Long-read sequencing resolves *TP53* allelic status in myeloid neoplasms

Determining *TP53* allelic status is essential for risk stratification in myelodysplastic neoplasms (MDS). In a cohort of 29 patients with multiple *TP53* mutations, long-read sequencing (LRS) demonstrated that monoallelic *TP53* inactivation is rare, whereas multi-hit patterns dominate. These findings support the continued use of conventional short-read sequencing (SRS) together with variant allele frequencies (VAF) analysis and cytogenetics as reliable tools in routine diagnostics.

The 5th edition of the World Health Organization (WHO) classification of myeloid neoplasms refined the terminology of MDS and emphasized the integration of genetic and morphologic criteria.[1,2] Among its newly defined entities is "MDS with biallelic *TP53* inactivation", which is also incorporated into the International Consensus Classification (ICC).[3] *TP53* mutations - hereafter referring to pathogenic/likely pathogenic variants - are among the most clinically significant genetic alterations in MDS. Although historically considered uniformly high-risk, recent evidence clearly distinguishes between monoallelic and biallelic *TP53* inactivation. Monoallelic *TP53* mutations - defined as a single mutation without concurrent deletion or copy-neutral loss of heterozygosity (cnLOH) - have been shown to confer outcomes comparable to *TP53* wild-type.[4] In contrast, biallelic *TP53* inactivation, typically resulting from multi-hit *TP53* alterations, confers adverse prognosis and poor response to allogeneic hematopoietic stem cell transplantation (allo-HSCT), the only potentially curative therapy for patients with MDS.[4]

Accordingly, identification of multiple *TP53* alterations has gained diagnostic and prognostic importance.[4-6] However, comprehensive assessment of the *TP53* allelic status remains challenging in routine laboratories, particularly in cases without deletion of 17p or cnLOH of 17p.[5,7] Standard SRS accurately detects individual mutations but cannot reliably phase variants separated by more than ~200 bp, thereby limiting its ability to distinguish monoallelic from biallelic *TP53* configurations.[8] In contrast, LRS enables phasing of variants across large genomic distances, overcoming this major limitation of conventional SRS.[9]

In this context, we applied LRS to a cohort of MDS patients harboring multiple *TP53* mutations, aiming to determine whether these mutations resided on the same allele (monoallelic) or on different alleles, indicative of multi-hit *TP53* inactivation.

The study cohort comprised 29 patients diagnosed with MDS, each carrying ≥2 *TP53* mutations identified by SRS (total mutations: N=62), with VAF ranging from 1% to 53% (Figure 1; *Online Supplementary Figure S1*). SRS panels used in the study provide full coverage of all coding exons, including ±3 bp of the flanking intronic regions of *TP53*. Patients were recruited from three hematology units in Denmark, and the study has been approved by the Regional Committee on Health Research Ethics (Ethics Commission: H-24080399).

Genomic DNA was obtained from two possible sources: mononuclear cells isolated from bone marrow or peripheral blood by density gradient centrifugation, or from whole bone marrow or peripheral blood samples. For each patient, DNA was extracted from only one source using standard protocols. DNA input for polymerase chain reaction (PCR) amplification was 100 ng (Figure 2; *Online Supplementary Table S1*).

PCR amplification of the full *TP53* coding region (exons 2-11; 7,640 bp) was performed using LongAmp® Taq 2X Master Mix. PCR products were verified by gel electrophoresis prior to library preparation according to the Native Barcoding Kit 96 V14 (SQKNBD114.96) protocol. Sequencing was conducted on Oxford Nanopore platforms. MinKNOW software (version 23.04.5) served as the device control interface, and a Phred quality threshold of 10 was applied to ensure data integrity.

Bioinformatic processing included alignment of reads to the hg38 reference genome, sorting and indexing with Samtools, and retention of full-length reads defined by coverage across both ends of the target region. Reads shorter than 7,300 nt were filtered out to remove PCR artefacts. Allele-specific analysis was performed using heterozygous single nucleotide polymorphism (SNP) to phase mutations and sort sequencing reads by individual variants, enabling distinction between monoallelic and multi-hit inactivation events. Variant interpretation utilized Integrative Genomics Viewer (IGV), with NM_000546.6 as the reference transcript.

All 62 *TP53* mutations initially detected by SRS were identified by LRS, demonstrating concordance between the two methods. Phasing of long reads enabled direct determination of allelic configuration. Among the 29 samples, only one (3.4%) exhibited monoallelic *TP53* inactivation, whereas the remaining 28 (96.6%) showed multi-hit configurations with mutations located on different alleles.

According to Bernard *et al.*[4] and the 5th WHO classification,[1] cumulative SRS-derived VAF exceeding 50% are consistent with biallelic *TP53* inactivation. Fourteen of the 28 multi-hit cases exhibited cumulative VAF above 50%, strongly suggesting that mutations co-occur within the same cellular population, despite inherent statistical uncertainty in VAF-based estimations. In the remaining

14 multi-hit cases, cumulative VAF remained below 50%, meaning that biallelic inactivation could not be definitively confirmed. These samples may therefore represent either true biallelic events or independent subclones carrying distinct *TP53* mutations, a distinction not resolvable using bulk sequencing alone. Although lower cumulative VAF reduces - but does not eliminate - the likelihood of subclonal mosaicism, definitive allelic resolution would require single-cell sequencing, which was beyond the scope of this study.[10-12] However, given that multi-hit *TP53* cases with cumulative VAF above 50% imply involvement of both alleles within the same clone, the predominance of transallelic configurations suggests that similar allelic patterns may extend to cases with lower VAF, even when formal WHO criteria for biallelic inactivation are not met.

Figure 3 provides an overview of *TP53* allelic configurations in the cohort. The upper panel summarizes allelic configurations among the 29 patients, while the lower panels show representative IGV screenshots illustrating two patterns of *TP53* inactivation: monoallelic mutations at low VAF and multi-hit mutations at higher VAF.

Using long-read phasing across the full *TP53* coding region, we demonstrate that *TP53* mutations in MDS are rarely located on the same allele, with multi-hit configurations representing the predominant allelic architecture. By enabling phasing across the entire *TP53* coding region, LRS provides insight into allelic configurations that cannot be resolved by conventional SRS. This is particularly relevant for *TP53*, where pathogenic variants may be distributed across distant exons, that lie beyond the phasing capability of standard diagnostic approaches.

While *TP53* mutations predominantly cluster within exons 5-8, corresponding to the DNA-binding domain, mutations outside this region have also been reported.[7,13] Unlike pre-

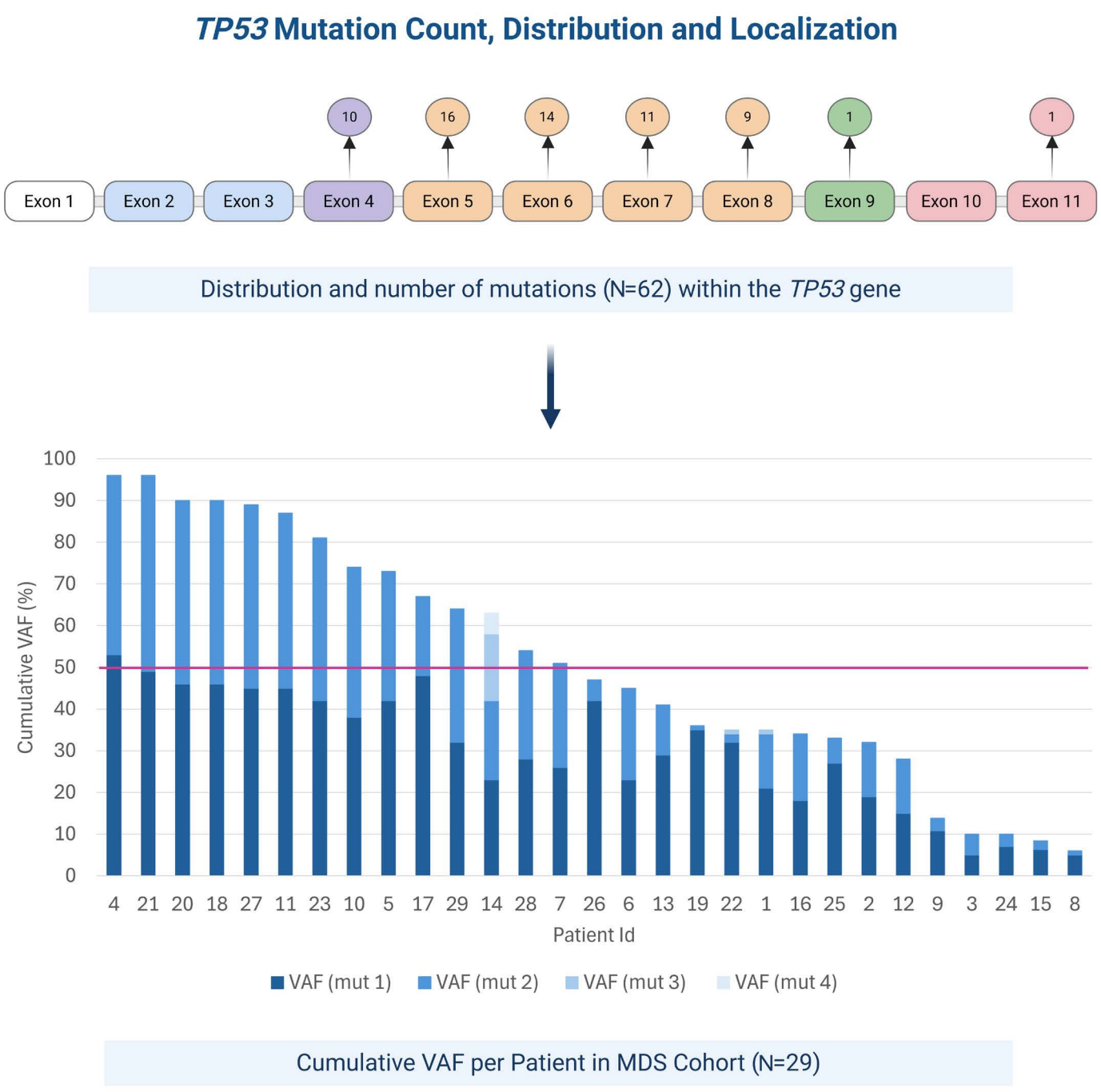


Figure 1. *TP53* mutation landscape in the study cohort. Data from 29 patients with myelodysplastic neoplasms (MDS) harboring 62 *TP53* mutations identified by short-read sequencing (SRS) are summarized. The upper panel shows the distribution and number of mutations across the *TP53* gene. Exons are depicted as colored blocks, with colors indicating functional domains. The lower panel displays the cumulative variant allele frequencies (VAF) per patient, previously determined using SRS. mut: mutated. Created in BioRender. https://BioRender.com/2ulsm0y.

vious studies that focused on short genomic intervals (e.g., 5-76 bp between mutations) or restricted analysis to the DNA-binding domain,[8,14] our approach encompassed the entire *TP53* coding sequence (exon 2-11), enabling a more comprehensive assessment. Of the 62 mutations identified, 12 were located outside the DNA-binding domain; notably, eight of these would have been misclassified as single-hit cases if only exons 5-8 been analyzed and one sample would have been classified as wild-type since both mutations were located in exon 4 (Id 29).

Given the poor prognosis associated with biallelic *TP53* inactivation, particularly in patients undergoing allo-HSCT, accurate assessment of allelic status is essential for risk stratification and treatment planning. As cytogenetic data were not included in this study, we cannot exclude the possibility that the one monoallelic case could represent biallelic inactivation if accompanied by del(17p) or cnLOH. However, as the primary aim of the study was to resolve allelic configuration by phasing, the absence of cytogenetic profiling does not affect our main findings.

Our findings indicate that *TP53* mutations in MDS are rarely located on the same allele - even when mutations

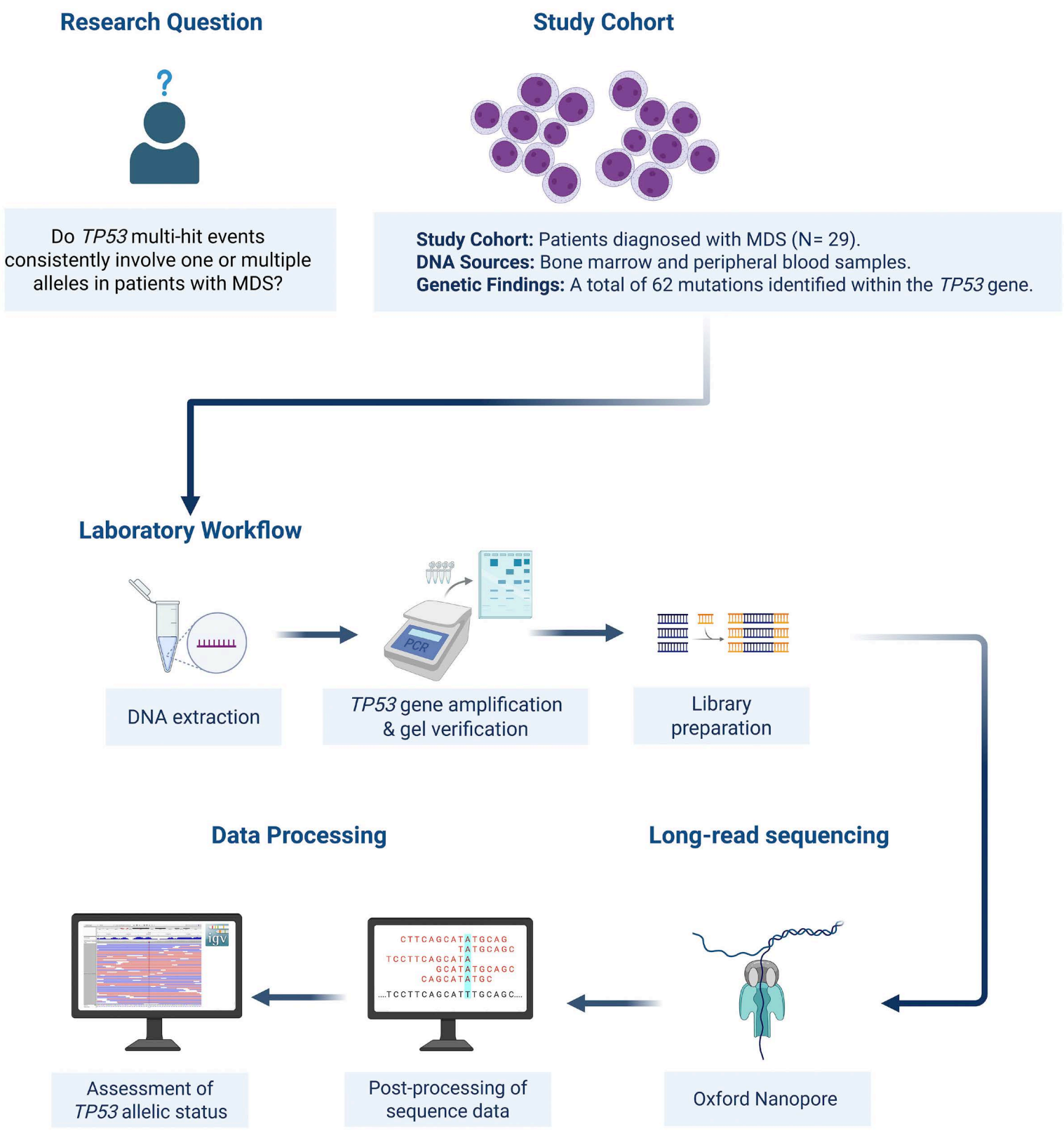


Figure 2. Study workflow. The figure outlines the experimental design. Twenty-nine patients with myelodysplastic neoplasms (MDS) and multiple *TP53* mutations (N=62) were analyzed using long-read sequencing (Oxford Nanopore platforms). The workflow included DNA extraction, *TP53* amplification, library preparation, sequencing, and alignment to the hg38 reference genome. Allelic status was determined by phasing full-length reads to distinguish monoallelic from multi-hit configurations using Integrative Genomics Viewer. Created in BioRender. https://BioRender.com/0gt5jxl.

are distributed across distant exons. The ability to phase mutations across long genomic distances provides novel insights into *TP53* biology and underscores the clinical relevance of allelic status.

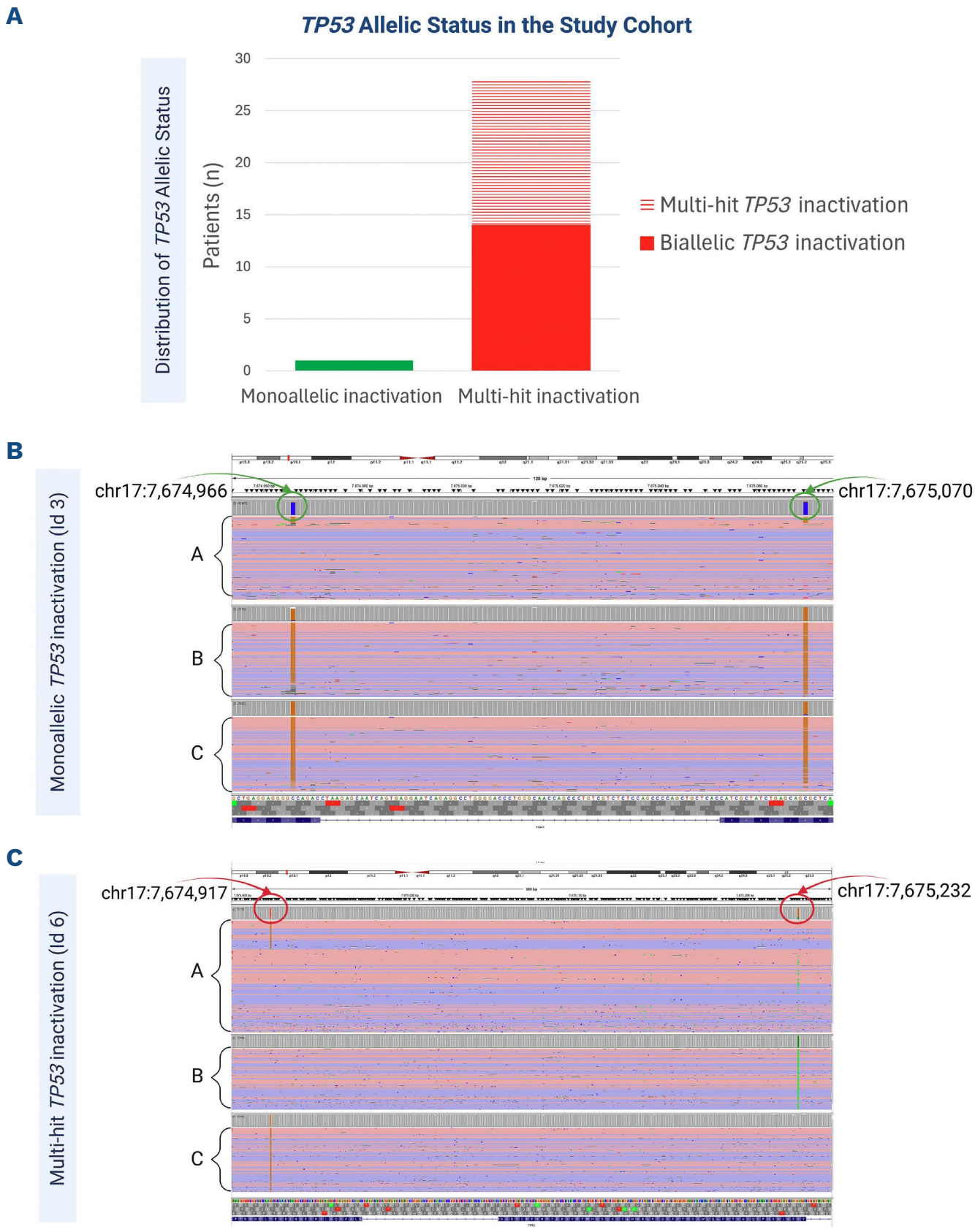


Figure 3. *TP53* allelic status in myelodysplastic neoplasms. The upper panel shows the distribution of *TP53* allelic configurations among 29 patients with myelodysplastic neoplasms. One patient (3.4%) exhibited monoallelic *TP53* inactivation, whereas the remaining 28 patients (96.6%) were classified as multi-hit, harboring *TP53* mutations on different alleles. According to Bernard *et al.*[4] and the 5th World Health Organization classification,[1] biallelic *TP53* inactivation was defined as cumulative variant allele frequencies (VAF) above 50% based on short-read sequencing. For the remaining 14 multi-hit samples, biallelic inactivation could not be excluded; these cases may represent either biallelic events or multiple independent clones carrying distinct *TP53* mutations. The lower panels show representative Integrative Genomics Viewer visualizations of two *TP53* inactivation patterns: monoallelic (Id 3: NM_000546.6: c.542G>C, p.(Arg181Pro), VAF: 5%, NM_000546.6:c.565G>C, p.(Ala189Pro), VAF: 5%) and multi-hit (Id 6: NM_000546.6: c.380C>T, p.(Ser127Phe), VAF: 23%, NM_000546.6: c.614A>C, p.(His205Pro VAF: 22%). Allelic status was determined by phasing full-length reads to distinguish mutations on the same allele from those on different alleles. Monoallelic inactivation (Id 3) involved 2 low-VAF mutations in the bulk sample, whereas multi-hit inactivation (Id 6) comprised 2 distinct mutations on separate alleles. NM_000546.6, hg38. (A) Total number of reads sorted by the first mutation. (B) Allele-specific analysis including BAM files filtered to include only reads harboring the second mutation. (C) Allele-specific analysis including BAM files filtered to include only reads harboring the first mutation. Created in BioRender. https://BioRender.com/mue1iac.

MDS cases with two or more *TP53* mutations are rare, which makes our cohort size limited. Nevertheless, a consistent pattern emerges, as 28 of the included 29 MDS cases exhibited multi-hit *TP53* mutations. Additional studies with larger cohorts will be required to support our findings.

Although current guidelines emphasize the importance of assessing *TP53* allelic status, this is, to our knowledge, not routinely implemented in most diagnostic laboratories, despite evidence demonstrating its clinical relevance. Our results indicate that routine diagnostic workflows do not necessarily require advanced sequencing technologies for accurate determination of *TP53* allelic status. Standard next-generation sequencing approaches, combining VAF assessment with cytogenetic profiling, appear sufficient to identify patients with biallelic *TP53* inactivation, supporting classification under the 5th WHO edition and informing allo-HSCT decision-making. Nevertheless, as demonstrated here, two *TP53* mutations may, in rare cases, reside on the same allele, justifying the use of LRS in selected patients.

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Disclosures

No conflicts of interest to disclose.

Contributions

MA and JC conceived and designed the study. MZ and LC developed the study concept, conducted the experiments, analyzed the data, and drafted the initial manuscript. LK, VS, and LG provided essential biological samples and scientific input to the study. UB and MH carried out bioinformatic data processing. All authors contributed to the revision and editing of the manuscript and approved the final submitted version.

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Data-sharing statement

The data supporting the findings of this study are available from the corresponding authors upon request, subject to ethical and legal restrictions.

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