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Mutational landscape of Juvenile Myelomonocytic Leukemia (JMML)—A real-world context

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Abstract

Introduction: Juvenile myelomonocytic leukemia (JMML) is a rare childhood neoplasm (<5% cases), which has been categorized under myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in the recent classification by the World Health Organization.

Methods: We developed a 51-gene (151.5kB) low-cost targeted myeloid panel based on single-molecule molecular inversion probes to comprehensively evaluate the genomic profile of Juvenile myelomonocytic leukemia (JMML).

Results: A total of 50 children with clinical and pathological features of JMML were sequenced at high coverage. Among the 50 patients, 44(88%) harbored mutations in one of the RAS/MAPK-pathway genes, most frequently in *NRAS* (32%), followed by *PTPN11* (28%) and *NF1* (22%). One-fifth of children had more than one mutation, with 5 cases harboring two RAS pathway mutations. Monosomy 7 was detected in 32% (16) patients, and five of these did not harbor any RAS pathway mutations. Children with monosomy 7 showed shorter overall survival compared with their wild-type counterparts ($P = .02$).

Conclusion: Our study highlights that comprehensive genomic profiling identifies at least one mutation in almost 90% of JMML patients. Performing genomic analysis at baseline might help in triaging children with JMML for allogenic stem cell transplant in resource-constrained settings.

KEYWORDS

dysplasia, JMML, molecular genetics, myeloid leukemia

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1 | INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare childhood neoplasm (<5% cases), which has been categorized under myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in the recent classification by the World Health Organisation.[1] It is characterized by a wide spectrum of clinical manifestations and is rapidly fatal if not appropriately treated.[2] The only current curative therapy for nonsyndromic de novo JMML patients is hematopoietic stem cell transplantation (HSCT). The clinical and pathologic factors are insufficient to predict the prognostic heterogeneity observed among JMML patients.[3]

Molecular diagnosis assumes paramount importance in the diagnosis of JMML as it lacks definite clinical features and morphological dysplasia, seen in adult MDS/MPN. Moreover, flow cytometric and karyotyping abnormalities are present in only a subset of cases.[4] It has been reported in many studies that mutations involving one of the genes in the RAS pathway (*NRAS*, *KRAS*, *c-CBL*, *PTPN11*, and *NF1)* are present in 85% of these cases.[5,6] Yet, around 15% of cases of JMML do not depict any molecular aberration in these five cardinal genes, which underscores the need for high-throughput techniques with larger panels to uncover the full mutational spectrum of JMML. Molecular evaluation could also aid in risk stratification of these patients toward hematopoietic stem cell transplant (HSCT).[7,8] This type of molecular triaging is especially relevant concerning the Indian subcontinent, where economic constraints prevail in a large section of the population.

In this study, we comprehensively evaluated the genomic profile of JMML that were referred to our hospital for diagnosis and treatment.

2 | MATERIALS AND METHODS

2.1 | Clinical Information and treatment protocol

Fifty (50) children with JMML confirmed based on WHO diagnostic criteria were included in this study. Peripheral blood/bone marrow aspirate samples of these patients procured as a part of the routine diagnostic workup were retrieved from the sample archive of our laboratory. The clinical information, other laboratory findings, and follow-up data were obtained from the electronic medical record system of the institution. For flow cytometry, samples were processed using lyse-stain-wash method with 10-color-5-tube antibody panel for acute leukemia as well as additional 10-color-3-tube panel for evaluation of myeloid, monocytic, and erythroid maturation (Table S1 and S2) The cells were acquired on a three-laser Navios instrument (Beckman Coulter, BC) with Navios acquisition software and were analyzed using a predefined template-based approach on Kaluza version-1.3 software. No extra samples or interventional procedures were undertaken or performed as a part of this study. Children with diagnosed JMML were started on workup for bone marrow transplant immediately on diagnosis. Oral combination chemotherapy including 6-mercaptopurine (6MP)(50 mg/m^2) or thioguanine (6TG) (40 mg/m^2), etoposide(50 mg/m^2) and cis-retinoic acid (100 mg/m^2) was started in the interim for symptomatic patients with the aim to halt progression/transformation till the time transplant could be done. However, patients for whom transplant did not materialize due to lack of resources/donor, the same combination chemotherapy was continued indefinitely, aiming for long-term symptom control.

2.2 | Samples

The samples used for this assay were peripheral blood/bone marrow aspirate samples, from which DNA was extracted using PureGene Gentra manual protocol (Qiagen) for DNA and automated RNA extraction using the Qiacube (Qiagen). The quality and quantity of the extracted nucleic acid were checked using Nanodrop spectrophotometry (Thermo Fischer Scientific Inc.) and Qubit Fluorometry (Thermo Fischer Scientific Inc.).

2.3 | Next-generation sequencing assay

2.3.1 | Detection of mutations using single-molecule molecular inversion probes (smMIPS)

We performed next-generation sequencing assay using a targeted gene panel comprising of 50 genes, covered in 1066 probes, based on the smMIPS principle.[9,10] The genomic footprint of the panel was 151.5 kb. The 50 genes included in this panel are known to be commonly implicated in myeloid malignancies, and based on the available data on COSMIC (Catalogue of Somatic Mutations in Cancer) and published literature, the hotspot regions in the coding regions of these genes were identified (Table S3). These 1066 probes capture the coding regions in these genes with a 5 bp flank covering the introns on either side. These 1066 probes were initially pooled in equimolar concentrations in 4 different pools, and these pools were subsequently balanced to ensure uniform coverage across the entire panel, indicating optimum capture efficiency. For the samples, 600 ng DNA was used as starting material, and after a 24-hour capture reaction, exonuclease treatment was performed, followed by amplification by polymerase chain reaction (PCR), incorporating dual sample-specific indices. After ensuring adequate amplification and quality checks at each step, a magnetic bead clean up and size selection were performed which generated sequencing ready fragments. These samples were then sequenced on an Illumina Miseq using the v2-300 cycle chemistry.

2.3.2 | Informatics and analysis of the sequenced data

The Fastq files which were generated were then demultiplexed using the MiSeq instrument demultiplexing software. Paired end assembly was performed using PEAR (v0.9.8). The assembled reads

were mapped to the human genome (build hg19) using burrows wheeler alignment software (v0.7.17), and further preprocessing was done using Picard (v2.1.1) and samtools (v1.3.1). Alignment files were further processed using different GATK (v3.8) walkers (RealignerTargetCreator, IndelRealigner, BaseRecalibrator). A. mpileup file was generated using samtools (v1.3.1). Variant calling was performed using Mutect2, Platypus (v.0.8.1) and Varscan2 (v.2.3.9) and Vardict. Variant files were annotated using annovar with population frequency databases (1000Genomes, Exome Aggregation Consortium) as well as the COSMIC database (Cosmic v83). These data were processed and collated with internal scripts. Variants were filtered by focusing on exonic regions (including splicing variants if any) followed by nonsynonymous and population frequency (<0.01) filtering. This was followed by in silico prediction using multiple tools (SIFT, PolyPhen2, CADD, PROVEAN, MutationTaster, MutationAssessor, M-CAP, FATHMM, LRT, and DANN).

2.3.3 | Statistical methods used for analysis of data

Descriptive statistics using measures of central tendency were employed to determine median and mean values. Clinical and biologic features were compared between groups using χ^2 test (for categorical variables), and Mann-Whitney *U* test (for continuous variables). The impact of an individual parameter on overall survival (OS) was evaluated using Kaplan-Meier method and compared using log rank test. Multivariate analysis was performed using the cox proportional-hazards regression analysis. Since only 5 (10%) children received allogenic stem cell transplant (ASCT) in our cohort, these children were censored at the time of receiving ASCT for survival analysis. The data were analyzed using MedCalc QC software (version 14.8.1, MedCalc).

3 | RESULTS

3.1 | Demographic, clinical, and laboratory features

We recruited fifty cases of already diagnosed JMML, who had attended our facility. The median age of our cohort was 2 years (range: 1.2-84 months), with a male preponderance; male to female ratio being 2.6:1 (36 males, 14 females). Clinically, dysmorphic facial features were observed in 2 cases, who did not harbor a syndromic *PTPN11* mutation, and 4 cases had café au lait spots on their limbs and back, and all four had *NF1* mutation, indicating a syndromic association (Table S4). The median hemoglobin concentration of the entire cohort was 8.8 g/dL (3.5-13.6 g/dL). The median platelet count was 44.5 × 10^9/L (6-227 × 10^9/L). The median peripheral blood absolute monocyte count was 2.7 × 10^9/L (1-13.2 × 10^9/L), and the median peripheral blood blast count was 5% (0%-16%). When baseline clinical and laboratory parameters were compared, children with one molecular alteration showed a trend of higher platelet count (Median 46 × 10^9/L vs 18 × 10^9/L, Mann-Whitney *P*-value = 0.07) compared to children with >1 molecular alteration (Table S5).

3.2 | Bone marrow morphology and Immunophenotype

Morphologic evaluation results of bone marrow aspirate were available in 36 cases as the others (n = 14) were diagnosed elsewhere. Flow cytometric immunophenotypic evaluation was performed in 30 cases. A representative flow cytometric evaluation study of a child depicting these abnormalities is shown in Figure 1. The detailed findings on bone marrow morphologic evaluation and flow cytometric immunophenotyping are described in Table 1.

3.3 | Next-generation sequencing

We performed targeted panel-based next-generation sequencing on fifty samples from patients diagnosed with JMML based on clinical and pathological findings. At least one molecular alteration was present in 44 (88%) of these children. Mutations in at least one of the RAS pathway genes were encountered in 43 (86%) of these patients. The most frequently mutated genes in our cohort were *NRAS* (16, 32%) and *PTPN11* (14, 28%), followed by *NF1* mutation in 22%[11] cases (Figure 2A). *KRAS* and *CBL* were mutated in 5 (10%) and 1 (2%) children. Most frequent non-RAS pathway gene to be mutated was *ASXL1* (3.6%).

Interestingly, 20%[10] of children had more than one mutation, with 5 cases harboring two RAS pathway mutations. Of these 5 patients, one patient harbored two coexistent mutations in the *NF1* gene, three patients had coexistent *PTPN11* and *NF1* mutations, and one patient had a coexistent *NF1 and NRAS* mutations. Additionally, two patients also had an *ASXL1* mutation, coexistent with *NF1* and *NRAS* mutations, respectively. Other non-RAS pathway mutations involved *ABL1*, *ATRX*, *SETBP1*, *SH2B3*, and *ZRSR2*genes. (Figure 2B) The median variant allele frequency of RAS pathway mutations detected was 40.75%. The variant allele frequencies of the mutations encountered in the cardinal genes involved in JMML are summarized in Table S6.

Monosomy 7 was detected in 32%[16] children, and six of these did not harbor any RAS pathway mutations. The six patients who did not harbor any mutations showed monosomy 7 on cytogenetics analysis by FISH and conventional karyotyping, hence meeting the WHO criteria for diagnosis of JMML. Additionally, an overview of all the mutations encountered, with number of mutations per patient, the type of mutation (missense, indel), the variant allele frequency, and co-occurrence of mutations has been depicted in Figure 2A,B.

3.4 | NRAS mutations

NRAS mutation was the most commonly encountered mutation, detected in 32% cases. These were clustered mainly in the G12, G13, and Q61 hotspots of exons 2 and 4 of the NRAS gene. Two of these cases harbored additional mutations: One child with

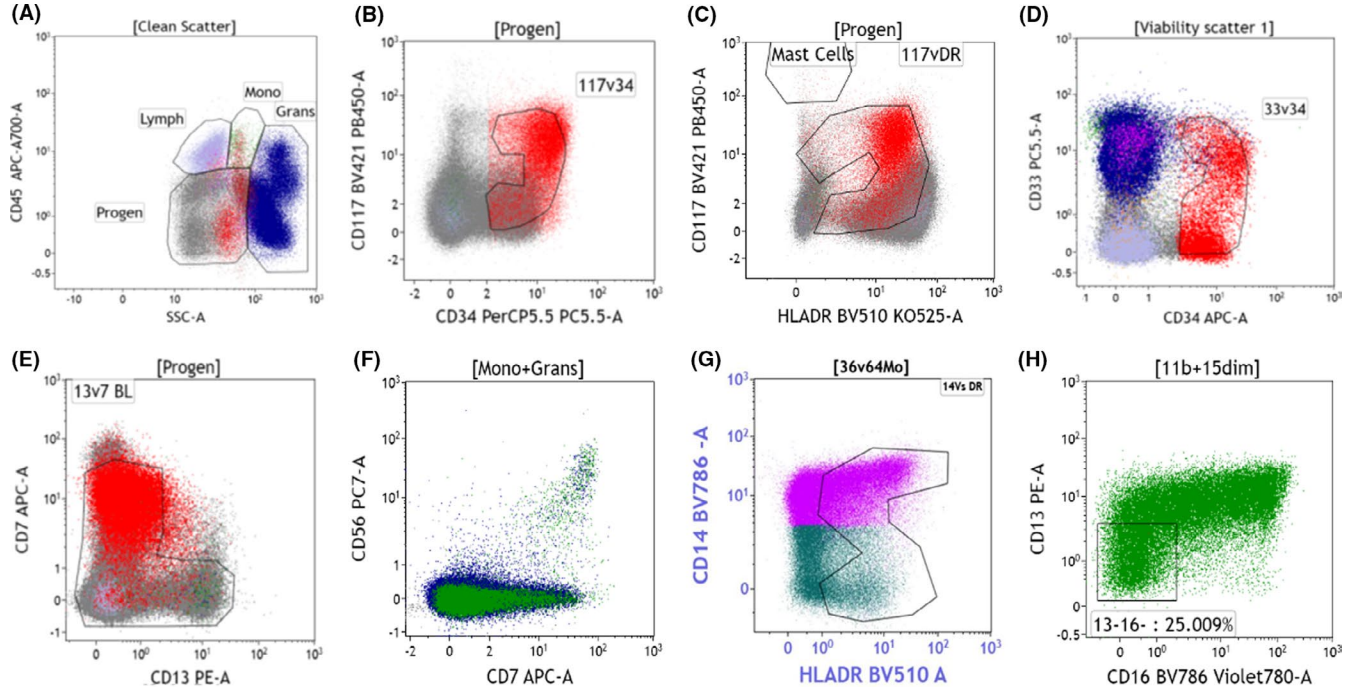

FIGURE 1 Flow cytometry plots of a representative case. A, CD45 vs SSC gating plot shows the CD45 dim blast population (colored in red). B, Progenitor gate: blasts are positive for CD 34 and CD 117. C, HLA-DR positivity in the blasts. D, CD33 in a subset of the blast population. E, Aberrant CD7 expression in the myeloid blast population. F, Aberrant CD7 expression in monocytes. G, Asynchronous maturation pattern in monocytes on CD14 vs HLA-DR plot. H, Asynchronous maturation of granulocytes with respect to CD13 vs CD16

an additional NF1 mutation, and the other with NF1 and ASXL1 mutations.

3.5 | PTPN11 mutations

PTPN11 mutation was detected in 28% cases, with most of them located in the E76,[4] A72,[3] D69,[3] and D61[2] codons. Two patients showed a duplication in the 503 codon. However, none of the patients clinically revealed any Noonan-like features.

3.6 | Follow-up and outcome

The follow-up data revealed that 34 (68%) of these patients had succumbed to the disease. This dismal outcome could be attributed to only five children receiving allogenic stem cell transplant, owing to economic constraints; and two of these five patients had succumbed to the disease. The median time period from diagnosis to death was 7.2 months in the cohort. The follow-up and outcome findings are depicted in Figure 3.

On survival analysis, none of the individual molecular mutation was associated with significant change in OS. However, children with monosomy 7 showed shorter overall survival, compared with their wild-type counterparts ($P = .018$, Hazard ratio (HR) 2.19, 95% confidence interval (CI) 1.02-4.69, median OS 6.33 months vs 8.6 months) (Figure 4). Children who harbored more than one molecular alteration did not show significant difference in overall survival as compared to children with one molecular alteration (Median OS 7.4 months vs 7.47 months, $P = .87$, HR = 1.08 95% CI = 0.41-2.86). Among the two children who succumbed to the disease despite receiving allogenic stem cell transplant, one child had coexistent *NRAS* mutation with monosomy 7, and the other child harbored multiple mutations in *PTPN11* and *NRAS*. The three children who are doing good without relapse/progression after allogenic stem cell transplant harbored mutation in *NF1*, *NF1*, and *CBL* respectively without monosomy 7. In A multivariate model that included age, hemoglobin, and PB blast% at baseline, monosomy 7 was the only parameter showing a trend toward predicting inferior overall survival ($P = .06$, HR 2.07, 95% CI 0.98-4.39). (Table S7).

Among the thirteen patients who are still on regular follow-up, two patients have relapsed with disease progression to acute myeloid leukemia and T/myeloid mixed phenotypic acute leukemia (MPAL), respectively. The patient who has progressed to acute myeloid leukemia harbored *NRAS* mutation at primary diagnosis of JMML with <20% blasts on peripheral blood and bone marrow. This patient relapsed as AML after a period of 23 months, during which he received etoposide-based chemotherapy. Transplant could not be performed owing to economic constraints. At relapse, genomics could not be performed and this child succumbed to the disease after 3 months. The other patient who relapsed as mixed phenotype acute leukemia (T/myeloid) had mutations in the *PTPN11* and *ZRSR2* genes at diagnosis. The mutation in *ZRSR2* might have been a germline polymorphism as the mother of the patient also tested positive

TABLE 1 Morphologic and immunophenotypic evaluation of children with JMML

Bone marrow aspirate morphologic evaluation (*n*-36)

Cellularity:
- 27.7% (10/36) samples showed hypercellular marrow
- 27.7% (10/36) samples showed normocellular marrow
- Rest of the samples (16/36, 44.4%) revealed dilute bone marrow aspirate

Blasts:
- Median (range) of blast count on morphology was 7.5% (2%-18%)
- 72.2% (26/36) samples showed blast count >5% of all nucleated cells

M:E ratio:
- Most of the marrows were myeloid preponderant with median M:E ratio of 6.2 (range 0.3-72)
- 58.3% (21/36) of the samples showed M:E ratio >4.5
- Erythroid hyperplasia (M:E < 1.5) was observed in 16.7% (6/36) samples

Monocytes:
- Median (range) monocyte count on bone marrow aspirate morphology was 7% (1%-26%)
- 30.6% (11/36) of bone marrow samples showed monocyte count ≥10% on morphology

Megakaryocytes:
- Megakaryocytes were adequate in 30.6% (11/36) of the samples.
- Reduced megakaryocytes were observed in 61.1% (22/36) samples.

Morphologic dyspoiesis:
- Morphologic features of dyspoiesis in erythroid, myeloid, and megakaryocytic lineages were noted in 13.9% (5/36), 24.2% (16/36), and 13.9% (5/36) of cases

Flow cytometric immunophenotyping (*n*-30)

Blasts:
- Median (range) myeloid blast percentage on flow cytometry was 3.1% (0.2%-12%)
- The myeloid blasts commonly expressed dim CD45 and moderate CD34, CD117, HLA-DR, CD13, CD33, and variable CD38
- Discernible immunophenotypic abnormalities in myeloid blasts were identified in 80% (24/30) samples
- Most common immunophenotypic aberrancies observed in abnormal myeloid blasts were:
  a. Aberrant expression of CD7 (70%, 21/30)
  b. Downregulation of CD13 (60%, 18/30)
  c. Bright overexpression of CD34 (23.3%, 7/30)
  d. Din-to-negative expression of CD38 (20%, 6/30)
  e. Downregulation of CD33 (13.3%, 4/30)
  f. Overexpression of CD11b (10%, 3/30)
  g. Co-occurrence of a CD13dimCD7+ aberrant phenotype was observed in 53.3% (16/30) of the samples

Lymphoid precursors:
- Immunophenotyping revealed proliferation of normal B-cell precursors/hematogones in 19.4% (7/36) cases
- In 3 (10%) cases, we observed <10% blasts (5.6%, 2.1%, and 5.9%, respectively) expressing a mixed T/Myeloid phenotype. These blasts expressed CD7 and variable cytoplasmic CD3 in addition to myeloid markers

Immunophenotypic evaluation of myeloid maturation:
- Immunophenotypic evidence of aberrant myeloid maturation was observed in 60% (18/30) cases
- Most common abnormalities included:
  a. Asynchronous maturation with respect to CD13 vs CD11b and CD13 vs CD16 (50%, 15/30)
  b. Low side scatter indicating hypogranularity (43.3%, 13/30)

(Continues)

TABLE 1 (Continued)

Immunophenotypic evaluation of monocytes:
- Median (range) proportion of monocytes among all viable cells on immunophenotyping was 11.4% (0.4%-40%)
- Immunophenotypic evidence of aberrant monocytic maturation was observed in 56.7% (17/30) cases
- Asynchronous maturation with respect to CD14 vs HLA-DR was the most common abnormality (267%, 8/30) in monocytic maturation

Immunophenotypic evaluation of erythroid maturation:
- Immunophenotypic evidence of abnormal erythroid maturation was observed in 10% (3/30, 2 cases showed downregulation of CD71 and 1 case showed abnormal dim expression of CD36) cases

for the same variant encountered in the patient. He relapsed after a period of 11 months, and targeted next-generation sequencing performed at relapse revealed a subclonal *TP53* mutation, and the same *ZRSR2* mutation detected at baseline. The *PTPN11* mutation was not detected at relapse.

4 | DISCUSSION

In this manuscript, we describe the genomic landscape of JMML and real-world outcomes. We describe the utility of single-molecule molecular inversion probes (smMIPS) to create a myeloid hotspot panel. As compared to amplicon-based techniques, smMIPs-based panels are a relatively low-cost library preparation choice, that is scalable and does not suffer from amplification bias.[10] In our experience, smMIPs-based workflows are also relatively simple and easily reproduced. Because of their modular nature, NGS panels can be expanded as new probes can be added to pools anytime. Although we do not demonstrate this here, smMIPS enable single-molecule consensus base calling by incorporating unique molecular barcodes permitting sensitive variant calling.[11]

Juvenile myelomonocytic leukaemia remains a rare childhood malignancy with dismal outcome, with only curative treatment being allogenic stem cell transplant. Although mutation in one of the cardinal five RAS pathway genes in JMML has been elucidated extensively,[5,6,12] the broader genetic spectrum of this disease has only recently been started to unravel.[8,13] Our cohort represents one of the largest single-center experiences and is the first of such studies from the Indian subcontinent.

The median age of our population was 2 years with 88% children harboring atleast one gene mutation and the rest had monosomy 7. Our finding of at least one RAS pathway mutation in 86% of children with JMML is similar to that reported by Stieglitz et al[8] In our cohort, *PTPN11* mutations were detected in 28% cases, of which none had any clinical features suggestive of Noonan's syndrome. It has been reported that nonsyndromic somatic mutations in *PTPN11* gene can be found in 35% cases with the most common mutations occurring in the E76 codon.[14] In our cohort, however, germline testing was not performed in all 14 cases, to confirm the somatic nature of the mutation. In six of these patients, the samples from both the

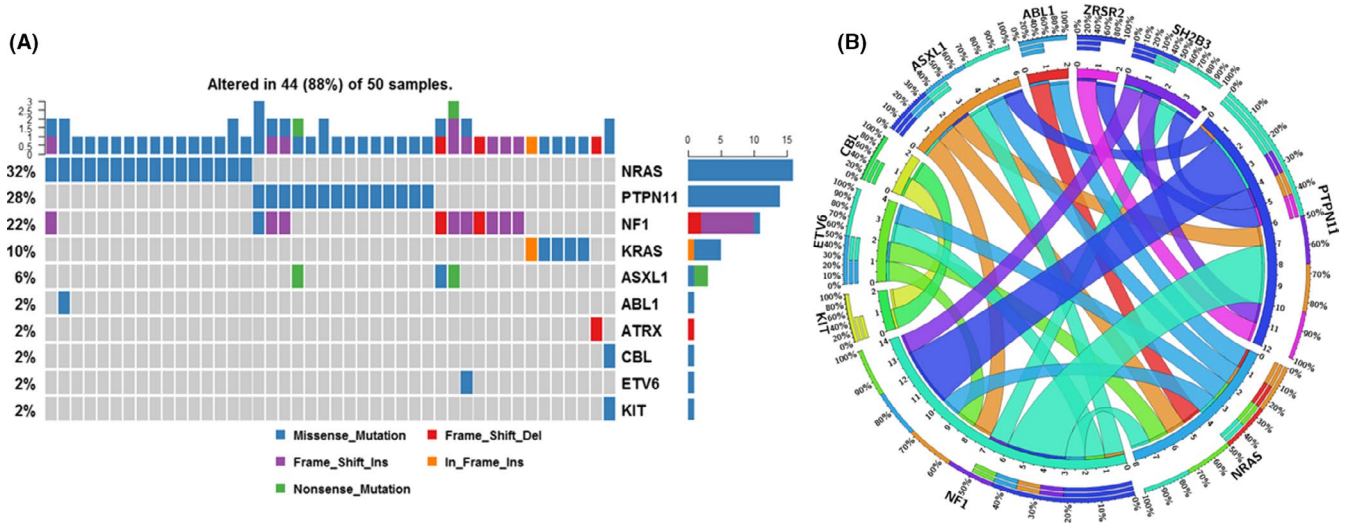

FIGURE 2 Molecular spectrum of JMML in the cohort. A, Oncoplot depicting various mutations detected on next-generation sequencing; B, Circos plot depicting co-occurring mutations

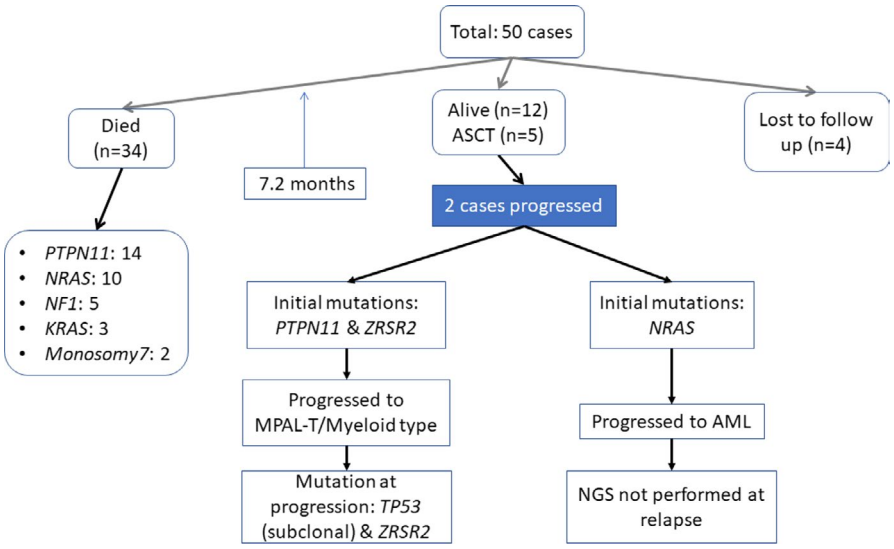

FIGURE 3 Outcome and follow-up of the children with JMML

parents of the proband were tested, which yielded negative results. Additionally, the patient who relapsed as MPAL (T/myeloid) had a *PTPN11* mutation at baseline, which was not detected at relapse, confirming that it was not present in germline.

There were 10% children who harbored more than 1 mutations, with two concurrent mutations in *NF1* and *NF1* with *PTPN11* mutations being the most common combinations. Mutations in more than one driver gene have been reported in 11% cases in literature and are associated with an overall adverse outcome.[7,8] Additionally, we found that these patients tend to be older when compared to those with 1 molecular alteration and also tend to show lower platelet count and higher blast% in peripheral blood, although these associations did not reach statistical significance in our cohort (Table S5). The plausible explanation for higher PB blast proportion in children with >1 molecular alterations could be due to the higher leukemogenic potential imparted by RAS pathway genes, which has been previously documented in AML cases.[15]

Juvenile myelomonocytic leukaemia is known to harbor germline mutations in genes including *NF1*, *PTPN11*, *CBL*, *ETV6*, *RUNX1*, and *GATA2*. However, we could not assess the germline nature of these mutations in our study owing to cost restraints and unavailability of skin fibroblast culture-based germline testing.

We also identified mutations in genes involved in gene splicing and polycomb repressive complex 2 (PRC2). One patient had a missense mutation in the *SH2B3* gene, which has been reported in 7% cases previously, with the potential of leukemic transformation.[8,16] Our case, however, had a missense exon 8 mutation which was predicted to be of uncertain significance using in silico prediction tools, and on follow-up is currently disease free for last 6 months. Two patients also harbored an additional *ASXL1*

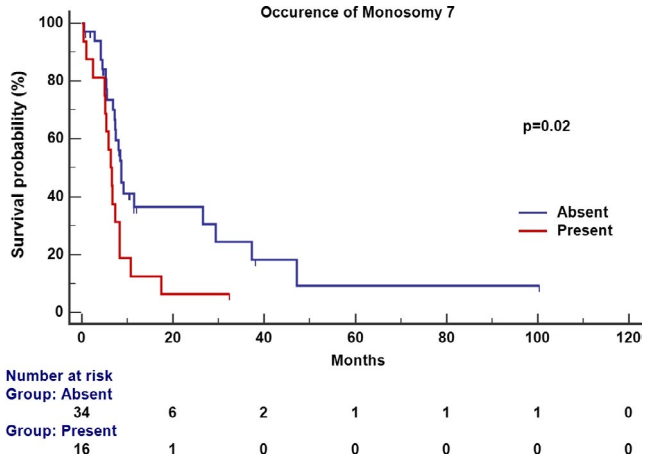

FIGURE 4 Survival analysis of children with JMML with respect to presence of monosomy 7

mutation which has also been documented in JMML patients, and known to portend an adverse outcome in these cases.[17] Spliceosome complex gene mutation involving the *ZRSR2* gene was found in only one patient, which was most likely a germline polymorphism, as it was also detected in the patient's mother. This patient later on relapsed as MPAL (T/myeloid) and retained the *ZRSR2* mutation.

A multitude of studies has started to find association with molecular aberration and outcome in children with JMML. One of the most consistent observations has been adverse outcome in children with *PTPN11* mutations, and these children generally require transplant.[18,19] More recently, children with higher number of molecular alterations have been shown to have poorer outcome.[8] Furthermore, recent studies have shown that DNA methylation studies could identify children with distinct outcome.[20] In our cohort, only monosomy 7 was associated with poorer OS ($P = .018$, HR 2.19, 95% CI 1.02-4.69, median OS 6.33 months vs 8.6 months). The lack of association of individual molecular alteration and significant change in outcome could be explained by the overall poorer outcome in our cohort, which is attributable to only a few children (5/50) receiving allogenic stem cell transplant due to economic constraints. Furthermore, we found out that two children, who succumbed to the disease even after receiving transplant, had high-risk molecular features like PTPN11 mutation, monosomy 7 and harbored more than one molecular alteration. On the contrary, three children who did not progress/relapse after transplant had single genetic lesion in our panel. These data seem to indicate that molecular triaging performed at baseline may help in selecting the children most likely to benefit in a resource-constrained setting.

This study is the largest single-center experience from Indian subcontinent and highlights broad genomic landscape of JMML using a fairly large targeted next-generation sequencing panel. Our study highlights that comprehensive genomic profiling identifies at least one mutation in almost 90% of JMML patients. Performing genomic analysis early in evaluation of JMML might help in triaging patients for allogenic stem cell transplant in resource-constrained settings.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

SN collected all the data and interpreted the NGS assay, and wrote the manuscript and performed statistical analysis of the data, **SHG** performed the wet-lab procedures for the next generation sequencing assay. **GC, PT, PGS, NP and SG** performed morphologic and flow cytometric evaluation, collected data and helped in the manuscript, **CD, SB, NM and GN** treated all the children and helped in the manuscript, **DS** performed FISH and karyotyping in order to detect chromosomal anomalies. **GC & SN** performed statistical analysis. **NP** conceptualized the study, supervised next generation assay and results, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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