

miR-486-5p: A Prognostic Biomarker for Chronic Myeloid Leukemia

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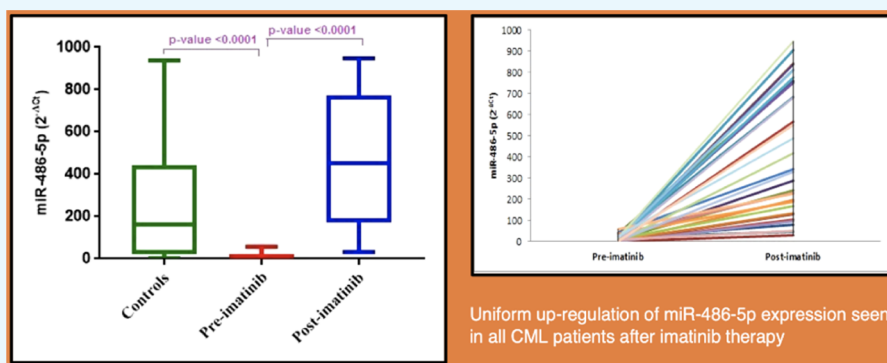
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ABSTRACT: MicroRNA miR-486-5p has been reported as a potential biomarker for diagnosis, prognosis, and as a therapeutic target in various cancers. In this study, we analyzed alterations in the expression of miR-486-5p in chronic Myeloid Leukemia (CML) patients. Initially, the expression of miR-486-5p was studied in the BCR-ABL1+ve CML K562 cell line by quantitative real-time polymerase chain reaction (qRT-PCR). The results indicated that the miR-486-5p expression was significantly upregulated in K562 cells after imatinib exposure, as compared to untreated K562 cells (p -value = 0.047). These observations were corroborated by a hospital-based study of the miR-486-5p expression in peripheral blood leucocytes of 36 CML patients in the chronic phase (CP) and compared with age and sex-matched healthy volunteers as control subjects. qRT-PCR-based quantification revealed significant downregulation of the miR-486-5p expression in newly diagnosed untreated CP-CML patients' samples ($2^{-\Delta C_t} = 13.19 \pm 14.41$) as compared to control samples ($2^{-\Delta C_t} = 254.5 \pm 274.8$) (p -value < 0.0001). Levels of miR-486-5p were found to be distinctly elevated in the post-imatinib treatment samples of CML patients ($2^{-\Delta C_t} = 469.7 \pm 312.9$) as compared to pre-treatment samples (p -value < 0.0001). CML patients' clinical and hematological responses to imatinib therapy (oral dose of 400 mg OD) were monitored for 12 months. The correlation of pre-treatment miR-486-5p levels with Sokal score indicated that patients with a higher expression of miR-486-5p had better prognoses. Patients with higher pre-imatinib miR-486-5p levels also showed a major hematologic response to imatinib in a shorter time and *vice versa*. To the best of our knowledge, this is the first report of alterations in the miR-486-5p expression in peripheral blood leucocytes of CML patients. Our observations support a tumor suppressor role of miR-486-5p in CML. The downregulation of the miR-486-5p expression may be critically important in the disease progression of CML patients. The upregulation of the miR-486-5p expression in post-imatinib exposure K562 cells and CML patients after 12 months of imatinib treatment suggests an onco-suppressor effector role of miR-486-5p in the BCR-ABL downstream signaling pathway. miR-486-5p can be explored as a novel biomarker for the early detection of CML.

1. INTRODUCTION

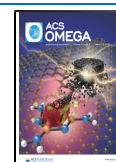
Cancer growth is determined not only by increased cell division but also by decreased cancer-cell attrition.¹ Many miRNAs are dysregulated in human cancers through various mechanisms including the amplification or deletion of miRNA genes, abnormal transcriptional control of miRNAs, epigenetic changes, and defects in the miRNA biogenesis machinery. miRNAs may function as either oncomirs or onco-suppressors. Many microRNAs have been reported as potential biomarkers for human cancer diagnosis, prognosis, and as therapeutic targets or tools, though it needs further investigation and

validation.² Alterations in the expression of miR-486-5p have been reported in different cancers. The reduced miR-486-5p expression has been reported in lung cancer,^{3–5} gastric cancer,⁶ hepatocellular carcinoma,⁷ colorectal carcinoma,⁸ esophageal

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squamous cell carcinoma,⁹ prostate cancer,¹⁰ and breast cancer.^{11,12} Ren *et al.*¹³ observed the aberrant expression of miR-486-5p in digestive system cancers.

MicroRNAs are key regulators of hematopoietic stem cell differentiation and their importance is further substantiated by observations of the deregulated expression of several microRNAs in hematological malignancies.^{14,15} As reported by Fu *et al.*,¹⁶ the miR-486-5p was first identified in the human fetal liver, an important site of hematopoiesis in prenatal life, but its role in hematopoietic regulation has not been explored. MicroRNAs have emerged as promising molecular biomarkers for early diagnosis and enhanced understanding of the molecular pathogenesis of cancers as well as certain diseases.¹⁷

Chronic myeloid leukemia (CML) results from the malignant transformation of primitive hematopoietic stem cells by the BCR-ABL fusion oncogene. The role of microRNAs in the regulation of leukemic stem cells in CML is poorly understood. Wang *et al.*¹⁸ showed miR-486-5p to be overexpressed in CML CD34⁺ progenitor cells and this enhanced their growth, survival, and response to tyrosine kinase inhibitors.

To the best of our knowledge, the miR-486-5p expression in CML patients' peripheral blood samples has not been investigated so far. This study was undertaken to analyze alterations in the expression of miR-486-5p in the CMLK562 cell line and CML patients' peripheral blood leukocytes before and after treatment with imatinib. Further, the correlation of the pre-treatment miR-486-5p expression level with the prognostic Sokal score of each CML patient was investigated and risk stratification was done. The association of miR-486-5p expression initial levels, that is, before imatinib therapy with the time taken to achieve a complete hematological response (THR) was also studied.

2. RESULTS

2.1. Experimental Study in the CML K562 Cell Line.

2.1.1. Expression of miR-486-5p in the CML K562 Cell Line and Control Normal Blood Monocytes. Comparison of corresponding $2^{-\Delta C_t}$ values for the expression of miR-486-5p in K562 cells treated with 4 μ M imatinib, untreated K562 cells, and control normal blood cells are shown in Table 1.

Table 1. $2^{-\Delta C_t}$ Values of miR-486-5p in CML K562 Cells (Untreated and Imatinib Treated) and Control Normal Blood Monocytes

miR-486-5p ($2^{-\Delta C_t}$)	mean \pm SD	median (interquartile range)	p-value
control blood cells	0.26 \pm 0.23	0.18 (0.078–0.334)	0.007
CML K562 cell line (untreated)	0.06 \pm 0.062	0.04 (0.016–0.051)	0.047
CML K562 cell line (imatinib-treated)	0.15 \pm 0.136	0.10 (0.048–0.257)	

A statistically significant downregulation of the miR-486-5p expression was observed in untreated K562 cells, in comparison to control blood cells from healthy volunteers (p -value = 0.007). On exposure of K562 cells to 4 μ M imatinib (10 times lower dose than standardized IC50 for imatinib) for 48 h, the upregulation of miR-486-5p was observed in comparison to untreated K562 cells (p -value = 0.047) (Figure 1).

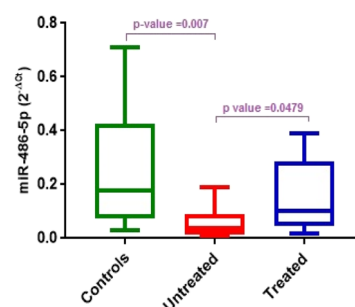


Figure 1. miR-486-5p expression in the CML K562 cell line (imatinib treated and untreated) and control normal blood monocytes.

2.2. Hospital-Based Study in CML Patients. 2.2.1. Patients' Recruitment. A total of 36 newly diagnosed cases of CP-CML were recruited (Figure 2).

2.2.2. Clinical and Hematological Parameters. The clinical and hematological parameters (Hb, TLC, DLC, and platelet count) of the CML patients were examined at the baseline and later during the follow up after the initiation of imatinib treatment, at 1.5 months, 3 months, 6 months, and 12 months (Table 2).

Time taken for achieving a complete hematologic response (THR) (as defined by The National CML Society—www.nationalcmlsociety.org/living-cml/response) was recorded for each patient (Table 3).

2.2.3. Expression of miR-486-5p in Peripheral Blood Leucocytes of CML Patients and Control Subjects. The expression of miR-486-5p was analyzed in peripheral blood leucocytes of CML patients (pre- and post-imatinib therapy) and healthy control subjects, using quantitative real-time polymerase chain reaction (qRT-PCR). Statistical analysis of miR-486-5p expression levels indicated a significant downregulation in pre-imatinib CML samples in comparison to the healthy control subjects (p -value < 0.0001) and an upregulation in post-imatinib CML samples in comparison to the pre-imatinib samples (p -value < 0.0001). A comparison of corresponding $2^{-\Delta C_t}$ values of the miR-486-5p expression between CML patients (pre- and post-imatinib treatment) and control subjects are shown in Table 4.

After imatinib therapy for 12 months, expression levels of miR-486-5p were distinctly elevated in the post-treatment CML patients as compared to pre-treatment samples and were even higher than the expression levels in the control subjects (Figure 3).

The upregulation of expression of miR-486-5p after imatinib treatment was uniformly observed in all the CML patients (35.61 mean-fold increase) (Figure 4).

2.2.4. Association of the miR-486-5p Expression with a Sokal Score in CML Patients. The risk stratification of CML patients was done.¹⁹ CML patients were categorized into two groups based on the median value of the miR-486-5p relative expression levels ($2^{-\Delta C_t}$ values) in pre-imatinib therapy blood samples (i.e., high expression and low expression groups). An analysis of the association of the miR-486-5p expression levels with pre-treatment prognostic Sokal scores showed a trend indicating better prognosis in CML patients with the higher miR-486-5p expression (Table 5). However, the correlation study of pre-treatment miR-486-5p expression levels in CML patients vis-à-vis Sokal score was not found to be statistically significant (p -value was 0.16).

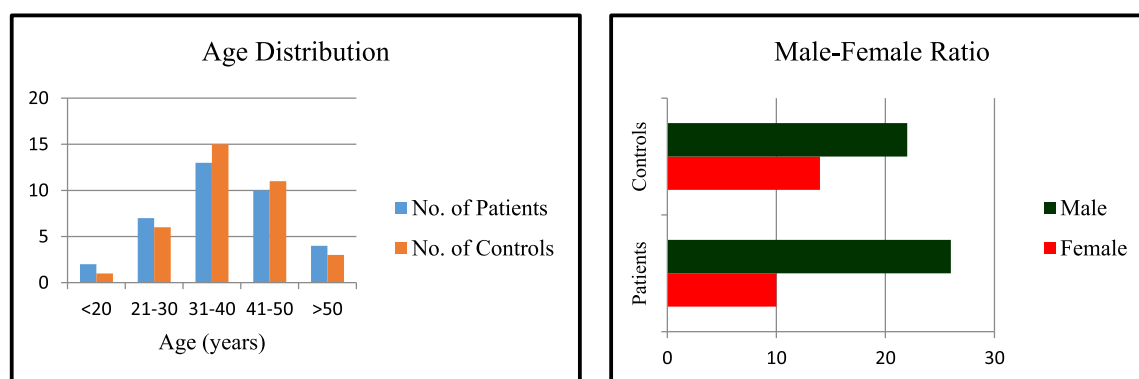


Figure 2. Demographic details of CML patients and control subjects.

Table 2. Clinical and Hematological Parameters of the CML Patients Examined at the Baseline and Later during the Follow Up after the Initiation of Imatinib Treatment, at 1.5 Months, 3 Months, 6 Months, and 12 Months

parameters ⇒ (N = 36) (*in peripheral blood sample)		Hb gm %	TLC (10 ³ /μL)*	platelet count (10 ³ /μL)*	eosinophils (%)*	basophils (%)*	blasts (%)*	spleen size (cms below costal margin)
at baseline before therapy	mean ± SD	9.8 ± 1.88	210 ± 117	301 ± 91	3.4 ± 2	4.8 ± 3.9	2.9 ± 2.2	6.6 ± 3.1
	median (inter-quartile range)	9.35 (8.6–11.2)	207 (103–261)	290 (239–403)	3 (2–4)	4 (2–6)	2 (2–3)	6 (5–7)
after 1.5 months of imatinib therapy	mean ± SD	10.5 ± 1.74	30 ± 74	280 ± 90	3.7 ± 2.4	4 ± 3.6	2.5 ± 5	splenomegaly not found in 14 patients
	median (inter-quartile range)	10.7 (9.17–11.5)	16 (9–27)	272 (228–350)	3 (2–5)	3 (2–6)	2 (0–3)	
after 3 months of imatinib therapy	mean ± SD	12.5 ± 1.69	21 ± 61	238 ± 114	2.6 ± 1.6	1.9 ± 3.3	0.9 ± 4.3	splenomegaly not found in 29 patients
	median (inter-quartile range)	12.7 (11.38–13.4)	11 (8–12)	205 (148–310)	2 (2–3)	1.5 (0–2)	0 (0–0)	
after 6 months of imatinib therapy	mean ± SD	12.7 ± 1.61	15 ± 39	214 ± 80	2.8 ± 1.5	0.8 ± 1.3	0.7 ± 3	splenomegaly not found in 32 patients
	median (inter-quartile range)	12.85 (11.6–13.6)	8 (6–10)	202 (153–267)	2.5 (2–3)	0.5 (0–1)	0 (0–0)	
after 12 months of imatinib therapy	mean ± SD	12.77 ± 1.55	13 ± 37	220 ± 117	2.3 ± 2	1 ± 2.8	0.7 ± 4	splenomegaly not found in 35 patients
	median (inter-quartile range)	13 (11.81–13.78)	6 (5–8)	201 (139–250)	2 (1–3)	0 (0–1)	0 (0–0)	

Table 3. Time Taken for Achieving Complete Hematologic Response (THR) for Each CML Patient

hematologic response (as per the National CML Society Guidelines) TLC (10 ⁹ /L) in the peripheral blood sample		duration of imatinib therapy (months)			
		≤1.5	≤3	3–6	6–12
		no. of patients (N = 36)			
major ≤ 12,000		12 (33.33%)	29 (80.56%)	32 (88.89%)	35 (97.22%)
partial (reduced but > 12,000)		24 (66.67%)	7 (19.44%)	4 (11.11%)	1 (2.78%)

Table 4. 2^{−ΔCt} Values of the miR-486-5p Expression in CML Patients (Pre- and Post-Imatinib Treatment) and Control Subjects

miR-486-5p (2 ^{−ΔCt})	mean ± SD	median (interquartile range)	p-value
		N = 36	
control subjects	254.5 ± 274.8	160.5 (23.58–375.48)	<0.0001
CML patients (pre-imatinib therapy)	13.19 ± 14.41	8.28 (1.54–18.03)	<0.0001
CML patients (post-imatinib therapy)	469.7 ± 312.9	453.3 (182.89–764.07)	

2.2.5. Association of the miR-486-5p Expression with THR in CML Patients. To study the association of the miR-486-5p expression in CML patients with the time taken to achieve a complete HTR, patients were divided into four groups (THR

≤1.5 months, THR 1.5–3 months, THR 3–6 months, and THR 6–12 months). The pre-imatinib treatment expression levels of miR-486-5p were compared with THR in each patient in the respective groups (Figure 5).

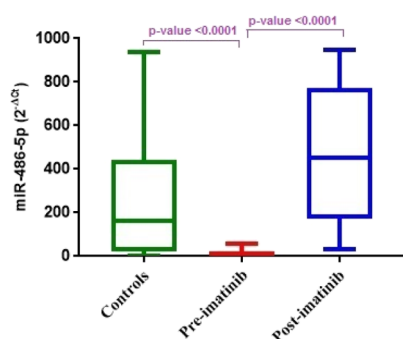


Figure 3. miR-486-5p expression in CML patients (pre- and post-imatinib therapy) and in control subjects.

It was observed that patients with a relatively higher expression of miR-486-5p showed complete HTR after imatinib treatment in a shorter duration and *vice versa*.

3. DISCUSSION

MicroRNAs, a class of small non-coding RNAs comprising 20–24 nucleotides in length, function as important regulators of gene expression, by post-transcriptional gene silencing. They control key biological processes, *viz.* cell proliferation, differentiation, and apoptosis, through the modulation of the expression of a wide spectrum of oncogenes and tumor suppressor genes. Expression profiling by microarrays has shown that the dysregulated expression of microRNAs in different cancers is the rule rather than an anomaly.

The miR-486-5p has been assigned conflicting roles, both as a tumor suppressor and as a tumor promoter in different solid cancers. miR-486-5p has been reported in many studies to be downregulated in a non-small cell lung cancer, with an inverse relationship between its expression and tumor progression, invasion, and metastases.^{3–5} Wang *et al.*³ identified ARHGAP5, a pro-tumorigenic gene to be a target gene of miR-486-5p in the non-small cell lung cancer. In gastric carcinoma, miR-486-5p was observed to function as a tumor suppressor by Oh *et al.*,⁶ who attributed its anti-oncogenic activity to targeting and inhibiting the anti-apoptotic OLFM4 gene. Liu *et al.*,⁸ in their study on colorectal carcinoma tissues, detected a comparative decrease in the miR-486-5p expression and validated the neuropilin-2 gene as its direct functional target. The expression of miR-486-5p was seen to be lowered in esophageal squamous cell carcinoma by Yi *et al.*⁹ They

Table 5. Association of the miR-486-5p Expression with the Sokal Score in CML Patients

miR-486-5p expression	Sokal score			p-value
	low risk	intermediate risk	high risk	
low expression N = 18	4 (11.11%)	13 (36.11%)	1 (2.78%)	0.16
high expression N = 18	9 (25%)	9 (%)	0	

demonstrated the anti-oncogenic action of miR-486-5p to be due to its negative effect on cellular migration. The expression of miR-486-5p was reported by Zhang *et al.*¹⁰ to be significantly decreased in the metastatic carcinoma prostate tissues in comparison to localized prostate carcinoma. This group also verified Snail, a key regulator of the epithelial–mesenchymal transition, as a target gene of miR-486-5p.¹⁰ In lymph node-positive breast-invasive ductal carcinoma tissues, Rask *et al.*¹¹ observed the miR-486-5p expression to be significantly lower as compared to lymph node-negative breast carcinomatous tissues. Zhang *et al.*,¹² in their study on breast cancer, noted that overexpression of miR-486-5p dramatically suppressed cell proliferation *in vitro* and *in vivo* and promoted apoptosis by targeting the PIM-1 oncogene.

In contrast to a plethora of studies in support of a tumor-suppressor role of miR-486-5p, only a few reports have shown an upregulation of miR-486-5p and its action as an oncogene in solid cancers. Goto *et al.*²⁰ observed that in stage III and IV renal cell carcinoma patients, the high miR-486 expression in tumors was associated with worse cancer-specific mortality. The miR-486-5p was found to be overexpressed by Mees *et al.*²¹ in invasive and metastatic pancreatic ductal carcinomas.

There is only one earlier study reported on the role of miR-486-5p in hematological malignancies. Wang *et al.*¹⁸ observed higher levels of the miR-486-5p expression in bone-marrow-derived CD34⁺ stem/progenitor cells from CML patients, particularly in the megakaryocyte-erythroid progenitor population, compared to normal peripheral blood CD34⁺ stem cells. They further showed that the overexpression of miR-486-5p enhanced the proliferation and survival of CML progenitor cells, which was related to both BCR-ABL kinase-dependent and kinase independent mechanisms. This group demonstrated that targeting of the FOXO1 and PTEN genes by miR-486-5p, through the regulation of PI3K/AKT signaling, led to the

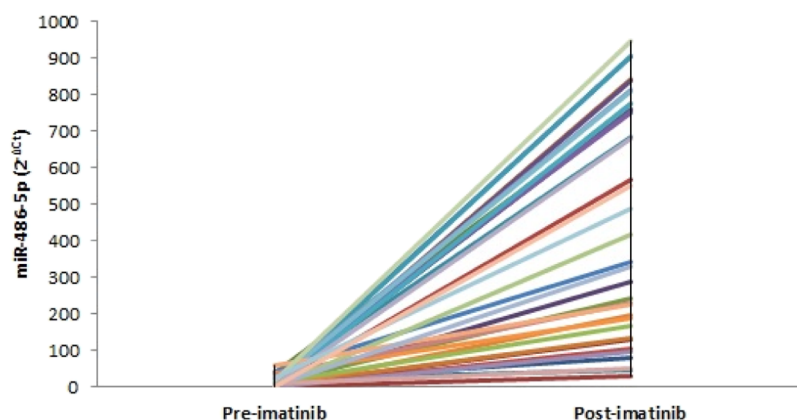


Figure 4. Uniform upregulation of the miR-486-5p expression seen in all CML patients after imatinib therapy.

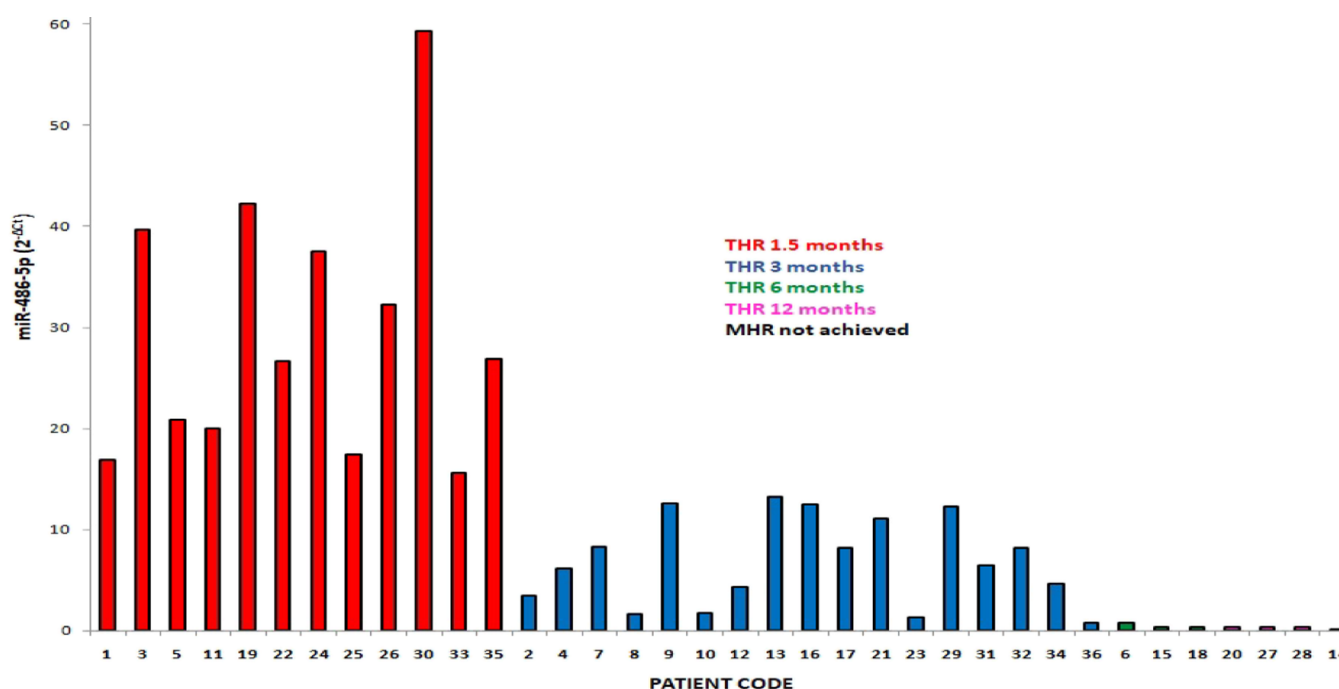


Figure 5. Association of the pre-treatment miR-486-5p expression ($2^{-\Delta C_t}$) in CML patients with THR.

growth-promoting effects of this miRNA in CML progenitor cells.

In the present study, a downregulation of miR-486-5p was noted in peripheral blood leucocytes of CP-CML patients in comparison to healthy volunteers. These findings were in sync with similar observations in the BCR-ABL1+ve K562 cell line, which support an onco-suppressor role of miR-486-5p in CML. To the best of our knowledge, this is the first report of the miR-486-5p expression pattern in CML patients' peripheral blood leukocytes.

Our observations are contrary to those of Wang *et al.*,¹⁸ who showed the induction of the miR-486-5p expression in bone marrow CD34⁺ stem/progenitor cells in CML. Alterations in the miRNAs expression have been observed during hematopoietic stem cell differentiation along specific lineages.²² While in our study the miR-486-5p expression in CML patients' peripheral blood leukocytes, which include immature cells at various stages of differentiation, has been analyzed, Wang *et al.*¹⁸ studied bone marrow CD34⁺ stem/progenitor cells in CML. The use of different cell populations could account for the variation in results.

Goto *et al.*²⁰ reported that the expression of miR-486-5p may be a potential prognostic factor in cancer. In the present study, correlation of pre-treatment miR-486-5p levels vis-à-vis Sokal score and THR indicated that patients with a higher expression of miR-486-5p had better prognosis and showed HTR to imatinib in a shorter duration and vice versa.

The distinctly elevated expression of miR-486-5p in CML patients following 12 months imatinib therapy, which was even higher than its expression in controls, was a novel and unique observation of our study. We hypothesize that this might be a consequence of the TKI activity of the imatinib targeting BCR-ABL1 fusion gene, which through its downstream effector pathways leads to the repression of miR-486-5p. Our results are similar to those reported by Yang *et al.*²³ that in lung cancer cell lines (H1299 and H1792 cells) treated with propofol

increased levels of miR-486 were observed that led to increased cell apoptosis while the miR-486 antagomir reversed this effect.

3.1. Proposed Pathway of miR-486-5p Action in CML.

The PI3K pathway is one of the important signaling cascades regulated by BCR-ABL activity in CML.²⁴ PI3K has two component subunits - p85 α and P110. p85 α , a protein encoded by *PIK3R1*, binds to activated (phosphorylated) protein-Tyr kinases (like BCR/ABL), through its SH2 domain, and acts as an adapter, mediating the association of the p110 catalytic unit with the plasma membrane. Activated p110 phosphorylates PIP2 to PIP3, which in turn binds to the γ domain of the serine/threonine kinase AKT that is frequently elevated in cancers.²⁵ The *PIK3R1* encoded protein p85 α has an important regulatory role in the activation of the PI3K-AKT pathway in different cancers including CML.

Huang *et al.*⁷ reported that in hepatocellular carcinoma, miR-486-5p inhibited the p85 α expression at both the mRNA and protein levels by binding to the 3'-UTR region of *PIK3R1*. The overexpression of miR-486-5p markedly suppressed the hepatocellular carcinoma cell proliferation, migration, and invasion *in vitro* and inhibited cell growth *in vivo*. Mechanistically, miR-486-5p was confirmed to target the *PIK3R1* expression directly, thereby suppressing the PI3K-AKT pathway activation.

The distinctly elevated expression of miR-486-5p in the post-imatinib CML samples in our study supports the hypothesis that BCR-ABL1 oncogene causes downregulation of miR-486-5p, which in turn targets mRNAs such as *PIK3R1* through the upregulation of miR-486-5p, leading to onco-genesis. Based on these findings, we propose the following pathway of malignant transformation in CML: BCR-ABL1 ↓ miR-486-5p | *PIK3R1* (p85) → PIP3 → AKT. Further work needs to be done to elucidate the molecular mechanisms underlying the dysregulation of miR-486-5p in CML and identification of its target genes.

4. CONCLUSIONS

There are many reports to demonstrate the role of miRNA to act as oncogenes or tumor suppressors in different cancers. We have reported here the alterations of the expression of miR-486-5p in CML patient samples. Our observations support the onco-suppressor role of miR-486-5p in CML. The distinct elevation of the expression of miR-486-5p in CML patients in response to imatinib might be associated with TKI activity of imatinib-targeting BCR-ABL1 fusion gene, which through its downstream effector pathways leads to the repression of the miR-486-5p expression. The validation of this pathway requires further studies. To the best of our knowledge, this is the first report of alterations in the miR-486-5p expression in peripheral blood leucocytes of CML patients.

5. EXPERIMENTAL SECTION

5.1. Ethics Statement. This study was carried out at Maulana Azad Medical College (MAMC) and the associated Lok Nayak Hospital, New Delhi, after obtaining approval from the Institutional Ethics Committee of MAMC, New Delhi. Informed consent of CML patients and healthy volunteers was taken before to their enrolment in the study.

5.2. Statistical Analysis. Statistical analyses were performed using Graph Pad Prism, version 7.03 software. Mann-Whitney-U test and Chi-square test were used, as appropriate, to compare the quantitative and qualitative data, respectively. A p -value < 0.05 was taken as statistically significant.

5.3. Experimental Study in the CML K562 Cell Line.

5.3.1. Maintenance of the CML K562 Cell Line. The BCR-ABL1+ve CML K562 cell Line was procured from the National Center for Cell Science, Pune, India. K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal Bovine Serum (FBS) containing 100 U/mL penicillin, 100 μ g/mL of streptomycin, 250 ng/mL of amphotericin, and 250 μ g/mL of gentamycin. The cultures were maintained at 37 °C, in 5% CO₂ in the air, in normal humidified conditions.

5.3.2. IC₅₀ of Imatinib in K562 Cells. Cell viability was assessed using the Trypan blue exclusion test. IC₅₀ for imatinib was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide] assay to be 40 μ M for K562 cells. A 10-times lower dose of imatinib, that is, 4 μ M was used as the treatment dose for the imatinib exposure of K562 cells.

5.3.3. Imatinib Exposure of K562 Cells. Two sets of T-25 flasks with K562 cells from the same passage number (3 flasks/set), each flask containing 500,000 cells seeded in 5 mL of complete RPMI 1640 media with 10% FBS and antibiotics, were prepared and placed in a CO₂ incubator. Cells were allowed to grow for 3 days, till they reached near confluency. After separation from the medium by centrifugation, K562 cells were washed with phosphate-buffered saline and seeded again in fresh T-25 flasks containing RPMI-1640 media with 2% FBS and antibiotics. In Set 1 flasks, imatinib at 4 μ M concentration was added, while K562 cells in Set 2 flasks were not exposed to imatinib.

5.3.4. Isolation of Peripheral Blood Monocytes from Healthy Volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by the Ficoll–Paque density gradient centrifugation, from whole blood samples of healthy volunteers. Monocytes were purified from PBMCs, based on their property of adherence to plastic surfaces, seeded in three

T-25 flasks with complete RPMI 1640 growth media (set 3), and used as normal control cells.

5.3.5. Estimation of the miR-486-5p Expression in K562 Cells (before and after Imatinib Exposure) and in Control Peripheral Blood Monocytes. All the 3 sets of T-25 flasks were kept in a CO₂ incubator for 48 h, after which aliquots of cells were drawn (in triplicate) for RNA extraction and further processing.

5.3.6. Total RNA Extraction. Total RNA (including miRNAs) was extracted from K562 cells (imatinib treated and untreated) and normal peripheral blood monocytes by the modified acid guanidinium thiocyanate–phenol–chloroform (AGPC) method²⁶ using the Trizol reagent (Invitrogen Life Technologies) by the standard protocol as per manufacturer's instructions.

5.3.7. Complementary DNA (cDNA) Synthesis. After the RNA integrity check, the polyadenylation of microRNAs was performed using poly(A) polymerase and ATP, and cDNA from poly(A) tailed microRNAs was synthesized using a oligo dT adapter primer and reverse transcriptase, by the Affinity Script qPCR First-strand cDNA synthesis kit (Agilent Technologies, USA). The oligo-dT adapter primer had a unique sequence at its 5' end that allowed the amplification of cDNAs by qRT-PCR.

5.3.8. qRT-PCR for Quantification of miR-486-5p. qRT-PCR was carried out using SYBR Green qPCR Mastermix (Thermo Fisher Scientific, USA) with miR-486-5p-specific forward primer (UCCUGUACUGAGCUGCCCCGAG) and a universal reverse primer (annealing temperature 58 °C), in Rotor-Gene Q (Qiagen) real-time PCR cycler. A melting curve was generated spanning a temperature range of 35–95 °C to ensure specificity of the PCR product. Ct values observed for miR-486-5p were normalized to the internal control RNA gene (RNU6B). The relative expression of miR-486-5p was expressed as the corresponding $2^{-\Delta C_t}$ values for K562 cells and control peripheral blood monocytes.

5.4. Hospital-Based Study in CML Patients. **5.4.1. Patients' Recruitment and Follow-Up.** A total of 36 newly diagnosed cases of CP-CML, in the age group of 18–70 years (male/female) who were to be initiated on imatinib therapy, were included in the study. Age- and sex-matched 36 healthy volunteers as the control subjects were also recruited (Figure 2). On inclusion in the study, patients underwent detailed clinical examination and hematological laboratory tests. The diagnosis of CML was confirmed by the detection of BCR-ABL transcripts by qualitative multiplex RT-PCR.

All the 36 CML patients recruited in the study were put on imatinib therapy, at an oral dose of 400 mg of OD. These patients were clinically followed and their HTR was monitored for the succeeding 12 months.

5.4.2. Estimation of the miR-486-5p Expression in Peripheral Blood Leucocytes of CML Patients and Control Subjects. For peripheral blood samples, 5 mL from each CML patient (before initiation of imatinib treatment) and control subject was collected in an ethylenediaminetetraacetic acid vial and kept in the erect position. After 15–20 min of standing of the blood sample, a buffy coat of peripheral blood leucocytes appeared in the top layers and were aspirated out. The isolated peripheral blood leucocytes after RBC lysis were used for the extraction of total RNA (including microRNAs), using the Modified AGPC method. The microRNAs were polyadenylated and cDNAs from the poly(A) tailed microRNAs was synthesized. The miR-486-5p expression in peripheral blood

leucocytes of CML (before and after imatinib therapy) and control subjects was quantified by SYBR Green I-based qRT-PCR. The protocols followed were the same as for the K562 cell line studies.

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