HSA-MIRNA-17 – A POTENTIAL ONCOMIR IN CML

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Abstract

I. **Background:** Chronic Myeloid Leukemia (CML) is the first disease to be linked to a specific gene alteration i.e. the BCR-ABL1 fusion gene. The BCR-ABL1 protein possesses a constitutively active tyrosine kinase activity, and Imatinib a tyrosine kinase inhibitor (TKI), is the first line treatment for CML. However Imatinib has been found unable to completely eliminate leukemic stem cells, which escape Imatinib induced apoptosis. Increased expression of MAPK pathway proteins in response to Imatinib therapy has been suggested as a probable cause of leukemic stem cells persistence. Oncomir-1 is a polycistronic micro RNA complex, which encodes six micro RNAs- miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1. Increased oncomir-1 expression which regulates MAPK pathway at various levels, and directly regulates pre-apoptotic proteins containing BH domain, has been suggested as a reason for disease persistence and relapse of CML. This study evaluated the pathogenetic role of miR-17 component of miR-17-92 polycistrorn in CML.

II. **Objective** – To measure the expression of microRNA 17 in peripheral blood leukocytes of newly diagnosed patients of Chronic Myeloid Leukemia and study the effect of standard care with Imatinib Mesylate on the expression level of above miR.

III. **Study design** – Hospital based Case-Control study.

IV. **Setting** – Department Of Biochemistry, Maulana Azad Medical College, New Delhi, Department Of Pathology, MAMC, New Delhi, Department of Medicine, LNJP Hospital, New Delhi.

V. **Subjects** – 35 newly diagnosed CML cases in chronic phase of disease and 35 age and sex matched healthy controls.

VI. **Methods** – The expression of microRNA 17 in peripheral blood mononuclear cells (PBMCs) of 35 CML cases in chronic phase, in comparison to 35 age and sex matched healthy control subjects was carried out. RNA was extracted from PBMCs of both CML patients and controls using Trizol reagent. Expression studies were performed by SYBR green based qRT-PCR and results were expressed as mean fold change. Expression of miR-17 was measured on two occasions i.e. in newly diagnosed untreated CML patients, and after 6 months of therapy with Imatinib.

VII. **Result** – The expression of miR-17 in PBMCs of untreated CML patients was up regulated with respect to healthy control subjects at statistically significant levels (p<0.001). A re-evaluation of miR19b levels after six months treatment with Imatinib, revealed statistically significant down regulation of expression, compared to the pre-therapy status (p <0.001).

VIII. **Conclusion** – The miR-17 functions as an oncomir in the pathogenesis of CML. The consistent down regulation of miR-17 expression in CML patients after Imatinib therapy, suggests that miR-17 could be a downstream component of BCR-ABL tyrosine kinase signaling pathway. The role of miR-17 in persistence of leukemic stem cells after therapy with TKIs, needs to be further dissected.

Key words – hsa-miR 17 ( Homo sapiens micro RNA 17 ), CML (chronic myeloid leukemia), Oncomir-1, micro RNA, RNA interference.
1. INTRODUCTION

CML (chronic myeloid leukemia) belongs to the group of myeloproliferative neoplasms, characterized by the presence of reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11). This reciprocal translocation generates the shortened chromosome 22 known as the Philadelphia (Ph) chromosome and the new fusion oncogene, called BCR-ABL. The corresponding fusion gene, BCR-ABL, is transcribed into an 8.5 kb mRNA, with two junction variants b2a2 (40% of cases) and b3a2 (55% of cases). It encodes a chimeric protein, p210, with constitutive tyrosine kinase activity. The b2a3 and b3a3 transcripts represent less than 5% of cases.

Annual incidence of CML is approximately 1-2 per 100,000, and CML accounts for 7-20% of adult leukemias. The impressive success of the imatinib as a first-line therapy in CML has been tarnished by problems such as disease persistence or relapse arising from different mechanisms, including duplications, mutations in the kinase domain of the BCR-ABL protein and mechanisms independent from BCR-ABL activity.

Stem cells may escape Imatinib-mediated apoptosis due to the inability of Imatinib to attack quiescent stem cells. In lieu of above mentioned clinical problems in the CML treatment, we need to explore those BCR-ABL signalling pathways that play a role in the progression of disease and decide Imatinib sensitivity, clinical course and relapse.

In the last decade a breakthrough was achieved in the field of epigenetic regulation of gene expression by non-coding RNAs. One type of non-coding RNA that is being researched most is micro RNA. Micro RNAs were found to regulate vast majority of cell signalling pathways in a cell type and context dependent manner in well integrated and complex way. miRs are functional non coding double stranded ribonucleic acids containing 18-24 nucleotides. They regulate roughly 60% of total gene expression through modifying mRNA biological characteristics either by destabilizing target mRNA or simply making its translation less efficient. The polycistronic microRNA cluster miR-17-92 encodes miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1. miR-17-92 has been strongly implicated in both solid and haematopoietic malignancies. Conditional knockout of the cluster revealed modulation of apoptosis as the predominant mechanism of action of miR-17-92. Over expression of miR-17-92 variant (miR-17-19b-1) induces proliferation, confers partial resistance against inhibition of c-MYC expression, and sensitizes to imatinib-induced cell death, suggesting a potential contribution of a BCR-ABL–c-MYC–miR17-92 pathway to the pathophysiology of CML.

This study analyzed the expression levels of miR17 in CML patients before and after the imatinib therapy.

2. METHODOLOGY

The study was carried out in the Department of Biochemistry in collaboration with the Department of Medicine and Department of Pathology, Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi. The Study was approved by Institutional ethics committee of MAMC, New Delhi.

35 new cases of CML with 35 healthy, age and sex matched controls were included in the study. A peripheral blood sample, 5mL from each case and control subjects was collected for molecular studies, in an EDTA vial by venipuncture. After 15-20 minutes buffy coat was carefully pipetted out in two 1.5mL microcentrifuge tubes (Eppendorf, USA) and the remaining blood sample was stored at -80 °C. Out of the two Eppendorf tubes one tube containing 200μL of buffy coat was immediately used for RNA extraction and other tube was stored at -80 °C for subsequent use.

2.1 RNA extraction and cDNA synthesis for BCR-ABL identification

RNA was extracted by modified acid guanidium thiocyanate-phenol-chloroform (TRIZOL) RNA extraction method from PBLs of CML cases. RNA from control samples was extracted by using Total RNA Mini Kit (Blood/Cultured Cell), Gene Aid Biotech Ltd., Taiwan. Detergents and chaotropic salt were used to lyse cells and inactivate RNase. The concentration and purity was checked by running 2 μL of each sample on 2% agarose gel. The ratio of the absorbance at 260 nm and 280 nm (A260/A280) was used to assess the purity of nucleic acids. For pure RNA A260/A280 ~ 1.7 to 2.0 was considered acceptable.

Extracted RNA was reverse transcribed to c-DNA by RT-PCR using the Verso cDNA synthesis kit (Thermo Scientific, EU). 200ng of RNA was used for each sample for cDNA synthesis as per the manufacturer’s instructions.
Human beta-actin housekeeping gene is used as an endogenous control and to check the quality of c-DNA synthesized. Diagnosis of CML was confirmed by Multiplex RT-PCR which allows simultaneous detection of all the BCR-ABL1 fusion gene transcripts in addition to normal BCR gene as an internal control. The sequence of oligonucleotide primers used for this multiplex PCR were adapted from Marjan Yaghmaie et al. The PCR products were resolved by 2% agarose gel electrophoresis and observed under UV illumination to confirm target amplicon size.

2.2 Micro RNA estimation -

Total RNA containing small microRNAs extracted from PBLs by Trizol reagent was used for micro RNA expression. (Paul D.Siebert and Alex Chenchik 1993) Polyadenylation of micro RNAs was performed using poly (A) polymerase and ATP. cDNAs from poly (A) tailed micro RNAs were synthesized, using oligo dT as a reverse transcription primer by AffinityScript QPCR cDNA Synthesis Kit provided by Agilent technologies (USA) as per manufacturer’s directions. SYBR green based qRT-PCR from the prepared cDNA was done for quantification of miRNA expression using miR-17 specific forward primer and a universal reverse primer.

Delta Delta Ct method was performed to quantify the gene expression for various miRNAs in CML patients and healthy controls. After initiation of Imatinib therapy, follow-up and response monitoring of patients were done for 6 months to 12 months.

2.4 Statistical analysis

Statistical analysis of data was performed using the SPSS 18 and Graph Pad version 6.0. Mann–Whitney u test AND Fisher’s exact test were used to know if any significant difference in demographic variables is present between selected cases and control subjects. Wilcoxon signed-rank test was used to analyse the association between pre and post treatment micro RNA expression within cases and Mann–Whitney U was used to know difference between micro RNA expression in pre-imatinib therapy cases and controls respectively. A p value <0.05 was considered statistically significant.

3. Results

3.1 Demographics

The age of the cases ranged from 18 to 70 years and that of controls ranged from 20 to 75 years. the mean age for cases was 38.20 ± 11.6 years while that of controls was 39.27 ± 16.00 years. There was no significant difference found in age distribution of cases and controls. The cases included 23 males and 12 females. On the other hand there were 22 males and 13 females in the control group. This difference in sex distribution between cases and controls was not significantly different. After initiation of Imatinib therapy, follow-up and response monitoring of patients was done for 6 months to 12 months i.e. the duration of the study, depending on the time point at which the patient was recruited in the study. Mean Duration of follow up was 8.05 ± 2.56 months.

3.2 micro RNA 17 quantification by Quantitative RT-PCR

- Expression of miR17 In peripheral blood mononuclear cells of newly diagnosed patients of Chronic Myeloid Leukaemia and healthy controls

Expression of miR-17 was analysed in CML cases and healthy controls and compared by evaluating 2^{-ΔΔCt} values respectively. A statistically significant (p<0.001) association was observed between the expression of micro RNA miR-17 in CML patients in respect to controls. miR17 was found to be elevated in CML patients in relation to healthy controls. Comparison of 2^{-ΔΔCt} value of miR-17 between CML patients and controls is shown in Table 1

<table>
<thead>
<tr>
<th>MiRNA-17</th>
<th>Mean</th>
<th>N</th>
<th>Std. Deviation</th>
<th>Std. Error Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mi-17 2^{-ΔΔCt} in Pre-Imatinib therapy sample of CML patients</td>
<td>2.6182</td>
<td>35</td>
<td>3.27466</td>
<td>.55352</td>
<td></td>
</tr>
</tbody>
</table>
Effect of standard care with Imatinib Mesylate on the fold change (FC) expression levels of miR-17

Expression levels of miR-17 was measured at two occasions, first at the beginning of study and second after six months of Imatinib therapy. These two levels were compared each other and a statistically significant down regulation was observed in post-imatinib sample. (p-value <0.001) (Table 2)

Table 2 Fold changes of miR-17 in pre-Imatinib and post-Imatinib patient samples.

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<td>miR17 FC1 (pre-Imatinib)</td>
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<td>miR17 FC2 (post Imatinib)</td>
<td>1.69</td>
<td>1.21</td>
<td>&lt;0.001</td>
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</table>

p-value calculated by Wilcoxon signed-rank test.

Figure 1. Box plot for $2^{\Delta\text{ct}}$ values of micro RNA 17 in pre-Imatinib sample of CML patients and healthy controls.

- **Effect of standard care with Imatinib Mesylate on the fold change (FC) expression levels of miR-17**

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Figure 2. Box plot for fold change of micro RNA 17 in pre-Imatinib sample and post-Imatinib sample. ΔCt values were calculated w.r.t. u6 sn RNA.
miR-17 belongs to a polycistronic miRNA complex miR-17-92. This complex was the first such polycistronic miRNA directly linked to tumorigenesis. Our results revealed significantly increased expression of micro RNA-17 in CML patients before the commencement of Imatinib therapy, in comparison to healthy controls that suggests its role in the pathogenesis of the disease, either by increasing the proliferation of progenitor cells or inhibiting the apoptosis through regulating expression of pro-apoptotic or anti-apoptotic proteins. These findings are consistent with previous studies identified amplification of 13q31-q32 in haematopoietic malignancies by conventional comparative genomic hybridization analysis. This 13q31-q32 contains the locus which is transcribed into miR-17-92 polycistron. Venturini L et al observed increased expression of microRNA-17 in proliferating CD-34+ cells in CML cell lines. The miR-17-92 cluster was shown to be essential for B cell development. Its absence led to increased levels of the pro-apoptotic protein Bim and inhibited B cell development at pro-B to pre-B transition. miR-17-92-deficient foetal livers showed a greatly reduced percentage and absolute number of pre-B cells and this reduction in pre-B cells in the mutant embryos (lacking miR-17-92 complex) was found associated with increased apoptosis.

Several known pro-apoptotic genes, including PTEN, E2F1 and BCL2L11 (Bim) are predicted targets of miR-17-92. miR-17-92 expression is regulated by c-MYC in K562 cells in accordance with earlier reports demonstrating binding of c-MYC to mir-17-92 regulatory sequences and its subsequent transcriptional activation. Bim is a particularly attractive candidate target of miR-17-92 given its role in controlling lymphocyte apoptosis and in suppressing Myc-induced B cell lymphomagenesis. It was reported that RAS/MAPK signalling may contribute to the survival of BCR-ABL positive cells under Imatinib selection pressure. Inhibition of other pathways downstream of BCR/ABL may contribute to Imatinib-mediated suppression of progenitor growth, including pathways downstream of Ras other than p42/44 MAPK. Other potential candidates include the PI-3K/AKT and Janus kinase–STAT (Jak-STAT) signalling pathways, as well as transcription factors such as c-Jun, c-Myc, and nuclear factor–kB (NF-kB), which have been shown to be important for transformation of CML cell lines. One study noted that the inhibition of ERK/MAPK, not the activation of JNK/SAPK is primarily required to induce apoptosis in chronic myelogenous leukemic K562 cells.

CRKL is a putative target of miR-17. CRKL is a major tyrosine-phosphorylated adapter protein in neutrophils from patients with chronic myelogenous leukemia. CRKL has been shown to activate the RAS and JUN kinase signalling pathways and transform fibroblasts in a RAS-dependent fashion. It is a substrate of the BCR-ABL tyrosine kinase and plays a role in fibroblast transformation by BCR-ABL. In addition, CRKL has oncogenic potential. A recent study indicates that miR-17 over expression in mice decreases cell proliferation, adhesion, and migration, raising a possibility that components of mir-17-92 can both positively and negatively regulate the same cellular process to achieve homeostasis. Together, these studies indicate that non-coding RNAs, specifically microRNAs, can modulate tumour formation, and implicate the mir-17–92 cluster as a potential human oncogene.
5. Conclusion

The study is important in showing the involvement of miR-17 in the initiation of CML. Treatment of CML patients with Imatinib reduced the expression level of miR-17 in PMN cells which might be a consequence of down regulated BCR-ABL tyrosine kinase activity that decreases the availability of C-MYC to act as a transcription factor for miRNA synthesis. Since the upregulation of miR-17 was detected in CML cases, miR-17 may be useful as a diagnostic and/or therapeutic target for the detection and/or treatment of CML after validation of results with studies involving larger sample size.

References