

miRNA–mRNA Profiling Reveals Prognostic Impact of *SMC1A* Expression in Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) with *NPM1* mutation is a disease driving genetic alteration with good prognosis. Although it has been suggested that *NPM1* mutation induces chemosensitivity in leukemic cells, the underlying cause for the better survival of *NPM1* mutated patients is still not clear. Mutant *NPM1* AML has a unique microRNA and their target gene (mRNA) signature compared to wild-type *NPM1*. Dynamic regulation of miRNA–mRNA has been reported to influence the prognostic outcome. In the present study, in silico expression data of miRNA and mRNA in AML patients was retrieved from genome data commons, and differentially expressed miRNA and mRNA among *NPM1* mutated ($n = 21$) and *NPM1* wild-type ($n = 162$) cases were identified to establish a dynamic association at the molecular level. In vitro experiments using high-throughput RNA sequencing were performed on human AML cells carrying *NPM1* mutated and wild-type allele. The comparison of in vitro transcriptomics data with in silico miRNA–mRNA expression network data revealed downregulation of *SMC1A*. On establishing miRNA–mRNA interactive pairs, it has been observed that hsa-mir-215-5p (logFC: 0.957; $p = 0.0189$) is involved in the downregulation of *SMC1A* (logFC: -0.481 ; $p = 0.0464$) in *NPM1* mutated AML. We demonstrated that transient expression of *NPM1* mutation upregulates miR-215-5p, which results in downregulation of *SMC1A*. We have also shown using a rescue experiment that neutralizing miR-215-5p reverses the effect of *NPM1* mutation on *SMC1A*. Using the leukemic blasts from AML patients, we observed higher expression of miR-215-5p and lower expression of *SMC1A* in *NPM1* mutated patients compared to wild-type cases. The overall survival of AML patients was significantly inferior in *SMC1A* high expressers compared to low expressers (20.3% vs. 58.5%, $p = 0.018$). The data suggest that dynamic miR-215-*SMC1A* regulation is potentially modulated by *NPM1* mutation, which might serve as an explanation for the better outcome in *NPM1* mutated AML.

Key words: miRNA–mRNA; *NPM1* mutation; Acute myeloid leukemia (AML); *SMC1A*

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by uncontrolled clonal proliferation of poorly differentiated cells of the myeloid lineage. Although more than 40 mutations in nucleophosmin (*NPM1*) have been reported, the most common mutations are characterized by tetranucleotide insertion in exon 12 accounting for 45%–60% of AML patients with normal karyotype (NK)^{1,2}. Although it is well established that *NPM1* mutation is an AML-driving lesion with good prognostic impact, the rationale behind this prognostication is not well defined. Some

studies suggested that *NPM1* mutation induces chemosensitivity in leukemic cells^{3–5}, but the precise cause is yet to be elucidated. Mutant *NPM1* AML has a unique microRNA (miRNA) and their target gene (mRNA) signature compared to wild-type *NPM1*^{6–10}.

In humans, most of the proteins coding RNAs are subject to miRNA–mRNA-mediated regulation. To reliably measure such interactions, prior knowledge of target mRNAs is required. However, with the advent of next-generation sequencing methods, it has now become possible to investigate these interactions globally. Dynamic regulation of miRNA–mRNA has been reported to

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influence the prognostic outcome in glioblastoma and breast cancer¹¹. Becker et al. described the gene expression signature of a series of older patients with NK-AML and *NPM1* mutations that featured upregulation of homeobox genes accompanied by higher expression levels of *miR-10a*, *miR-10b*, *miR-196a*, and *miR-196b*¹². The miRNA–mRNA interaction to influence the prognosis in *NPM1* mutated AML needs to be investigated. A recent study suggested that the molecular interaction of *SMC1A* with miRNA and long noncoding RNA could be used as a promising therapeutic target to mitigate the progression of AML¹³. The *SMC1A* has also been shown to promote cell proliferation, migration, and metastasis in various solid tumors^{14–20}. The *SMC1A* belongs to the cohesin complex family of genes contributing toward chromosomal segregation. Recurrent mutation of *SMC1A* and the other members of cohesin complex genes affects the prognosis in AML. It has also been suggested that genetic alterations in *SMC1A* may contribute in leukemogenesis through premature sister chromatid separation^{21,22}.

In this study we aim to evaluate the pairwise correlations of differential expression between miRNA and mRNA, and if the strength of differential (negative or positive) regulation of an miRNA on its target gene can be modulated by *NPM1* mutation in AML. The miRNA and mRNA expression data of AML patients from genome data commons (GDC) was analyzed for miRNA–mRNA interactions that were subsequently divided into two groups (*NPM1* mutated and wild type). This in silico data were validated in vitro using human AML cell line OCI-AML3 (*NPM1*^{mut}) carrying *NPM1* mutation and the OCI-AML3-siNPM1mut cells (*NPM1*^{mut}wild-type-mimic) using high-throughput RNA sequencing. The findings from in silico and in vitro analyses were further validated using leukemia cell lines and biological material from an independent cohort of AML patients. We observed that miR-215-5p mediates the downregulation of *SMC1A* in the presence of *NPM1* mutation.

MATERIALS AND METHODS

Transcriptomics Analysis From GDC: In Silico Analyses

The miRNA, mRNA (genes), and clinical information were obtained from TCGA (The Cancer Genome Atlas) and TARGET (Therapeutically Applicable Research to Generate Effective Treatments) database of GDC portal. A total of 988 and 200 AML cases from TARGET and TCGA, respectively, were identified. One hundred ninety-one cases were selected in which miRNA and mRNA expression data were available along with clinical information. Based on available clinicobiological data, cases were divided into *NPM1* mutated ($n = 21$) and *NPM1* wild-type ($n = 162$) groups. For 183 cases, high-throughput sequencing (HT-seq) count files of miRNA

and mRNA were obtained and differentially expressed miRNA and mRNA with p value and false discovery rate (FDR) were identified using EdgeR Bioconductor package v3.20.9²³. All the data derived from GDC are referred as in silico analyses.

High-Throughput Transcriptional Profiling (RNA Sequencing): In vitro Analyses Knockdown of NPM1 Mutation in OCI-AML3 Cells

Human AML cell line OCI-AML3 (*NPM1*^{mut}) carrying endogenous heterozygous *NPM1* type A mutation and the OCI-AML3-siNPM1mut cells (*NPM1*^{mut}wild-type-mimic) were used for in vitro validation tests for miRNA and mRNA expression profiling. The mutated allele of *NPM1* in OCI-AML3 cells was knocked down by reverse transfection using the small interfering RNA (siRNA) (GE Healthcare Dharmacon, Lafayette, CO, USA) that specifically targets the mutated *NPM1* allele but not the wild-type allele to generate OCI-AML3-siNPM1mut cells. Sequences of the siRNAs are provided in Supplementary Table 1a (available at <https://drive.google.com/open?id=12EmifB1BFJCwbG6-Cp39kQhPnlgxHLDdb>).

Evaluation of NPM1 Mutation Knockdown Efficiency

Following reverse transfection, RNA was isolated from *NPM1*^{mut}wild-type-mimic and *NPM1*^{mut}scramble siRNA cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), complementary DNA (cDNA) synthesis (Thermo Fisher Scientific), and polymerase chain reaction (PCR) using LightCycler 96 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, DEU) with previously described primers and probes for *NPM1* mutation quantification²⁴. Abelson (*ABL*) gene was used as an endogenous control to assess the quality of cDNA. Cell lysate preparation and Western blot analyses were performed according to standard procedures. The mouse monoclonal antibody recognizing specifically *NPM1*^{mut}scramble (#PA1-46356; Thermo Fisher Scientific) was used. The monoclonal β -actin antibody (#A5316; Sigma-Aldrich, St. Louis, MO, USA) was used as a control.

RNA sequencing of NPM1^{mut} Scramble and NPM1^{mut} Wild-Type-Mimic

Total RNA was isolated from *NPM1*^{mut}wild-type-mimic and *NPM1*^{mut}scramble cells using TRIzol reagent (Thermo Fisher Scientific). Trilink CleanTag Small RNA kit (Trilink Biotechnologies, San Diego, CA, USA) was used to prepare small RNA library. The small RNA is first captured using specific RNA adapters that ligate to the 3' and 5' ends of the small RNA. These adapters are each about 60 bases long, and they ligate to the small RNA, which is around 18–24 base pairs (bp) long. These adapter-ligated fragments (around 140 bases long) of

small RNA are then reverse transcribed, converting them to cDNA. The small RNA converted to adapter-ligated cDNA fragments were then amplified using PCR. The small RNA libraries thus prepared were checked on tapestation and sequenced on Illumina HiSeq 2500 in 1×50 single end run.

Data Analysis Pipeline. For mRNA-seq, quality of the fastq files was checked using FastQC for base and sequence quality score distribution. The paired-end reads were aligned to reference human genome Hg19 using genes and transcript (GTF) file from Ensembl by running the STAR 2.4.1 program²⁵. The aligned reads were used for estimating the expression of the genes and transcripts using cufflinks-2.2.1. The expression values are reported in FPKM (fragment per kilo per million) units for each of the genes and transcripts. The differential expression analysis is performed using DESeq2 and is reported as \log_2 fold change²⁶.

Similarly, for miRNA-seq, quality of the fastq files was checked using FastQC for base and sequence quality score distribution. The reads were aligned to the reference human genome (Hg19) and miRBase database using Bowtie 2²⁷. The aligned reads were used for estimating the expression of the miRNA using miRcat UEA Small RNA Workbench v3.2²⁸.

Independent Validation of NPM1 Mutation Effect on miR-215 and SMC1A Expression

Cell lines, Plasmids, and Transfection. Human myelogenous leukemia cell line K562 (generously provided by Professor Pelicci, IFOM Milan, Italy) was used for in vitro validation. The plasmids (pEGFP-C1) containing NPM1 mutant and NPM1 wild-type cDNA insert (provided by Prof. B. Falini, Perugia, Italy) were electroporated into K562 cells using Gene Pulser Xcell™ Electroporation Systems (Bio-Rad, Hercules, CA, USA), and cells were labeled as K562^{CTRL} (only cells), K562^{NPM1-wt} (plasmid with wild-type NPM1), and K562^{NPM1-mut} (plasmid with NPM1 mutant type A). Following electroporation, GFP-positive cells were sorted using FACS Aria III (BD, Franklin Lakes, NJ, USA). RNA from sorted cells was isolated, and mutant NPM1 was confirmed by Sanger sequencing. Total RNA and miRNA were extracted from K562^{CTRL}, K562^{NPM1-wt}, and K562^{NPM1-mut} cells using miRNeasy Mini kit (Qiagen, Venlo, Netherlands) as per the manufacturer's instructions. RQ-PCR analysis of miR-215-5p was carried out using the miRCURY LNA PCR Kit (Qiagen, Venlo, Netherlands). The *SMC1A* expression analysis was performed using SYBR green-based assay using in-house-designed primers (Supplementary Table 1a; available at <https://drive.google.com/open?id=12EmifBIBFJCwbG6-Cp39kQhPnlxHLDdb>). The template variation across samples was normalized using endogenous controls of miR-103-3p and -actin for miRNA and mRNA,

respectively. The comparative threshold cycle (2^{-CT}) method was used to analyze the data.

Inhibition of miR-215-5p Resulted in Upregulation of SMC1A. The miR-215-5p inhibitor (Cat. No. MIH01561; ABM Canada) was used to rescue the expression of *SMC1A* in OCI-AML3 cells, which carries an endogenous *NPM1* mutation type A. A total of 0.5×10^6 OCI-AML3 cells (in duplicates) were transfected with 0.1 and 0.5 μg of miR-215-5p inhibitor and incubated at 37°C for 48 h as per the manufacturer's instruction. Following incubation, total RNA was isolated using TRIzol method from inhibitor-treated and control cells. The cDNA for miR-215-5p and *SMC1A* expression was synthesized and quantified by real-time PCR in triplicates.

Analysis of the Prognostic Impact of SMC1A Expression in AML: Patient Samples, cDNA Synthesis, and RQ-PCR

To study the impact of *SMC1A* expression, survival analysis was performed in an independent cohort of AML patients. Bone marrow samples from 67 patients (*NPM1* wild type $n = 49$, and *NPM1* mutated $n = 18$) at the time of diagnosis were collected. The median age of the cohort was 27 years (range: 15–58 years). Out of 67 patients, 4 patients died during induction, 5 were refractory to the treatment, while the follow-up data were not available for 2 patient, and hence these cases were excluded from *SMC1A* expression and overall survival (OS) analysis. All patients provided written informed consent in accordance with the Declaration of Helsinki, and the Ethics Committee of Tata Memorial Centre (TMC), Mumbai, approved the study (DCGI registration number: IECIII: ECR/149/Inst/MH/2013/RR/2016 approved the study; approval reference number: 179/2015). Total RNA from AML blasts (1 μg) was used for cDNA synthesis using high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). The SYBR green-based RQ-PCR assays for *SMC1A* expression were carried out using LightCycler 96 real-time PCR system. According to the median values of *SMC1A/ABL1* expression, AML patients were divided into high expressers and low expressers. Results of *SMC1A/ABL1* expression data were analyzed for their impact on OS. In addition to AML patients recruited at TMH ($n = 56$), we also included *SMC1A* expression data of AML patients ($n = 179$) from GDC for OS analyses.

Statistical analysis was performed using IBM SPSS v21. OS was defined as the time from the start of induction therapy to time of last follow-up or death. Results of the *SMC1A* and miR-215-5p expression were analyzed for their impact on OS using the Kaplan–Meier method. Quantitative variables were analyzed by Student's *t*-test. All statistical analysis was two sided, and a value of $p < 0.05$ was considered statistically significant.

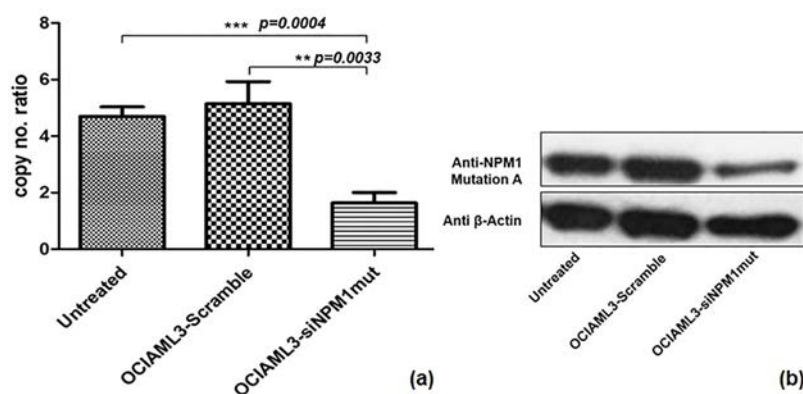


Figure 1. Quantitative expression of *NPM1* mutation A by RQ-PCR (a) and Western blot (b). ***p* 0.01, ****p* 0.001.

RESULTS

miRNA and mRNA Expression of NPM1 Wild-Type and Mutated Patients: In Silico Data

To understand the effect of *NPM1* mutation on miRNA–mRNA network, public domain gene expression repository was used to correlate with functional assay derived from *NPM1*^{mut}scramble and *NPM1*^{mut}wild-type-mimic. Out of 200 TCGA AML cases, miRNA-seq and mRNA-seq data were available for 103 and 151 AML cases, respectively. Concerning TARGET database (*N* = 988), 265 miRNA-seq and 179 RNA-seq were reported. To study the miRNA–mRNA interaction within the same patients, 85 cases were identified from TCGA and 106 cases from TARGET having miRNA and mRNA expression along with clinicobiological information. The read counts data for miRNA expression consist of 1,881 miRNA, while mRNA expression data consisting of 55,640 mRNA were extracted from 191 cases derived from TCGA and TARGET. In clinicobiological files of 191 cases, *NPM1* mutation status was not reported for 8 cases. Hence, these cases were excluded from the analyses. The differentially expressed miRNA and mRNA are reported in Supplementary Tables 1b and 1c, respectively (available at <https://drive.google.com/open?id=12EmifB1BFJCwbG6-Cp39kQhPnlgxHLDdb>).

Knockdown of NPM1 Mutant Allele and RNA Sequencing

RQ-PCR using plasmid calibration curve has allowed accurate assessment of *NPM1* mutation A copies/ 10^4 ABL1

in OCI-AML3 versus OCI-AML3-si*NPM1*mut cells. We observed 73.5% of reduction of *NPM1* mutation A expression in siRNA-mediated knockdown cells (Table 1). Western blot data revealed 64% repression efficiency of mutated *NPM1* (Fig. 1). The data were analyzed using ImageJ software²⁹. In vitro differentially expressed miRNA and mRNA are reported in Supplementary Table 1d and 1e, respectively (available at <https://drive.google.com/open?id=12EmifB1BFJCwbG6-Cp39kQhPnlgxHLDdb>) in *NPM1*^{mut}scramble compared to *NPM1*^{mut}wild-type-mimic. Based on the cutoff of 1.5 log fold change, 27 miRNA were upregulated while 33 were downregulated. Similarly, differentially expressed mRNA revealed 2,983 upregulated and 807 downregulated genes as a result of *NPM1* mutant allele silencing.

Identification of Genes Targeted by miRNAs Associated With Mutated NPM1: miRNA–mRNA Interactive Pairs

The differential miRNA expression between two groups (*NPM1* wild-type and mutant AML) has been reported in Table 2. With respect to GDC dataset, the list of miRNAs was generated based on the difference in the miRNA expression (\log_2 fold change) between the two groups with a value of *p* < 0.05. The \log_2 fold change was used to categorize the miRNAs in *NPM1* wild-type and mutant groups. Two miRNAs (hsa-mir-7976 and hsa-mir-6718) were downregulated, while four (hsa-mir-215, hsa-mir-450a-1, hsa-mir-5087, and hsa-mir-3912) were upregulated in *NPM1* mutated AML compared to *NPM1* wild-type AML.

Table 1. Knockdown Efficiency of *NPM1* Mutation A Using Specific siRNA

Sample	<i>NPM1</i> Mutation A/ 10^4 ABL1 Copy Number	% of <i>NPM1</i> Mutation A Expression (siRNA-Treated Cells $\times 100$ /Scramble-Treated Cells)	% of <i>NPM1</i> Mutation A Knockdown
Treated cells (siRNA 60 nM)	12,000		
Treated cells (scramble siRNA 60 nM)	45,000	26.7	73.3

Table 2. miRNA Expression in Acute Myeloid Leukemia (AML) Patients With *NPM1* Mutation Compared to *NPM1* Wild-Type AML

miRNA	Log ₂ FC	<i>p</i> Value	FDR
hsa-mir-363	1.9336	0.0002	0.0105
hsa-mir-1291	-1.3753	0.0004	0.0176
hsa-mir-607	2.3239	0.0014	0.0405
hsa-mir-7976	-1.1299	0.0020	0.0541
hsa-mir-382	-1.9266	0.0027	0.0623
hsa-mir-3912	1.0942	0.0031	0.0677
hsa-mir-6718	-1.2294	0.0045	0.0841
hsa-mir-3911	2.7209	0.0047	0.0851
hsa-mir-5087	1.8014	0.0081	0.1154
hsa-mir-4786	1.0669	0.0088	0.1203
hsa-mir-450a-1	1.4821	0.0134	0.1545
hsa-mir-215	0.9579	0.0189	0.1765
hsa-mir-6747	1.2568	0.0339	0.2443
hsa-mir-449a	1.9596	0.0536	0.3393

FC, fold change; FDR, false discovery rate.

To obtain the target genes of 14 miRNAs associated with *NPM1* mutation, the miRTar database (<http://mirtar.mbc.nctu.edu.tw/human/>) was used. The miRTar database also gives the mature miRNA, which is known to form a pair with the target genes³⁰. Given the fact that one miRNA can regulate the expression of multiple genes, we focused miRNA interactive analyses on the genes that are known to be modulated in leukemia. The comprehensive panel of 54 genes of Illumina's TruSight myeloid leukemia was used. These genes were searched in the list of target genes associated with 14 miRNAs from the miRTar database. Out of 14 miRNAs, 5 miRNAs (hsa-miR-363, hsa-mir-607, hsa-mir-382, hsa-miR-215, and hsa-mir-449a) form pair with 13 genes from TruSight Myeloid Sequencing panel. The differential expression of 13 genes and their corresponding miRNAs has been reported in Table 3.

On establishing miRNA–mRNA interactive pairs, it was observed that hsa-mir-215-5p (LogFC: 0.9579 and *p*-value: 0.0189, Table 2) is involved in the downregulation of *SMC1A* (LogFC: -0.4816 and *p* = 0.0464) in *NPM1* mutated AML.

Effect of *NPM1*^{mut} on miR-215-5p and *SMC1A* Expression

Expression of *NPM1* type A mutation in K562 cells was verified using Sanger sequencing (Fig. 2a). K562^{*NPM1*-mut} cells showed significantly higher expression of miR-215-5p compared to K562^{CTRL} cells. The upregulation of miR-215-5p was approximately 1.45-fold in K562^{*NPM1*-mut} cells in comparison to K562^{CTRL} cells (*p* = 0.0050) (Fig. 2b). We also observed significant downregulation of *SMC1A* expression in K562^{*NPM1*-mut} cells compared to *NPM1*^{*NPM1*-wt} cells (*p* = 0.0010) and *NPM1*^{CTRL} cells (*p* = 0.0029) (Fig. 2c). We further checked the effect of miR-215-5p inhibition on *SMC1A* expression in OCI-AML3 cells comprising endogenous *NPM1* type A mutation. Compared to control, we have shown 1.85- and 2.66-fold reduction in the levels of miR-215-5p after 0.1 and 0.5 μg of inhibitor treatment, respectively (Fig. 3a). On analyzing the effect of miR-215-5p inhibition on the expression of *SMC1A*, we observed 1.20- and 1.90-fold increase in *SMC1A* transcript levels (Fig. 3b).

SMC1A and miR-215-5p Expression in AML Patients: Survival Analyses

Among 65 analyzed cases, the mean *SMC1A* expression value in *NPM1* wild type (*n* = 47) was 2.99 (range: 0.56–20.33), while in *NPM1* mutated patients (*n* = 18), the mean value was 1.14 (range: 0.08–3.79). We observed significantly lower expression of *SMC1A* in *NPM1* mutated patients compared to *NPM1* wild-type (*p* = 0.0018) (Fig. 4a). We carried out expression study

Table 3. miRNA–mRNA Interactive Pairs Derived From In Silico Analyses of *NPM1* Mutated AML

miRNA	Mature miRNA	Genes (mRNA)	Log2 FC	<i>p</i> Value	
hsa-miR-363	hsa-miR-363-3p	<i>NRAS</i>	0.0614	0.7581	
hsa-mir-607	hsa-mir-607	<i>NPM1</i>	0.2240	0.1686	
		<i>PTEN</i>	-0.3414	0.0846	
		<i>SRSF2</i>	-0.0263	0.8232	
hsa-mir-382	hsa-miR-382-5p	<i>PTEN</i>	-0.3414	0.0846	
		<i>SF3B1</i>	-0.1508	0.4729	
		<i>SMC3</i>	-0.1740	0.3946	
hsa-miR-215	hsa-miR-215-5p	<i>SMC1A</i>	-0.4816	0.0464	
		hsa-miR-215-3p	<i>BCOR</i>	0.1747	0.3525
			<i>CDKN2A</i>	0.6033	0.1625
		<i>PHF6</i>	-0.2824	0.3182	
		<i>RAD21</i>	-0.1296	0.4879	
hsa-mir-449a	hsa-mir-449a	<i>WT1</i>	0.0509	0.9041	
		<i>NOTCH1</i>	-0.0187	0.9394	

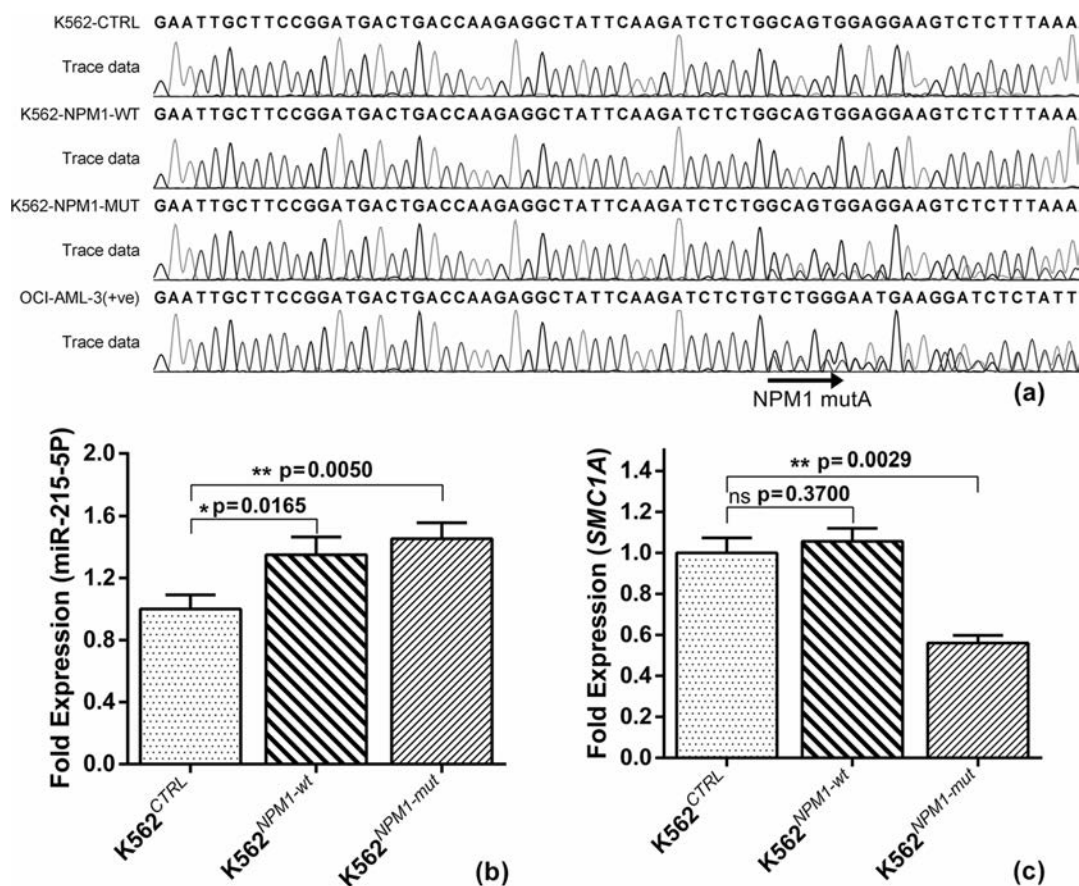


Figure 2. (a) Induced expression of *NPM1* type A mutation in K562 leukemia cells. (b) Expression of *NPM1* type A mutation modulated expression of miR-215 and (c) *SMC1A* in K562^{CTRL}, K562^{NPM1-wt}, and K562^{NPM1-mut} cells. Ct = difference in threshold cycles of *SMC1A* with respect to *ABL1* (endogenous control). **p* 0.05, ***p* 0.01.

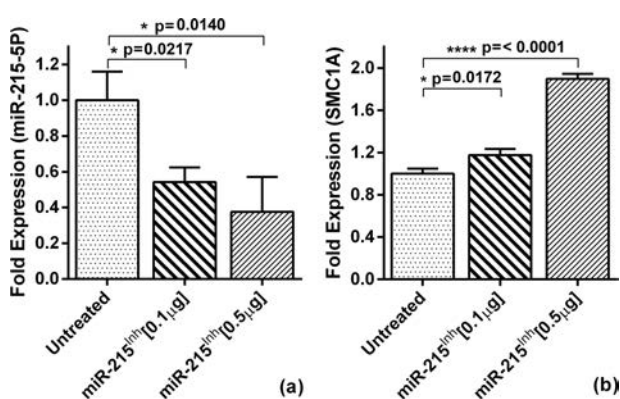


Figure 3. (a) Quantitative real-time polymerase chain reaction (PCR) expression of miR-215-5p and (b) *SMC1A* in OCI-AML3 cells treated with miR-215-5p inhibitor. Ct = difference in threshold cycles of *SMC1A* with respect to *ABL1* and miR-103-3p, respectively (endogenous control). **p* 0.05, *****p* 0.0001.

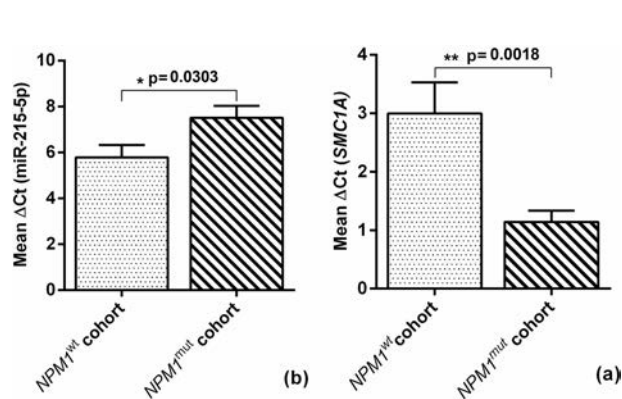


Figure 4. (a) Quantitative real-time PCR expression of *SMC1A* and (b) miR-215-5p in AML patients accrued at Tata Memorial Centre. Ct = difference in threshold cycles of *SMC1A* and miR-215-5p with respect to *ABL1* and miR-103-3p, respectively (endogenous control). **p* 0.05, ***p* 0.01.

of miR-215-5p in 26 patients (*NPM1* wild-type $n = 12$ and *NPM1* mutated $n = 14$) for which biological material was available. The mean miR-215-5p expression value in *NPM1* wild-type was 5.78 (range: 3.47–8.54), while in *NPM1* mutated patients, the mean value was 7.51 (range: 4.19–11.07). We observed significantly higher expression of miR-215-5p in *NPM1* mutated patients compared to *NPM1* wild-type ($p = 0.03$) (Fig. 4b).

For the purposes of clinical correlation, we examined the utility of high *SMC1A/ABL1* expression as a prognostic marker to predict survival outcome in AML. The survival data derived from TMC cohort ($n = 56$) have shown that low *SMC1A/ABL1* expressers were doing better compared to high expressers ($p = 0.018$ at 3 years 58.5% vs. 20.3%) (Fig. 5a). The results were corroborated with the AML patients from GDC ($n = 179$), where OS of high *SMC1A/*

ABL1 expressers was inferior compared with low *SMC1A/ABL1* expressers ($p < 0.0001$ at 6 years 23.6% vs. 53.6%) (Fig. 5b). Similar findings were observed when data from GDC and TMC cohorts pooled together to assess the survival benefits in *SMC1A* low expressers (Fig. 5c). With respect to hsa-miR-215 expression and survival analyses, we observed superior OS of high hsa-miR-215 expressers compared with low expressers, but the difference was not statistically significant (Fig. 5d).

DISCUSSION

The dynamic miRNA–mRNA regulation in AML is potentially modulated by *NPM1* mutation. In this study, the differential miRNA–mRNA expression data in AML patients with and without *NPM1* mutation were derived from an in silico approach. Subsequently,

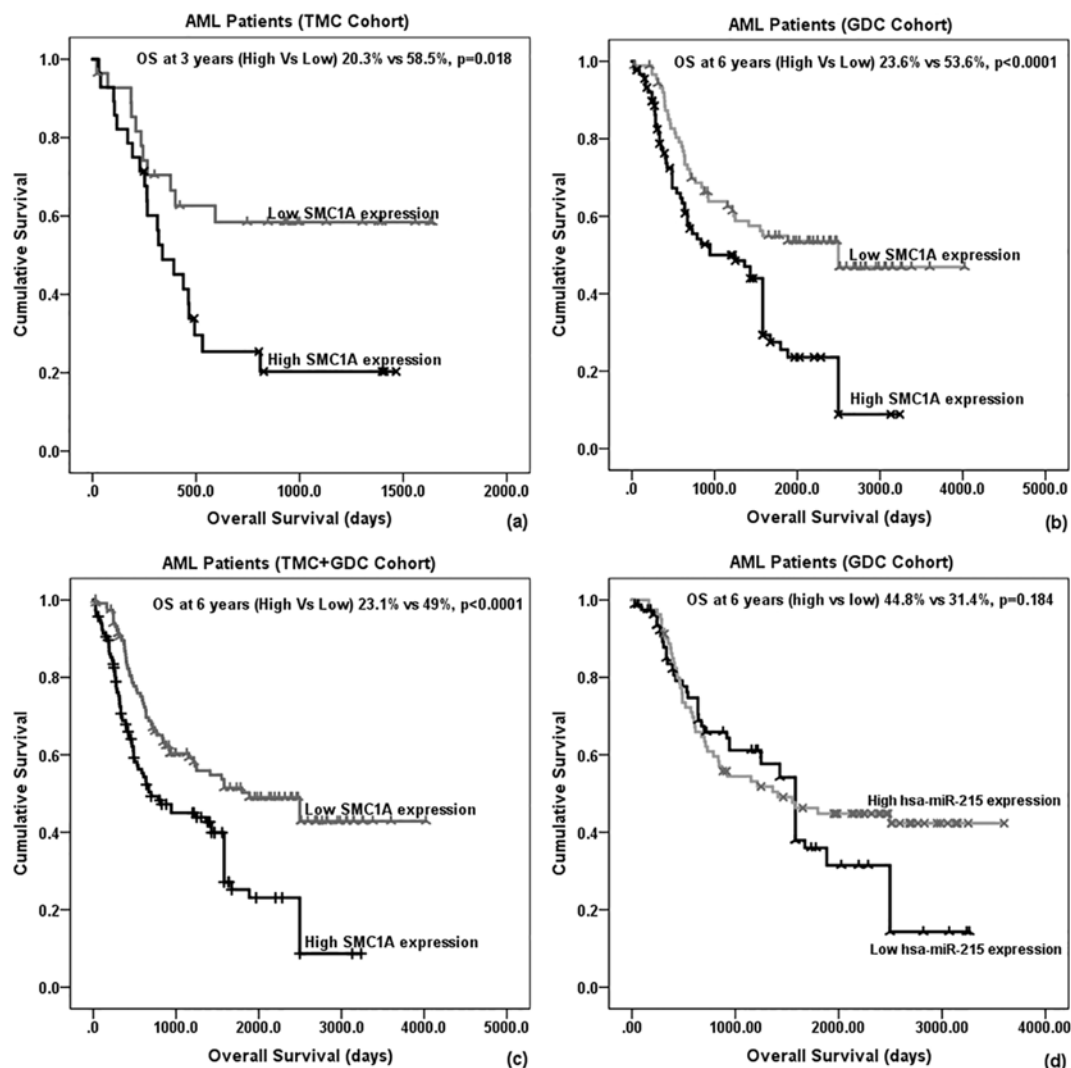


Figure 5. *SMC1A* and hsa-miR-215 median expression versus survival outcome in (a) Tata Memorial Centre (TMC) cohort (*SMC1A*), (b) GDC cohort (*SMC1A*), (c) TMC + GDC cohort (*SMC1A*), and (d) GDC cohort (hsa-miR-215).

in vitro experiments using high-throughput RNA sequencing were performed on OCI-AML3 and OCI-AML3-si*NPM1*mut cells. The comparison of in vitro transcriptomics data with in silico miRNA–mRNA expression network data revealed that hsa-miR-215-5p downregulates *SMC1A* expression in the presence of *NPM1* mutation. Furthermore, biochemical data including expression analyses also revealed that *NPM1* mutation status play a major role in altering the expression of *SMC1A* and miR-215-5p, which was shown in K562 cells and using AML patients' samples (Figs. 2 and 4). We have also shown using a rescue experiment neutralizing miR-215-5p reverse the effect of *NPM1* mutation on *SMC1A*. The *SMC1A* expression in AML is reported to influence event-free survival and OS³¹; however, the limited number of AML cases precludes to draw any conclusion. The impact of *SMC1A* expression on survival was analyzed in AML patients from GDC portal as well as an independent cohort of AML patients who were recruited at our center. Findings obtained from both the analyses have confirmed the low *SMC1A* expression is associated with an improved outcome in AML (Fig. 5).

On analyzing quantitative expression data of *SMC1A* in *NPM1* wild-type and mutated AML patients, we observed significantly high *SMC1A* expression in wild-type patients ($p = 0.0018$) (Fig. 4A). However, we could not perform survival analyses based on *NPM1* mutation status due to the limited number of *NPM1* positive cases in our cohort. The *SMC1A* belongs to the family of cohesin complex, which comprises *SMC1A*, *SMC3*, *RAD21*, *STAG2*, and *STAG1*. These proteins form a ring structure that regulates chromosome segregation. Therefore, cohesin complex is an essential structure during cell division. AML blasts are known to have increased proliferation potential due to unregulated cell division. The mutations of the genes in cohesin complex have been described in 13% of AML patients³². Thol and colleagues have shown a strong correlation between the mutated cohesion gene and *NPM1* mutations (57% of cohesin gene mutated patients had an *NPM1* mutation)²¹. Recently, Patel et al. also demonstrated that most of the *NPM1*-mutated AML patients harbor concurrent mutations in genes involved in the regulation of DNA methylation, RNA splicing, and the cohesin complex³³. On analyzing the functional effect of *SMC1A* mutations, Mannini et al. observed that the mutations of *SMC1A* do not affect *SMC1A* expression³⁴. However, it has been proposed that altered *SMC1A* can influence the dynamic relationship between the cohesin complex and chromatin, leading to altered expression of proto-oncogenes or tumor suppressor genes. It has been demonstrated that *SMC1A* overexpression is associated with colorectal cancer (CRC) development and can be used as a potential therapeutic target in CRC^{16,35}.

Several different miRNAs have been associated with *NPM1* mutated AML; however, strong upregulation of miR-10a-5p and miR-10b-5p has been frequently reported^{6,36}. Garzon and colleagues reported that AML with *NPM1* mutation shows distinct miRNA, mRNA, and miRNA–mRNA signatures⁶. Chiu et al. recently identified nine miRNA–mRNA regulatory pairs that could predict patients' outcome in *NPM1* mutated AML³⁷.

To the best of our knowledge, no study has shown the miR-215-5p association with *NPM1* mutation. Within AML, it has been reported that miR-215-5p is significantly downregulated in *FLT3*-ITD mutated AML cases. However, it is yet to be established that miR-215-5p adversely affects the survival independent of other biomarkers in AML.

CONCLUSION

The downregulation of *SMC1A* mediated by miR-215-5p suggests its role behind the better prognosis of *NPM1* mutated AML compared to AML with wild-type *NPM1*. Considering the small cohort of patients in the present study, future studies are warranted to confirm these findings in a larger cohort of AML patients.

ACKNOWLEDGMENTS: This work was supported by a grant from IEC, Tata Memorial Centre Mumbai to S.K.H. N.K. was supported by a grant from the Lady Tata Memorial Trust Mumbai. The authors are grateful to Prof. F. Lo-Coco from Rome and Prof. P.G. Pelicci, Milan, Italy, who provided *NPM1* mutation-specific antibody and leukemia cell lines. Thanks to bioinformatic centre ACTREC for in silico analyses. Author contributions: N.G., R.K., and S.A. contributed to in silico and in vitro experiments, data acquisition, and data analyses. A.G. and T.G. performed RQ-PCR and data analyses. A.K.V. and N.K. revised the manuscript critically for intellectual content. S.K.H. designed the study and wrote the paper. The authors declare no conflicts of interest.

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