(2): 215-225.

Seitz, H., J. S. Tushir and P. D. Zamore (2011). "A 5'-uridine amplifies miRNA/miRNA* asymmetry in Drosophila by promoting RNA-induced silencing complex formation." Silence **2**: 4.

Sempere, L. F., S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky and V. Ambros (2004). "Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation." Genome Biol **5**(3): R13.

Shah, A. A., P. Leidinger, N. Blin and E. Meese (2010). "miRNA: small molecules as potential novel biomarkers in cancer." Curr Med Chem **17**(36): 4427-4432.

Shelly, C., L. Petruzzelli and R. Herrera (1998). "PMA-induced phenotypic changes in K562 cells: MAPK-dependent and -independent events." Leukemia **12**(12): 1951-1961.

Shi, L., Y. Wang, Z. Lu, H. Zhang, N. Zhuang, B. Wang, Z. Song, G. Chen, C. Huang, D. Xu, Y. Zhang, W. Zhang and Y. Gao (2017). "miR-127 promotes EMT and stem-like traits in lung cancer through a feed-forward regulatory loop." Oncogene **36**(12): 1631-1643.

Si, M. L., S. Zhu, H. Wu, Z. Lu, F. Wu and Y. Y. Mo (2007). "miR-21-mediated tumor growth." Oncogene **26**(19): 2799-2803.

Siomi, H. and M. C. Siomi (2010). "Posttranscriptional regulation of microRNA biogenesis in animals." Mol Cell **38**(3): 323-332.

Soifer, H. S., J. J. Rossi and P. Saetrom (2007). "MicroRNAs in disease and potential therapeutic applications." Mol Ther **15**(12): 2070-2079.

Soverini, S., A. Hochhaus, F. E. Nicolini, F. Gruber, T. Lange, G. Saglio, F. Pane, M. C. Muller, T. Ernst, G. Rosti, K. Porkka, M. Baccarani, N. C. Cross and G. Martinelli (2011). "BCR-ABL kinase domain mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet." Blood **118**(5): 1208-1215.

Starega-Roslan, J., P. Galka-Marciniak and W. J. Krzyzosiak (2015). "Nucleotide sequence of miRNA precursor contributes to cleavage site selection by Dicer." Nucleic Acids Res **43**(22): 10939-10951.

Starega-Roslan, J., J. Krol, E. Koscianska, P. Kozlowski, W. J. Szlachcic, K. Sobczak and W. J. Krzyzosiak (2011). "Structural basis of microRNA length variety." Nucleic Acids Res **39**(1): 257-268.

Stark, A., J. Brennecke, R. B. Russell and S. M. Cohen (2003). "Identification of Drosophila MicroRNA targets." PLoS Biol **1**(3): E60.

Su, X., D. Chakravarti, M. S. Cho, L. Liu, Y. J. Gi, Y. L. Lin, M. L. Leung, A. El-Naggar, C. J. Creighton, M. B. Suraokar, I. Wistuba and E. R. Flores (2010). "TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs." Nature **467**(7318): 986-990.

Sun, H. L., R. Cui, J. Zhou, K. Y. Teng, Y. H. Hsiao, K. Nakanishi, M. Fassan, Z. Luo, G. Shi, E. Tili, H. Kutay, F. Lovat, C. Vicentini, H. L. Huang, S. W. Wang, T. Kim, N. Zanesi, Y. J. Jeon, T. J. Lee, J. H. Guh, M. C. Hung, K. Ghoshal, C. M. Teng, Y. Peng and C. M. Croce (2016). "ERK Activation Globally Downregulates miRNAs through Phosphorylating Exportin-5." Cancer Cell **30**(5): 723-736.

Suresh, S., L. McCallum, W. Lu, N. Lazar, B. Perbal and A. E. Irvine (2011). "MicroRNAs 130a/b are regulated by BCR-ABL and downregulate expression of CCN3 in CML." J Cell Commun Signal **5**(3): 183-191.

Szafranska, A. E., T. S. Davison, J. Shingara, M. Doleshal, J. A. Riggenbach, C. D. Morrison, S. Jewell and E. Labourier (2008). "Accurate molecular characterization of formalin-fixed, paraffin-embedded tissues by microRNA expression profiling." J Mol Diagn **10**(5): 415-423.

Takahashi, R. U., H. Miyazaki, F. Takeshita, Y. Yamamoto, K. Minoura, M. Ono, M. Kodaira, K. Tamura, M. Mori and T. Ochiya (2015). "Loss of microRNA-27b contributes to breast cancer stem cell generation by activating ENPP1." Nat Commun **6**: 7318.

Tan, G. C., E. Chan, A. Molnar, R. Sarkar, D. Alexieva, I. M. Isa, S. Robinson, S. Zhang, P. Ellis, C. F. Langford, P. V. Guillot, A. Chandrashekran, N. M. Fisk, L. Castellano, G. Meister, R. M. Winston, W. Cui, D. Baulcombe and N. J. Dibb (2014). "5' isomiR variation is of functional and evolutionary importance." Nucleic Acids Res **42**(14): 9424-9435.

Telonis, A. G., R. Magee, P. Loher, I. Chervoneva, E. Londin and I. Rigoutsos (2017). "Knowledge about the presence or absence of miRNA isoforms (isomiRs) can successfully discriminate amongst 32 TCGA cancer types." Nucleic Acids Res **45**(6): 2973-2985.

Tetzlaff, M. T., A. Liu, X. Xu, S. R. Master, D. A. Baldwin, J. W. Tobias, V. A. Livolsi and Z. W. Baloch (2007). "Differential expression of miRNAs in papillary thyroid carcinoma compared to multinodular goiter using formalin fixed paraffin embedded tissues." Endocr Pathol **18**(3): 163-173.

Tokumaru, S., M. Suzuki, H. Yamada, M. Nagino and T. Takahashi (2008). "let-7 regulates Dicer expression and constitutes a negative feedback loop." Carcinogenesis **29**(11): 2073-2077.

Torrezan, G. T., E. N. Ferreira, A. M. Nakahata, B. D. Barros, M. T. Castro, B. R. Correa, A. C. Krepischi, E. H. Olivieri, I. W. Cunha, U. Tabori, P. E. Grundy, C. M. Costa, B. de Camargo, P. A. Galante and D. M. Carraro (2014). "Recurrent somatic mutation in DROSHA induces microRNA profile changes in Wilms tumour." Nat Commun **5**: 4039.

Tyagi, S., C. Vaz, V. Gupta, R. Bhatia, S. Maheshwari, A. Srinivasan and A. Bhattacharya (2008). "CID-miRNA: a web server for prediction of novel miRNA precursors in human genome." Biochem Biophys Res Commun **372**(4): 831-834.

Ueda, Y., S. Hirai, S. Osada, A. Suzuki, K. Mizuno and S. Ohno (1996). "Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf." J Biol Chem **271**(38): 23512-23519.

Van Assche, E., S. Van Puyvelde, J. Vanderleyden and H. P. Steenackers (2015). "RNA-binding proteins involved in post-transcriptional regulation in bacteria." Front Microbiol **6**: 141.

van Rooij, E. and S. Kauppinen (2014). "Development of microRNA therapeutics is coming of age." EMBO Mol Med **6**(7): 851-864.

Vaz, C., H. M. Ahmad, R. Bharti, P. Pandey, L. Kumar, R. Kulshreshtha and A. Bhattacharya (2013). "Analysis of the microRNA transcriptome and expression of different isomiRs in human peripheral blood mononuclear cells." BMC Res Notes **6**: 390.

Vaz, C., H. M. Ahmad, P. Sharma, R. Gupta, L. Kumar, R. Kulshreshtha and A. Bhattacharya "Analysis of microRNA transcriptome by deep sequencing of small RNA libraries of peripheral blood." BMC Genomics **11**: 288.

Vaz, C., H. M. Ahmad, P. Sharma, R. Gupta, L. Kumar, R. Kulshreshtha and A. Bhattacharya (2010). "Analysis of microRNA transcriptome by deep sequencing of small RNA libraries of peripheral blood." BMC Genomics **11**: 288.

Vaz, C., A. S. Mer, A. Bhattacharya and R. Ramaswamy (2011). "MicroRNAs modulate the dynamics of the NF-kappaB signaling pathway." PLoS One **6**(11): e27774.

Vella, M. C., E. Y. Choi, S. Y. Lin, K. Reinert and F. J. Slack (2004). "The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR." Genes Dev **18**(2): 132-137.

Vella, M. C., K. Reinert and F. J. Slack (2004). "Architecture of a validated microRNA::target interaction." Chem Biol **11**(12): 1619-1623.

Venturini, L., K. Battmer, M. Castoldi, B. Schultheis, A. Hochhaus, M. U. Muckenthaler, A. Ganser, M. Eder and M. Scherr (2007). "Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells." Blood **109**(10): 4399-4405.

Vickers, K. C., P. Sethupathy, J. Baran-Gale and A. T. Remaley (2013). "Complexity of microRNA function and the role of isomiRs in lipid homeostasis." J Lipid Res **54**(5): 1182-1191.

Voorhoeve, P. M., C. le Sage, M. Schrier, A. J. Gillis, H. Stoop, R. Nagel, Y. P. Liu, J. van Duijse, J. Drost, A. Griekspoor, E. Zlotorynski, N. Yabuta, G. De Vita, H. Nojima, L. H. Looijenga and R. Agami (2007). "A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors." Adv Exp Med Biol **604**: 17-46.

Wahid, F., A. Shehzad, T. Khan and Y. Y. Kim (2010). "MicroRNAs: synthesis, mechanism, function, and recent clinical trials." Biochim Biophys Acta **1803**(11): 1231-1243.

Wang, F., X. Ren and X. Zhang (2015). "Role of microRNA-150 in solid tumors." Oncol Lett **10**(1): 11-16.

Wang, J., J. Chen and S. Sen (2016). "MicroRNA as Biomarkers and Diagnostics." J Cell Physiol **231**(1): 25-30.

Wang, X. (2014). "Composition of seed sequence is a major determinant of microRNA targeting patterns." Bioinformatics **30**(10): 1377-1383.

Wang, Y., R. Rathinam, A. Walch and S. K. Alahari (2009). "ST14 (suppression of tumorigenicity 14) gene is a target for miR-27b, and the inhibitory effect of ST14 on

cell growth is independent of miR-27b regulation." J Biol Chem **284**(34): 23094-23106.

White, E., M. Schlackow, K. Kamieniarz-Gdula, N. J. Proudfoot and M. Gullerova (2014). "Human nuclear Dicer restricts the deleterious accumulation of endogenous double-stranded RNA." Nat Struct Mol Biol **21**(6): 552-559.

Wiesen, J. L. and T. B. Tomasi (2009). "Dicer is regulated by cellular stresses and interferons." Mol Immunol **46**(6): 1222-1228.

Winter, J., S. Jung, S. Keller, R. I. Gregory and S. Diederichs (2009). "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nat Cell Biol **11**(3): 228-234.

Wu, L., J. Fan and J. G. Belasco (2006). "MicroRNAs direct rapid deadenylation of mRNA." Proc Natl Acad Sci U S A **103**(11): 4034-4039.

Wyman, S. K., E. C. Knouf, R. K. Parkin, B. R. Fritz, D. W. Lin, L. M. Dennis, M. A. Krouse, P. J. Webster and M. Tewari (2011). "Post-transcriptional generation of miRNA variants by multiple nucleotidyl transferases contributes to miRNA transcriptome complexity." Genome Res **21**(9): 1450-1461.

Xie, M., M. Li, A. Vilborg, N. Lee, M. D. Shu, V. Yartseva, N. Sestan and J. A. Steitz (2013). "Mammalian 5'-capped microRNA precursors that generate a single microRNA." Cell **155**(7): 1568-1580.

Yamamoto, K., S. Ito, H. Hanafusa, K. Shimizu and M. Ouchida (2015). "Uncovering Direct Targets of MiR-19a Involved in Lung Cancer Progression." PLoS One **10**(9): e0137887.

Yang, J. S. and E. C. Lai (2011). "Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants." Mol Cell **43**(6): 892-903.

Yates, L. A., C. J. Norbury and R. J. Gilbert (2013). "The Long and Short of MicroRNA." Cell **153**(3): 516-519.

Yue, D., H. Liu and Y. Huang (2009). "Survey of Computational Algorithms for MicroRNA Target Prediction." Curr Genomics **10**(7): 478-492.

Zarif, M. N., M. Soleimani, H. Abolghasemi, N. Amirizade, E. Arefian and A. Rahimian (2013). "Megakaryocytic differentiation of CD133+ hematopoietic stem cells by down-regulation of microRNA-10a." Hematology **18**(2): 93-100.

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

Zhang, H., Y. Hou, L. Xu, Z. Zeng, S. Wen, Y. E. Du, K. Sun, J. Yin, L. Lang, X. Tang and M. Liu (2016). "Cytoplasmic Drosha Is Aberrant in Precancerous Lesions of Gastric Carcinoma and Its Loss Predicts Worse Outcome for Gastric Cancer Patients." Dig Dis Sci **61**(4): 1080-1090.

Zhang, J., S. Li, L. Li, M. Li, C. Guo, J. Yao and S. Mi (2015). "Exosome and exosomal microRNA: trafficking, sorting, and function." Genomics Proteomics Bioinformatics **13**(1): 17-24.

Zhu, D. X., L. Fan, R. N. Lu, C. Fang, W. Y. Shen, Z. J. Zou, Y. H. Wang, H. Y. Zhu, K. R. Miao, P. Liu, W. Xu and J. Y. Li (2012). "Downregulated Dicer expression predicts poor prognosis in chronic lymphocytic leukemia." Cancer Sci **103**(5): 875-881.

Zhu, D. Y., X. N. Li, Y. Qi, D. L. Liu, Y. Yang, J. Zhao, C. Y. Zhang, K. Wu and S. Zhao (2016). "MiR-454 promotes the progression of human non-small cell lung cancer and directly targets PTEN." Biomed Pharmacother **81**: 79-85.

Zid, B. M., A. N. Rogers, S. D. Katewa, M. A. Vargas, M. C. Kolipinski, T. A. Lu, S. Benzer and P. Kapahi (2009). "4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila." Cell **139**(1): 149-160.

List of publications

1. Ramachandran, S. S., **P. Muiwo**, H. M. Ahmad, R. M. Pandey, S. Singh, S. Bakhshi, L. Kumar, A. Bhattacharya and Y. K. Gupta (2017). "miR-505-5p and miR-193b-3p: potential biomarkers of imatinib response in patients with chronic myeloid leukemia." Leuk Lymphoma **58**(8): 1981-1984.

2. Ahmad, H. M., **P. Muiwo**, R. Muthuswami and A. Bhattacharya (2017). "FosB regulates expression of miR-22 during PMA induced differentiation of K562 cells to megakaryocytes." Biochimie **133**: 1-6.

3. Ahmad, H. M., **P. Muiwo**, S. S. Ramachandran, P. Pandey, Y. K. Gupta, L. Kumar, R. Kulshreshtha and A. Bhattacharya (2014). "miR-22 regulates expression of oncogenic neuro-epithelial transforming gene 1, NET1." FEBS J **281**(17): 3904-3919