RESEARCH ARTICLE

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Association of Specific Gene Mutations with Immunoglobulin Heavy-Chain Variable Region and Chromosomal Alterations in Chronic Lymphocytic Leukemia Patients in India

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Abstract

Chronic lymphocytic leukemia (CLL) is a less common hematological malignancy in Indian people. It accounts for less than 5% of all leukemias. Information on genomic alteration in CLL is limited immunoglobulin heavy-chain variable region (*IGHV*) mutational status is considered the most reliable prognostic marker. In this study, we performed mutation analysis of significantly mutated genes of CLL and correlated them with the *IGHV* mutational status and cytogenetic alterations. We included 97 patients in this study; 36 were *IGHV* hypermutated, and 61 were *IGHV* unmutated. We observed frequent mutations in *TP53* (16.4%), *ATM* (19.5%), *SF3B1* (18.5%), and *NOTCH1* (14.2%). *NOTCH1* mutations were significantly observed in patients with unmutated *IGHV*. We observed that patients with no mutations in *ATM, NOTCH1,* or *TP53* had chromosomal alterations (del 11q, del 13q, del 17q, and trisomy 21) identified by FISH. Our results have shown mutations in essential genes and their association with *IGHV* status. Overall, specific gene mutations, *IGHV* status, and chromosomal alterations can provide information on prognosis.

Keywords: Chronic lymphocytic leukemia- IGHV mutational status- borderline mutated

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Introduction

Chronic lymphocytic leukaemia (CLL) is a molecularly heterogeneous disease resulting from the clonal proliferation of mature B lymphocytes. CLL is a clonal B-cell lymphoproliferative disorder with an accumulation of small, mature, CD5+CD23+ neoplastic lymphocytes in the peripheral blood, bone marrow, spleen, and other lymphoid tissues [1, 2]. CLL is divided into two broad prognostic subgroups based on the immunoglobulin heavy variable region (*IGHV*) mutations [3]. Patients with *IGHV* mutation status (*IGHV* M CLL) have favourable prognosis outcomes when compared with patients having no mutation in *IGHV* (*IGHV*-UM CLL) [4]

Next-generation sequencing has helped characterize the CLL genomic landscape, providing insights into its biology. Information obtained from NGS has transformed clinical decision-making and prediction of early relapses. It has been observed that a typical CLL genome comprises ~2000 molecular lesions, of which only ~ 20 are non-synonymous and ~5 are gross structural abnormalities [5]. In total, ~40 driver genes have been identified in CLL. Novel mutations have been identified in CLL and the most common mutations are observed in NOTHCH1 and *SF3B1*. We evaluated nine significantly mutated genes (*TP53, ATM, SF3B1, BRAF, MYD88, BIRC3, NOTCH1*, *BTK*, and *PLCG2*) in CLL from [6-8].

Cytogenetic alterations are known to affect outcomes. The regularly evaluated alterations 13q14, 13q34, 11q22 (*ATM*), 17p13 (*TP53*) regions, and trisomy 12 [9], were also studied. In this study, *IGHV* status, clinically significant mutations in the nine most commonly affected genes, and cytogenetic abnormalities were analysed. In this study, we observed mutations in essential genes and their association with *IGHV* status which could be unique to the Indian population.

Materials and Methods

Patients

Patients were recruited from 8 cancer centres, and the study was conducted at the Rajiv Gandhi Cancer Institute and Research Centre. In this study, treatment-naive adult CLL patients were included. The approval to conduct the study was obtained from the institutional ethics board. The study was performed per the Helsinki Declaration.

RNA extraction: RNA was extracted from peripheral blood or bone marrow aspirate. The RNA is extracted using the RNeasy Mini Kit (Qiagen, Germany). as per manufacturer recommendations and quantified using Qubit fluorometry (Thermofisher Scientific, Life Technologies, USA).

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IGHV Library preparation

The *IGHV* mutation status is assessed using the Oncomine BCR IGH-LR assay from Thermofisher Scientific. Extracted RNA is reverse transcribed to cDNA, followed by PCR amplification. This assay uses multiplex Ion Ampliseq primers to target the FR1 region of the variable gene and the constant gene segments of the IGH-VDJ rearrangements, resulting in a ~415 bp amplicon and followed by purification using magnetic beads (AMPure XP, Agencourt 1X). The libraries are again amplified, followed by two rounds of magnetic bead purification with 0.5X and 1.2X beads.

Sequencing

The prepared libraries are sequenced on the Ion Torrent S5 Plus machine (Thermofisher Scientific, Lifetechnologies, USA).

Data Analysis

The quality metrics were evaluated in the torrent suite. The evaluation was based on the 'Read classification" as unproductive, productive, or revived productive reads. The spectral typing plots are analyzed to identify the largest clone, and the attributes of the same are recorded, including the variable domain and joining regions that are mutated, the percentage of mutation, and the Shannon diversity. The cumulative mutation rate of >2% is *IGHV* mutated, and <2% is *IGHV* unmutated. A frequency of 2% is considered borderline, which is recorded in the report. The sequence of the region is run on the ARRest/Assign subsets tool, a web-based tool to ascertain the stereotypy of the CLL case being analyzed. Any involvement of the V3-21 region is separately recorded owing to the adverse prognosis distinctly associated with this region.

Next-generation sequencing in CLL patients

DNA extraction: The DNA was extracted after RBC lysis using the DNeasy kit by Qiagen (Germany) as per manufacturer recommendations. The DNA was quantified using Qubit fluorometry from Thermo Fisher Scientific (Life Technologies, USA).

DNA library preparation

Multiplex Ion Ampliseq custom panel was designed for DNA-based sequencing, interrogating single nucleotide variations, and insertions and deletions in the following genes: *TP53, ATM, SF3B1, BRAF, MYD88, BIRC3, NOTCH1, BTK,* and *PLCG2*. The libraries are prepared by PCR amplification followed by purification and size selection using 1.5X (AMPure XP, Agencourt) and sequenced on an Ion S5 Plus machine (Thermofisher Scientific, Life Technologies, USA).

Data Analysis

Quality check was done on Torrent Suite v5.10 and the variants identified were visualized on IGV to ascertain the validity of the call. The variants were determined against the tumor cellularity, and variants with an allele frequency of less than 5% were not considered.

Fluorescence in-situ hybridization

Fluorescence-in-situ hybridization was done using Metasystem probes LSI *ATM* (11q22.3) Spectrum Green/ LSI *TP53* (17p13.1) Spectrum Orange to look for deletion, CEP 12 Spectrum Green for trisomy, and LSI DLEU1 Spectrum Orange/LSI 13q34(LAMP1) Spectrum aqua for deletion.

Statistical Analysis

The statistical analysis was performed using SPSS version 23 (IBM). Descriptive statistics were used to calculate the frequency distribution, and the Chi-squared test was used to determine the association of the *IGHV* status with alterations in FISH. A p-value of <0.05 was considered significant.

Results

A total of 97 treatment-naive CLL patients were included in this study. The median age was 65 years (males: 87.6%, n=85; females: 12.4%, n=12. The demographic features were obtained from the patient information record (Table 1). Patients were classified based on *IGHV* mutation status. 37.1% (n=36) were *IGHV* mutated, and 63.9% (n=61) patients were *IGHV* unmuted (Table 2). In this study, we did not observe any statistically significant association between demographics and chromosomal alteration status with the *IGHV* status.

IGHV characterization and stereotype: In *IGHV*-mutated patients, the most common alteration detected was in *SF3B1* (25%). Among *SF3B1* alteration, the most common variant detected was the K700E mutation (n=4), followed by G742D (n=2). Table 3 lists the mutations identified in other genes in *IGHV*-mutated patients.

One patient was identified with concurrent *SF3B1* variants, specifically K700E and G742D. Furthermore, the *TP53* gene was shown to have the second most common co-occurring genomic mutation seen in six patients. Mutations in *ATM* and *BIRC3* were seen in four (11.1%) patients each and *NOTCH1* in one (2.8%) case. Nevertheless, due to the small number of samples, none of these showed a statistically significant correlation with the *IGHV* mutant status. In the *IGHV* unmutated cohort (n=61), the most common genomic alteration detected was in the *ATM* gene in 15 patients (24.6%), followed by *NOTCH1* in 12 (19.7%) patients. The association of *NOTCH1* with the unmutated *IGHV* status was statistically significant (p<0.03). Mutations were identified in *SF3B1* and *TP53* in 9 patients (14.8%), *BIRC3* in 5 patients (8.2%), MYD88 in 2 (5.6%) patients, and PLCG2 in 1 (1.6%) patient (Table 4) (Figures 1, 2,3).

Discussion

This is the first and largest study to characterize the genomic landscape of Indian CLL patients, along with *IGHV* mutation status. The limitation of our study is the sample size and number of genes analysed. However, we have included putative driver genes specific to CLL as a significant strength. Approximately 80% of CLL patients carry at least one of four common chromosomal

Table 1. Characteristics of the Cohort According to IGHV Mutation Status (N = 97, CLL patients)

	Patients N = 97 (%)	IGHV Hypermutated n = 36 (%)	IGHV Unmutated N= 61 (%)	P-value
Age (years)				
Median (Range)	65 (33 - 91)	65 (45 - 82)	64 (33 - 91)	
Gender				
Male	85 (87.6)	32 (88.9)	53 (86.9)	0.52
Female	12 (12.4)	4 (11.1)	8 (13.1)	
FISH result				
Normal	48 (49.5)	16 (44.4)	32 (52.5)	0.69
11q deletion	5 (5.2)	1 (2.8)	4 (6.6)	
13q deletion	9 (9.3)	5 (13.9)	4 (6.6)	
Both 11q & 13q deletion	8 (8.2)	3 (8.3)	5 (8.2)	
17p deletion	17 (17.5)	8 (22.2)	9 (14.8)	
Trisomy 12	10 (10.3)	3 (8.3)	7 (11.5)	

Table 2. IGHV Alleles Observed in the Cohort (N = 97, CLL Cases)

Allele	Number of cases	Allele	Number of cases
IGHV1		IGHV3-30*04 IGHJ6	1
IGHV1-2-02 IGHJ4	2	IGHV3-33*01-IGHJ2	1
IGHV1-2 IGHJ6	2	IGHV3-33 IGHJ4	2
IGHV1-3*01 IGHJ2	1	IGHV3-33*01-IGHJ2	1
IGHV1-3*01 IGHJ4	1	IGHV3-33*01-IGHJ6	1
IGHV1-3*01 IGHJ6	1	IGHV3-43*01 IGHJ6	1
IGHV1-24-IGHJ4	1	IGHV3-43D IGHJ4	1
IGHV1-46 IGHJ4	1	IGHV3-48*04 IGHJ2	2
IGHV1-69*01 IGHJ3	1	IGHV3-48*04 IGHJ4	1
IGHV69*04 IGHJ6	1	IGHV3-48-IGHJ6	1
IGHV1-69*01 IGHJ6	9	IGHV3-48 IGHJ4	1
IGHV1-69*06 IGHJ4	1	IGHV3-53 IGHJ4	3
IGHV1-69*01 IGHJ4	1	IGHV3-64*01 IGHJ6	4
IGHV1-69*06 IGHJ4	1	IGHV3-64*01 IGHJ6	1
IGHV2		IGHV3-64D*06 IGHJ4	1
IGHV2-5 IGHJ4	1	IGHV3-73 IGHJ5	1
IGHV2-5*01 IGHJ4	2	IGHV3-74*01 IGHJ4	3
IGHV2-05*01 IGHJ6	1	IGHV4	
IGHV2-70*01 IGHJ3	1	IGHV4-34*01 IGHJ4	3
IGHV3		IGHV4-34*01 IGHJ5	2
IGHV3-7 IGHJ6	1	IGHV4-34*01 IGHJ6	1
IGHV3-7*01 IGHJ4	1	IGHV4-39*01/07 IGHJ4	1
IGHV3-9*01 IGHJ6	3	IGHV4-39 IGHJ4	1
IGHV3-11*03 IGHJ4	3	IGHV4-39*07/*01-IGHJ3	1
IGHV3-13*01 IGHJ4	1	IGHV4-59 IGHJ3	1
IGHV3-15*01 IGHJ4	1	IGHV4-59*01 IGHJ4	2
IGHV3-20*01-IGHJ5	1	IGHV4-59*01 IGHJ6	2
IGHV3-21 IGHJ4	2	IGHV4-61 IGHJ4	1
IGHV3-21*01/04 IGHJ3	1	IGHV5	
IGHV3-21 IGHJ6	1	IGHV5-10-1 IGHJ4	1
IGHV3-23 IGHJ4	2	IGHV5-51*01 IGHJ6	1
IGHV3-23 IGHJ6	3	IGHV7	
IGHV3-23*01 IGHJ3	1	IGHV7-4-1 IGHJ4	2
IGHV3-30 IGHJ5	1	IGHV7-41*02 IGHJ4	2

Table 3. Gene Mutations Classification According to IGHV status

Genes	Patients n=97	IGHV Hypermutated n = 36 (37.1%)	IGHV Unmutated n = 61 (62.9%)	P value
ATM				
Mutated	19 (19.6)	4 (11.1)	15 (24.6)	0.11
Wild type	78 (80.4)	32 (88.9)	46 (75.4)	
BIRC3				
Mutated	9 (9.3)	4 (11.1)	5 (8.2)	0.72
Wild type	88 (90.7)	32 (88.9)	56 (91.8)	
MYD88				
Mutated	2 (2.1)	2 (5.6)	0 (0)	0.13
Wild type	95 (97.9)	34 (94.4)	61 (100)	
NOTCH1				
Mutated	13 (13.4)	1 (2.8)	12 (19.7)	0.03
Wild type	84 (86.6)	35 (97.2)	49 (80.3)	
PLCG2				
Mutated	1 (1)	0 (0)	1 (1.6)	1
Wild type	96 (99)	36 (100)	60 (98.4)	
TP53				
Mutated	15 (15.5)	6 (16.7)	9 (14.8)	0.8
Wild type	82 (84.5)	30 (83.3)	52 (85.2)	
SF3B1				
Mutated	18 (18.6)	9 (25)	9 (14.8)	0.21
Wild type	79 (81.4)	27 (75)	52 (85.2)	

alterations: deletion 13q14, deletion 11q22-23, deletion 17p12, and trisomy 12. However, in our study, the numbers do not concord, as the FISH testing was done elsewhere, and hence, numerous missing data points falsely reduce the prevalence.

IGHV mutation status is known to be an independent prognostic marker. The prevalence of mutated *IGHV* genes (defined as a <98% identity compared with the germ line nucleotide sequence) is higher among newly diagnosed and asymptomatic CLL patients (~60%). In contrast, the prevalence of unmutated *IGHV* genes (defined as ≥98% identity compared with the germ line nucleotide sequence) is higher among progressive (~50%-60%) and relapsed/refractory (~70%-80%) CLL patients [10]. However, this is discordant with this study's findings as all patients included were treatment-naive, yet the prevalence of unmutated patients was significantly higher than the hypermutated patients.

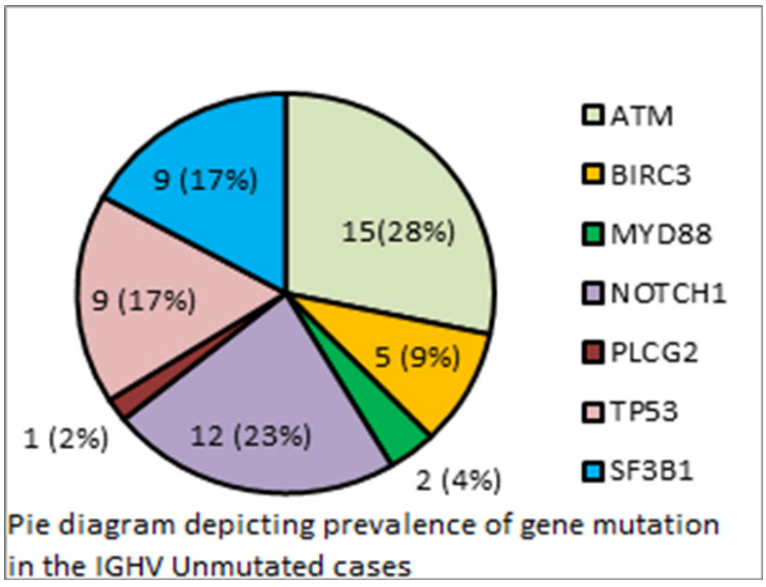

Figure 1. Pie Diagram Depicting Prevalence of Gene Mutation in the IGHV Unmutated Cases

Table 4. Gene Variants Observed in the IGHV Hypermutated and Unmutated CLL Patients (N = 97 Cases)

Hypermutated cases

ATM	*BIRC3*	*TP53*	*SF3B1*
p.Leu2767His (VUS)	p.Gln547AsnfsTer21 (2 cases)	p.Arg273His	p.Lys700Glu (4 cases)
p.Gln2730Ter	p.Gln547fs	p.Arg249Met; Arg248Trp	p.K700E;T663I
p.Leu2952Val (VUS)	p.Arg434Glnfs*12	p.Asn247Ile	p.Gly742Asp
		p?(c.673-2A>T)	
p.Ser1492Tyr	p.Lys596Glnfs*18	c.919+1G>A	p.Lys700Glu;p.Gly742Asp
	NOTCH1	p.Arg249Gly (2 cases)	p.His662Asp
	p.Pro2514Argfs*4		p.Lys666Glu

Unmutated cases

ATM	*BIRC3*	*NOTCH1*	*TP53*
p.Tyr1442fsTer5	p.Cys588Ser	c.*371A>G	p.Arg248Gln
p.Arg23*	p.Ile531Ter	p.Asn2143Alafs*104	p.Ile251ThrfsTer7
p.Cys2930Arg			
p.Val2441_Arg2443 del (VUS)	p.Leu444TyrfsTer2	p.Gln2416*	p.Arg249Gly
	p.Cys560Arg		
p.Asp841Ala	p.Arg555HisfsTer12	p.Pro2514Argfs*4	p.Gly245Ala
p.Phe2732Leu		p.Gln2444*	
p.Tyr2019Cys	p.Glu429GlyfsTer7	p.P2514Rfs*4 (5 cases)	c.772G>A
p.Ser1165Gly(VUS)		p.Gly2126Arg	p.Arg282Trp
p.Arg1150Thr		p?(c.*371A>G)	c.772G>A
p.Asp1637Gly (VUS)	*MYD88*	p.His2241Tyr (VUS)	p.Arg209Lysfs*6
p.Arg2443Gln	p.Leu252Pro (2 cases)		p.Tyr220Cys
p.Tyr2144Ter			
p.Tyr2404Ser (VUS)		*PLCG2*	*SF3B1*
p.Arg3008His		p.Arg1001His	p.His662Asp (2 cases)
p.Thr1242TyrfsTer4			p.Lys700Glu (4 cases)
c.8788T>C			p.Gly742Asp (2 cases)
p. Trp3055Arg			p.Lys666Asn

Apart from the mutation status, the usage of specific *IGHV* genes may also affect prognosis, including progression (i.e., *IGHV*3-21 in stereotyped subset 2) and transformation to Richter syndrome (*IGHV*4-39 in stereotyped subset 8). The most commonly detected allele was *IGHV*1-69*01 IGHJ6 (seen in 9 patients), whereas the 3-21 and 4-39 were reported in 3 patients each. However, information on the outcomes of these patients was unavailable during the period of this study.

The mutational landscape of Indian CLL also differs from that of the Western population. At presentation, the *TP53* gene is disrupted in 4% to 8% of unselected CLL by deletions, mutations, or a combination. In this study, 15.5% of patients harbored a *TP53* mutation at diagnosis,

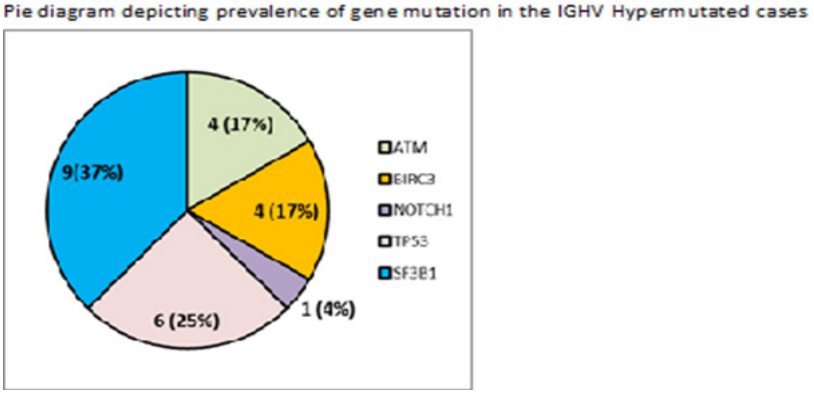

Figure 2. Pie Diagram Depicting Prevalence of Gene Mutation in the IGHV Hypermutated Cases

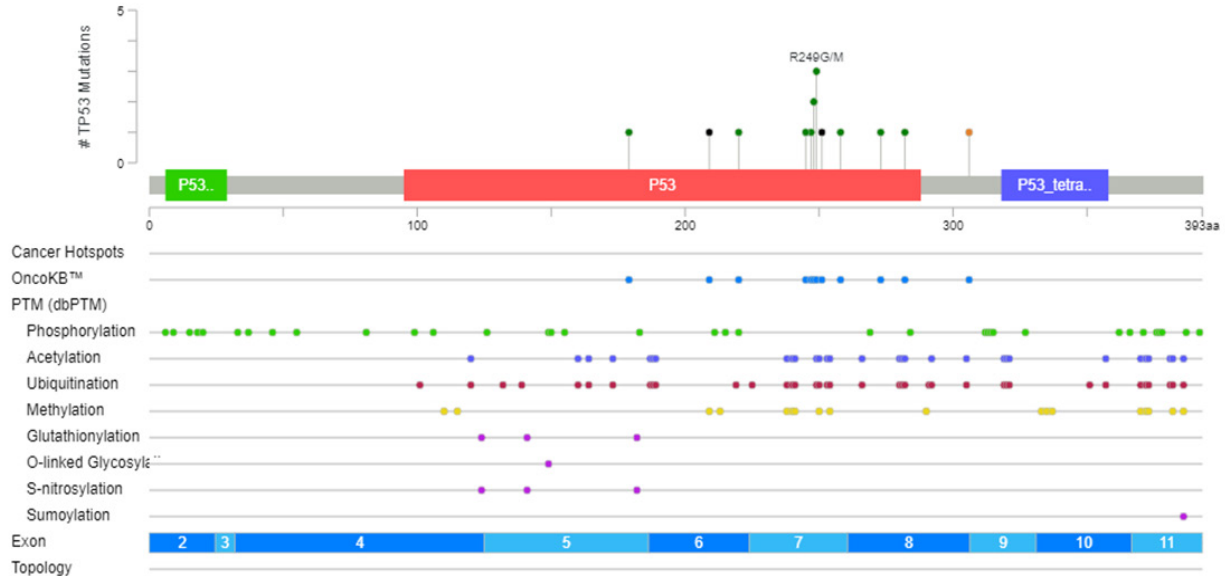

Figure 3. Lolly Plot Showing the *TP53* Mutation and It association with Various Physiologic Processes

irrespective of the mutation status. Missense mutations in *TP53* have been reported to be located in the DNA binding domain of the gene, involving exons 4-10. This is concordant with the study's findings, where, apart from 2 patients, all were missense variants.

ATM occurs in <10% of newly diagnosed CLL, whereas its prevalence rises to ~20% at the time of first treatment and 30% at disease relapse. In our study, *ATM* was detected in 19.6% of patients at diagnosis. Mutations of the *SF3B1* and *NOTCH1* genes are observed in 10% to 15% of newly diagnosed CLL, whereas their prevalence rises to ~20% in progressive or relapsing CLL. In our study, *SF3B1* & *NOTCH1* were detected in 18.6% and 13.4% of patients, respectively.

Concerning the correlation of mutation load with the *IGHV* status, it has been reported that the mean number of clonal mutations in the unmutated cohort is higher, reported to be 1.3-4.2/Mb, compared to the mutated subgroup: 1-2/Mb. The findings in our cohort support the same conclusion. However, they cannot be quantified in terms of mutation/Mb due to the small size of the NGS panel.

The differences may be attributed to geographic and racial differences, as most of these studies have been reported from the West. The prevalence and pattern of the *IGHV* stereotype are unique in Indian patients; this is possibly due to exposure to various bacterial and viral infections. This could contribute to establishing *IGHV* stereotypes and antibody class switching early in our population. The limitation of our study is the unavailability of clinical information to correlate survival status with the molecular findings because of patient recruitment of 8 cancer centres. This comprehensive study in CLL patients delineates the mutation status and molecular alterations in the treatment of naïve Indian CLL to a large extent, which has paved the way for future research into the biology of Indian CLL, which may also show differential responses to standard-of-care therapies recommended by international guidelines. This will warrant changes in policymaking for optimal patient care.

Author Contribution Statement

All authors contributed equally in this study.

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None.

References

1. Kipps TJ, Stevenson FK, Wu CJ, Croce CM, Packham G, Wierda WG, et al. Chronic lymphocytic leukaemia. Nat Rev Dis Primers. 2017;3:16096. https://doi.org/10.1038/nrdp.2016.96.
2. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005;352(8):804-15. https://doi.org/10.1056/NEJMra041720.
3. Huang YJ, Kuo MC, Chang H, Wang PN, Wu JH, Huang YM, et al. Distinct immunoglobulin heavy chain variable region gene repertoire and lower frequency of del(11q) in taiwanese patients with chronic lymphocytic leukaemia. Br J Haematol. 2019;187(1):82-92. https://doi.org/10.1111/bjh.16051.
4. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated ig v(h) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94(6):1848-54.
5. Gaidano G, Rossi D. The mutational landscape of chronic lymphocytic leukemia and its impact on prognosis and treatment. Hematology Am Soc Hematol Educ Program. 2017;2017(1):329-37. https://doi.org/10.1182/asheducation-2017.1.329.
6. Chauzeix J, Pastoret C, Donaty L, Gachard N, Fest T, Feuillard J, et al. A reduced panel of eight genes (atm, sf3b1, notch1, birc3, xpo1, myd88, tnfaip3, and tp53) as an estimator of the tumor mutational burden in chronic lymphocytic leukemia. Int J Lab Hematol. 2021;43(4):683-92. https://doi.org/10.1111/ijlh.13435.
7. Jeromin S, Weissmann S, Haferlach C, Dicker F, Bayer K, Grossmann V, et al. Sf3b1 mutations correlated to cytogenetics and mutations in notch1, fbxw7, myd88,

xpo1 and tp53 in 1160 untreated cll patients. Leukemia. 2014;28(1):108-17. https://doi.org/10.1038/leu.2013.263.
8. Stilgenbauer S, Schnaiter A, Paschka P, Zenz T, Rossi M, Döhner K, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: Results from the cll8 trial. Blood. 2014;123(21):3247-54. https://doi.org/10.1182/blood-2014-01-546150.
9. Baliakas P, Espinet B, Mellink C, Jarosova M, Athanasiadou A, Ghia P, et al. Cytogenetics in chronic lymphocytic leukemia: Eric perspectives and recommendations. Hemasphere. 2022;6(4):e707. https://doi.org/10.1097/hs9.0000000000000707.
10. Wang J, Yan Y, Xiong W, Song G, Wang Y, Zhao J, et al. Landscape of immunoglobulin heavy chain gene repertoire and its clinical relevance to lpl/wm. Blood Adv. 2022;6(13):4049-59. https://doi.org/10.1182/bloodadvances.2022007279.