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Long-read sequencing and the boundary conditions for multi-hit reassurance in *TP53*-mutated myelodysplastic syndrome. Comment on: “Long-read sequencing resolves *TP53* allelic status in myeloid neoplasms”

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Contributions

Kok-Siong Poon conceived the letter, performed the literature and figure review and wrote the manuscript.

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The report by Zeuthen and colleagues, demonstrating that long-read sequencing (LRS) confirms trans (biallelic) *TP53* configuration in 28 of 29 (96.6%) myelodysplastic syndrome (MDS) patients with two or more *TP53* mutations detected by short-read sequencing (SRS),¹ together with the accompanying editorial by Mittelman and Shlush,² offers the first direct empirical test of a concern about *TP53* allelic classification that has largely remained theoretical: how often does SRS-based “multi-hit” calling misrepresent the true allelic configuration underlying *TP53* inactivation, the entity now formally recognized in the WHO fifth edition and International Consensus Classification of myeloid neoplasms.^{3,4} Their finding that only 1 patient (3.4%) harboured a cis, monoallelic configuration is genuinely reassuring, and the editorial’s conclusion that routine SRS combined with variant allele frequency (VAF) and cytogenetic assessment remains adequate for most patients is a pragmatic message diagnostic laboratories will welcome. Several observations within the same dataset, however, highlight a more circumscribed reading of the “back to square one” conclusion than the editorial’s framing suggests.

First, the study’s own mutation compendium is itself evidence for a distinct, coverage-related blind spot that the phasing result does not resolve. Of the 62 *TP53* mutations identified, 12 (19%) fell outside the canonical DNA-binding domain (exons 5-8); 8 of these would have been misclassified as single-hit, and one patient would have been called wild-type entirely, had analysis been restricted to exons 5-8.¹ This is an assay-design problem independent of phasing, and it is precisely the limitation of routine clinical panels that restrict *TP53* sequencing to hotspot exons. The reassurance that SRS-detected multi-hit calls are usually true biallelic events presupposes gene-wide coverage of exons 2-11 in the first place, consistent with current diagnostic guidance⁵ and as achieved in this study.¹ A laboratory using a hotspot-restricted panel cannot extend this reassurance to its own results.

Second, and more fundamentally, this coverage gap raises doubt about the sensitivity of hotspot-restricted SRS itself, not only about mis-phasing calls already made. A patient reported as single-hit on a panel limited to exons 5-8 has not had a true negative result for a second variant because roughly 40% of the coding sequence was never interrogated. Zeuthen et al.'s own data illustrate the scale of this effect: on a hotspot-only panel, 8 of their patients with genuine multi-hit disease would have been called single-hit and one wild-type.¹ If this rate generalises, the widely cited estimate that roughly one-third of *TP53*-mutated MDS is monoallelic may itself be inflated by assay blind spots rather than true biology with direct prognostic consequences given that single-hit and biallelic disease are managed differently.⁸ Zeuthen et al.'s cohort cannot test this directly, since patients were enrolled only after two or more mutations were already identified on a full-coverage panel¹ while no published cohort has systematically re-sequenced hotspot-defined single-hit cases gene-wide to check how many are secretly biallelic. This supports reflexing apparent single-hit cases where management hinges on the distinction to gene-wide sequencing with long-read phasing performed in the same step, since the missed-variant and phasing problems are distinct limitations that share a common fix.

Third, variant interpretation itself is a determinant of allelic status that the paper leaves largely unexamined. The authors restrict their analysis to variants they describe as pathogenic or likely pathogenic but do not report whether classification followed a standardized tiering framework, such as the joint AMP/ASCO/CAP consensus recommendation,⁶ nor how a case would have been handled in which one of two co-occurring alterations was a variant of uncertain significance (VUS) rather than a confirmed pathogenic variant. Whether a VUS is counted toward multi-hit status, excluded pending orthogonal evidence, or flagged descriptively directly determines whether a patient is reported as monoallelic or biallelic, independent of whether phasing itself is technically resolved. Current curation frameworks

increasingly incorporate VAF pattern as its own line of evidence precisely because bulk VAF alone cannot separate a genuine second hit from an unrelated subclone or an unclassified variant.⁷ Without an explicit variant-classification standard, the reassuring multi-hit ratio reported here cannot be assumed to generalize to cohorts in which VUS-containing cases are not first filtered out.

Fourth, sequencing depth and analytical sensitivity for low-VAF variants are not reported. Cohort VAFs ranged from 1% to 53%,¹ yet the manuscript states no mean or per-amplicon read depth for LRS, nor a formal limit of detection on the Nanopore platform used. The reported 62-of-62 concordance¹ is inherently one-directional: it confirms LRS did not miss variants already known from SRS, not that LRS independently detects or phases low-VAF variants without prior knowledge of their position. Variant read depth, not only qualitative concordance should be reported.

Fifth, the “square one” conclusion applies unevenly within the multi-hit group itself with only 14 of 28 multi-hit cases (50%) had cumulative VAF above 50% with this threshold conventionally taken as supportive of biallelic inactivation.⁸ For the remaining 14, biallelic inactivation could not be confirmed and subclonal mosaicism could not be excluded without single-cell sequencing,¹ leaving half of this cohort’s multi-hit designations as ambiguous as before phasing was attempted.

Finally, the single monoallelic (cis) case was not assessed for del(17p) or copy-neutral loss of heterozygosity since no cytogenetic data were collected.¹ Hence, a monoallelic call by sequencing alone cannot exclude biallelic inactivation occurring at the structural level.^{5,9} This reinforces the editorial’s own caveat that LRS or targeted cytogenetic testing should be reserved for patients in whom the VAF is ambiguous or in whom multi-hit status will materially alter management rather than treated as dispensable across the board.²

Zeuthen et al.'s data are a valuable reality check as they sharpen rather than close the phasing question with specifying the boundary conditions, full gene-wide coverage, standardised variant classification, adequate sequencing depth and high cumulative VAF, under which their reassurance holds.¹ Laboratories should treat these as prerequisites rather than options, reflex apparent single-hit cases to gene-wide sequencing where management depends on the distinction and reserve confirmatory phasing for the ambiguous, clinically consequential minority these data help define.

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