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Mutational Analysis of BGLT3 Gene and Its Prognostic Significance in CML

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ABSTRACT

Beta globin locus transcript 3 (BGLT3) is a candidate lncRNA with a tumor suppressive role in Chronic myeloid leukaemia (CML), a stem cell disorder characterized by presence of Philadelphia chromosome (Ph) with unique translocation between chromosomes 9 and 22 [t (9;22) (q34; q11)] resulting in BCR-ABL fusion protein with aberrant tyrosine Kinase activity. It is reported that BCR-ABL represses *BGLT3* expression in MYC-dependent manner and silencing of the BGLT3 tumor suppressor is required BCR-ABL-mediated cellular transformation. In view of this, the current study was planned to carry out mutation analysis of BGLT3 gene exon1 region in CML patients. Genomic DNA isolated from blood samples of 42 recruited CML patients was used for PCR amplification followed by sequencing. The results revealed 3 novel mutations: NR_121648.1: n.203T>G, NR_121648.1: n.205T>A, NR_121648.1: n.432G>T in patients with all the three clinical phases. Further, in silico analysis and functional enrichment analysis revealed prominent target mRNAs and miRNAs in CML pathways. In view of the significant observations, it is important to study the functional role of BGLT3 in BCR-ABL mediated CML pathogenesis for identification of BGLT3 as effective target for therapy. Future functional studies on these novel mutations in BGLT3 are wanted to evaluate therapeutic and prognostic significance.

Keywords: BGLT3 gene, CML, BCR-ABL protein, Tyrosine Kinase Inhibitors, Long non-coding RNAs

INTRODUCTION

Haematopoiesis is a multilayered regulatory process of differentiation giving rise to lineages of blood cells, and maintenance[1,2]. Genetic variations lead to dysregulated hematopoiesis with perturbation in the differentiation regulation and bone marrow microenvironment. Chronic myeloid leukaemia (CML) is a stem cell disorder characterized by presence of Philadelphia chromosome (Ph) with unique translocation between chromosomes 9 and 22 [t (9;22) (q34; q11)]. The translocation event results in the fusion gene that encodes bcr-abl oncoprotein with aberrant tyrosine kinase known to activate proliferative signaling pathways[3,4]. CML is a triphasic disease with manifesting clinically in chronic phase, progressive accelerated phase and terminal blast phase. Targeted therapies to CML by Imatinib mesylate and second generation Tyrosine Kinase Inhibitors (TKI) have been successful in majority but subset of patients do not respond due to primary or secondary resistance leading to CML relapse[5]. Further, CML persistence at stem cell level is a major concern warranting long-term management therapy and is associated with chronic side-effects. Although BCR-ABL is the initiating event, CML maintenance and progression is driven by BCR-ABL dependent and independent mechanisms and identifying the critical regulators of CML progression and relapse is to design effective therapies for CML cure. Recent *in vitro* studies had reported RNA regulators in BCR-ABL driven pathways, mainly long non-coding RNAs (LncRNAs) and microRNAs (miRNAs).

Beta globin locus transcript 3 (BGLT3) is a candidate lncRNA with a tumor suppressive role reported in CML studies[6]. Inhibition of BCR-ABL in K562 CML cell lines and was found to be linked to upregulation of *BGLT3.* Further, it is known to associated with Imatinib induced apoptosis and reduced survival of CML cells. In this study, Guo et al. (2015) also concluded that BCR-ABL represses *BGLT3* expression in MYC-dependent manner and silencing of the BGLT3 tumor suppressor is required BCR-ABL-mediated cellular transformation. Transgenic mice studies on CML also revealed that expression of *BGLT3* inhibits primary bone marrow cell transformation and proliferation in transgenic mice. BGLT3 is implicated in PIK3 pathway and is known to correlate with the PTEN activity via interactions of its target miRNAs such as miR-17, miR-93, miR-20a, miR-20b, miR-106a and miR-106b. The mechanistic role of BGLT3 in PTEN regulation was also indicated in Imatinib treatment indicating a strong association of BGLT3 with CML origin and progression.

BGLT3 lncRNA is encoded by the gene mapped to the chromosome 11p15.4 spanning 1.6 kb long comprising of one exon resulting in a single transcript. *BGLT3* is located between Y- globin HBG1 and HBD which code for foetal and adult globin genes respectively. *BGLT3* is located within *BCL11A* locus, a master regulator of foetal haemoglobin switching. This locus contains the motifs RID1 and RID2 to which the transcription factor COUP-TFII are said to be involved in the regulation of ɣ-globin genes. The switching of the beta globin genes from ɣ-globin for erythroid cells developmental lineages involves the functional interactions of BCLIIA and transcription factors such as the GATA 1, COUPII and several others. The repression brought about by the BCLII gene also involves BGLT3 repression[7].The BGLT3 long non coding RNA acts like enhancer sequences and switches the globin gene expression according to erythropoietic cell developmental stages[8]. CML progression is known to be associated with the oncogenic biogenesis of miRNA 17 gene family members. The miRNA 17~92 cluster and its paralogs 106b ~ 25 and 106a~363[9,10,11]. There are limited studies on the specific role of BGLT3 in CML pathogenesis. Invitro studies have demonstrated its putative role and strongly indicated it as one of the target genes especially in Imatinib resistance CML.

In view of the functional significance of BGLT3, the present study was planned to investigate the exonic mutations of BGLT3 gene in CML patients and understand their role in perturbing miRNA interactions in BCR-ABL mediated pathway through their target gene annotations.

STUDY DESIGN AND METHODOLOGY

The current study has been approved by Institutional and Hospital research and ethical committee. The study consisted of 42 primary diagnosed CML patients recruited from Nizams Iinstitute of Medical Sciences (NIMS), Hyderabad during the period of 2010 to 2018. From each patient, detailed epidemiological and clinical information was recorded as per structured proforma and 4ml of blood sample was collected in EDTA vacuatainers.DNA was isolated from the samples by salting out method (Lahari DK et al) and stored in -80˚c. The purity was checked by nanodrop (Thermo Fisher Nanodrop Lite) and the samples were maintained -20^{0}C. The samples after standard working dilution were subjected to PCR amplification using 96 well plate (Applied Biosystems SimpliAMP) thermal cycler consisting the reaction mixture: 10x PCR Buffer, 25mMMgcl2, 25mM dNTP mix, 20pmM of each forward and reverse primers, 0.25-0.5U of Taq DNA polymerase. The BGLT3 specific primers were designed to amplify exon 1 region with **Forward primer:5' TTCACTGGTACGCAGGGTTT 3'** and the **Reverse primer: 5' TCCAGCATCAACTTGGAAGA 3'.** The PCR reaction was carried out with initial and final denaturation at 95˚c for 5min, 95˚c for 1min 32 cycles, annealing at 52˚c for 40sec, extension and final extension at 72˚c for 1 min and 72˚c for 5 min. The amplified PCR products were electrophoresed on 2% gel with containing 0.5µl of ethidium bromide and visualized on UV transilluminator (Bio-Rad). The amplified PCR products of 990 base pairs were sequenced by sanger sequencing (IRA Biotech, Hyderabad). Chromatogram analysis was done using the chromatogram viewer (FinchTV 1.4.0).

Functional gene enrichment analysis of BGLT3 gene binding deriving the miRNAs protein targets from miRbase and enrichment analysis performed using the Enricher gene set enrichment analysis web server.

RESULTS AND DISCUSSION

The study conducted in 42 CML patients at clinical stages of chronic phase (15), accelerated phase (17), and blast crisis phase (10) were screened for mutations in BGLT3 exon 1. The three variations NR_121648.1: n.203T>G, NR_121648.1: n.205T>A, NR_121648.1: n.432G>T were observed among three phases of CML (53.3%, 52.9%, 50.0%) with the chronic phase showing 203T>G, 205T>A variations, accelerated phase patients showing all three variations 203T>G, 205T>A, 432G>T and the blast crisis patients were observed with 203T>G, 205T>A variation (Table 1.1, Figure 1).

Table 1.1: BGLT3 variations observed among clinical phases of CML cases

CML Phase (N=42)	No. of cases with variation.n (%)	Nature of variation	
		Homozygous n	Heterozygous n
Chronic phase (15)	8(53.3)-203T>G,205T>A	4 with 203T>G	4 with203T>G8 with 205T>A
Accelerated phas (17)	9(52.9)-203T>G,205T>A 2(11.7)-432G>T	2 with 432G>T	9 with 203T>G
Blast crisis (10)	5(50)-203T>G,205T>A	NIL	5 with 203T>G5 with 205T>A

The variations 203T>G, 205T>A were equally observed among the males and females and all the three variations were observed among the age group 20-40 years with 203T>G, and 205T>A observed in 16 patients, and 432G>T variation observed only in one patient, in the age group <20 years one patient having all the three mutations was seen and in the age group >40 years five patients with 203T>G, 205T>A variations were observed.

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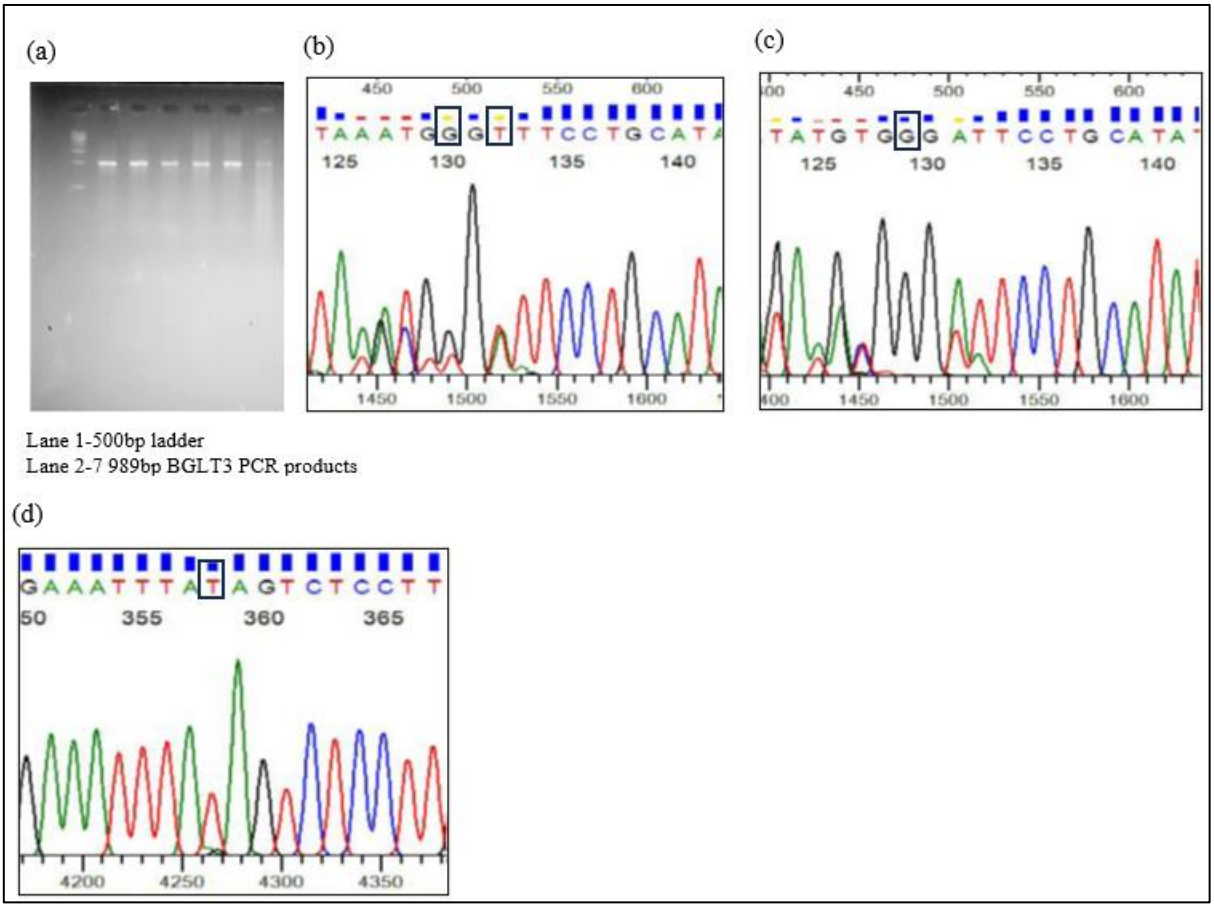

Figure 1: Figure showing the chromatogram pictures of BGLT3 gene with mutation sites

(a) Gel picture showing the amplification of BGLT3
(b) Chromatogram picture of BGLT3 showing heterozygous 203T>G, 205T>A
(c) Chromatogram picture of BGLT3 showing homozygous 203T>G
(d) Chromatogram picture of BGLT3 showing homozygous 432G>T

With regard to the clinical responses, complete Hematological response was observed in 12 patients, one patient showed partial response, and about 4 patients were non responders with 203T>G, 205T>A variation. Also with respect to cytogenetic responses, complete response was shown by 7 patients, partial response was seen in 5 patients, no response was seen in 4 patients having 203T>G, 205T>A variation and one patient with 432G>T mutation showed partial response. Among the molecular responders the complete response was seen in 8 patients with 203T>G, 205T>A variation. From rural area category, 13 patients showed 203T>G, 205T>A and in one patient 432G>T variation was seen but in urban area living 9 patients were observed with 201T>G, 205T>A variation. Among the diet group 20 non-vegetarian patients were observed with 203T>G, 205T>A variation and two patients with 432G>T, 2 patients of the vegetarian group showed 203T>G, 205T>A variation (Table 1.2).

Table 1.2: BGLT3 Exon 1 Variations among CML cases with respect to Age, Sex, Habit, Diet, Phase and Clinical responses.

Parameter	Variable	n = 22	%	203T>G(n)	205T>A(n)	432G>T(n)
Age	<20 Years	1	4.54	1	1	1
	20-40 Years	16	72.72	16	16	1
	>40 Years	5	22.72	5	5	0
Sex	Male	11	50	11	11	2
	Female	11	50	11	11	-
Habit	Rural	13	59.09	13	13	2
	Urban	9	40.90	9	9	-
Diet	Vegetarian	2	9.09	2	2	-
	Non vegetarian	20	90.09	20	20	2
Phase	Chronic phase	8	36.36	8	8	-
	Accelerated phase	9	40.90	9	9	2
	Blast crisis	5	22.72	5	5	-
Haematological response (n=17)	Complete response	12	70.58	12	12	-
	Partial response	1	5.88	1	1	-
	No response	4	23.52	4	4	-
Cytogenic response (16)	Complete response	7	43.75	7	7	-
	Partial response	5	31.25	5	5	1
	No response	4	25.00	4	4	
Molecular response (8)	Complete response	8	100	8	8	-
	No response	NIL	-	-	-	-

When the phase vs clinical responses comparison was made to assess the drug response between the phase of the CML cases complete responses were seen among the all the three phases with the major chronic phase patients responders but atleast two patients were observed with no response only among the accelerated with 203T>G, 205T>A, 432G>T and blast crisis with 203T>G, 205T>A (Table 1.3).

Table 1.3: BGLT3 Exon1phase vs clinical responses among the CML cases

Phase	Complete responders. CHR (N=12) CCR(N=7) CMR(N=8)	Partial responders PHR (N=1) PCR(N=5) MMR(N=0)	Non responders NHR (N=4) NCR(N=4) NMR(N=0)	Variation
Chronic phase	CHR (n=7) CCR(n=4) CMR(n=6)	PHR(n=0) PCR(n=1) MMR(n=0)	NHR(n=0) NCR(n=0) NMR(n=0)	203T>G,205T>A
Accelerated phase	CHR (n=3) CCR(n=2) CMR(n=2)	PHR(n=1) PCR(n=3) MMR(n=0)	NHR(n=2) NCR(n=2) NMR(n=0)	203T>G 205T>A 432G>T
Blast crisis phase	CHR (n=2) CCR(n=1) CMR(n=0)	PHR(n=0) PCR(n=1) MMR(n=0)	NHR(n=2) NCR(n=2) NMR(n=0)	203T>G 205T>A

Bioinformatic analysis of the miRNAs miR-106a, miR-17, miR-20a, miR-20b, miR-93, miR-106b detected a common 8mer sequence sharing complimentary to the PTEN mRNA and 4mer sequences found complimentary with BGLT3 which can be non canonical base pairing gene and are found at the mutated sites of 203T>G, 205T>A (Figure 2).

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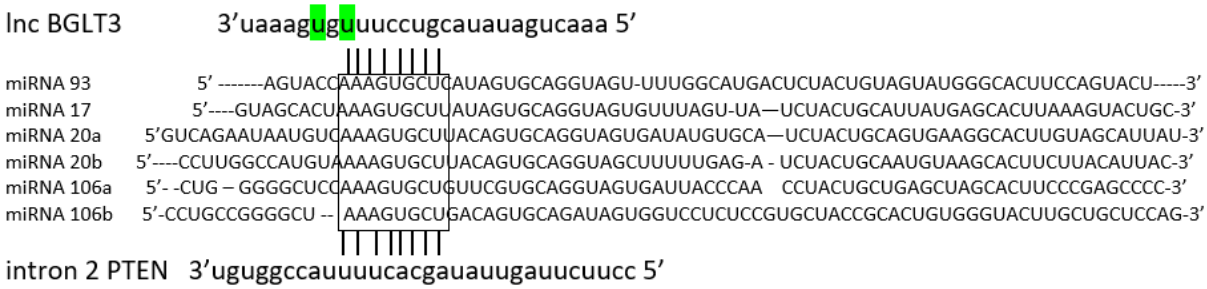
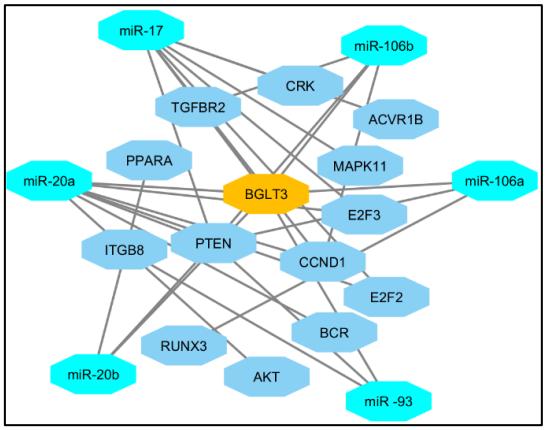


Figure 2: (a) showing the sequence alignment of BGLT3 target miRNAs and complimentary sites in BGLT3 and PTEN mRNA

To validate the BGLT3 sponging oncogenic miRNAs pathogenic emergence, as they were BCR-ABL driven and involved in progression of the CML, their putative targets analysis by gene enrichment was done using the online tool Enricher and to comprehend the underlying BCRABL driven activation of different signalling pathways of progression and relapse (Table 2.1, Figure 3).

Table 2.1 KEGG gene enrichment pathway analysis of target mRNAs of miRNA binding to BGLT3

LNCRNA	MIRNA	Reference	MRNA	KEGG pathway
BGLT3	miR-106a	K. Machova Polakova, T et al 2011, Koschmieder, S., & Vetrie, D. 2018, Guo, G. et al 2015	RUNX3, PTEN	WNT signalling pathway, PD-L1 expression and PD-1 check point pathway in cancer
	miR-106b	K. Machova Polakova, T et al 2011, Koschmieder, S., & Vetrie, D. 2018,Guo, G. et al 2015	TGFBR2, CCND1, PTEN	FOXO signalling
	miR-20a	K. Machova Polakova, T et al 2011, Koschmieder, S., & Vetrie, D. 2018, Guo, G. et al 2015	E2F2, E2F3, BCR, CCND,AKT, PTEN	CML pathway
	miR-20b	K. Machova Polakova, T et al 2011, Koschmieder, S., & Vetrie, D. 2018Guo, G. et al 2015	PTEN, PPARA	PPAR signalling pathway
	miR-93	Guo, G. et al 2015	PTEN, INETEGRIN B8	PI3K-AKTsignaling pathway Focal adhesion
	miR-17	Guo, G. et al 2015	E2F2, E2F3, CRK, MAPK11, CCND1, ACVR1B, PTEN	CML, miRNAs in cancer



(a)

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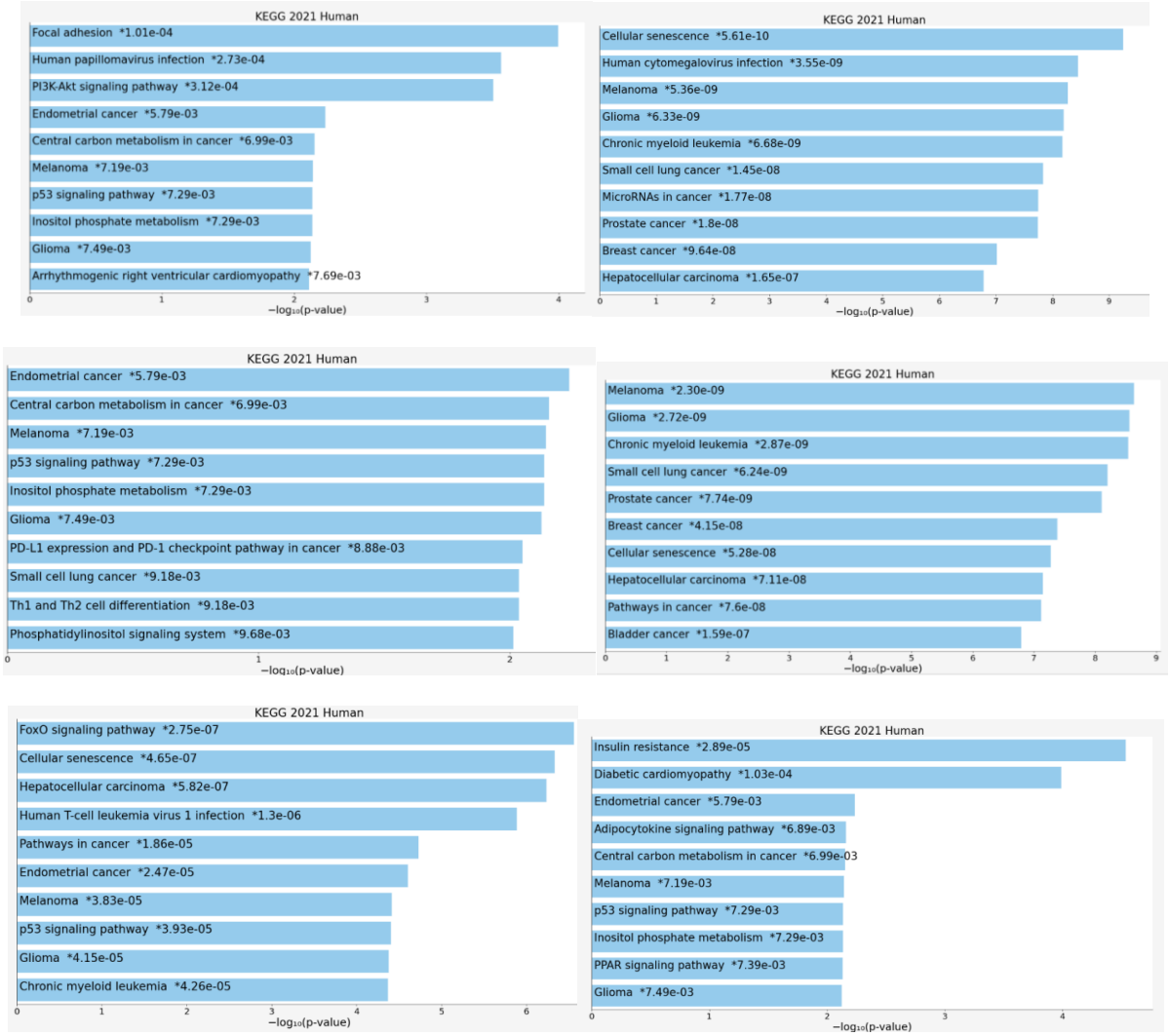

(b)

Figure 3: a Cytoscape network of BGLT3 - miRNAs – mRNAs
b KEGG pathway gene enrichment analysis of miRNAs 93, 17, 20a, 20b, 106a, 106b targeting lncBGLT3

The KEGG enrichment have given the significant output of signalling pathways of cancer, micro RNAs in cancer pathway, and CML pathways (Figure 3). Although the frequency of the identified mutations seem to be rare in our population, they could be actionable targets given their functional significance in CML. The impact of these mutations in CML progression and relapse need to be evaluated. Computational analysis of identified mutations had revealed important miRNA binding sites associated with PIK3 pathway indicating the scope of targeting CML via BGLT3 mediated miRNA. The study of these mutations in the follow up cases particularly in resistant CML could throw light on their clinical significance. Future studies need to be planned in these directions to validate its clinical utility.

CONCLUSIONS: The results report three mutations in BGLT3 gene among CML patients in our cohort: NR_121648.1: n.203T>G, NR_121648.1: n.205T>A, NR_121648.1: n.432G>T. These mutations are novel. In silico and gene enrichment analysis of mRNAs of BGLT3 sponging miRNAs revealed signaling pathway targets whose functional validation is warranted. BGLT3 can be promising target of BCR-ABL mediated pathways in CML.

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Conflict of Interest Statement: All authors have nothing else to disclose.

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