Title: Expanding the reach of paired DNA and RNA sequencing: Results from 450,000 consecutive individuals from a hereditary cancer cohort

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Paired DNA and RNA sequencing has shown promise in improving detection and interpretation of DNA variants identified across a variety of clinical indications. Expansion of genes eligible for RNA sequencing, adjustments to assay design, increases in the number of cases and controls tested, and refinement of guidelines for application of RNA evidence can all contribute to improved utility of RNA sequencing. Here we report outcomes of paired DNA and RNA sequencing in light of these improvements among 450,000 individuals undergoing hereditary cancer multigene panel testing (MGPT).

Results of MGPT including concurrent DNA and RNA sequencing performed between April 2019-December 2023 were retrospectively reviewed. The positive rate calculated includes pathogenic and likely pathogenic variants (PVs) and excludes monoallelic variants in genes associated with recessive conditions and moderate risk PVs (*APC* p.I1307K and *CHEK2* p.I157T). Variant classifications were compared before and after application of RNA evidence to calculate impact on positive yield. Medically significant upgrades were defined as those resulting from a newly detected intronic PV and reclassifications from uncertain significance to PV. Genes with no RNA-related reclassifications were further evaluated for rationale.

A total of 455,378 cases were included in this study. The overall positive rate was 9.9%, consisting of 46,027 PVs identified in 45,202 individuals. Medically significant upgrades based on RNA evidence were made to 1,838 variants, resulting in a 4.2% relative increase in positive yield. Among genes with at least 100 PVs reported, the greatest impact on positive yield due to RNA was observed in *LZTR1* (130 of 809 PVs; 19.1% relative increase in yield), *RAD51C* (80 of 691; 13.1%), *CDH1* (21 of 258; 8.9%), *ATM* (388 of 4,865; 8.7%), *APC* (57 of 780; 7.9%), and *PTEN* (19 of 277; 7.4%). Reclassifications based on RNA evidence were made in 53 of the 85 genes evaluated. Reasons for lack of RNA reclassifications included: loss of function via haploinsufficiency was not the established mechanism for disease (27 of 32 genes; 84.4%), extreme rarity of disorder (<0.01% of tested individuals) (4 of 32; 12.5%) and technical limitations (1 of 32; 3.1%).

In this study, on average, 1 in 25 pathogenic variants were dependent on RNA evidence. Discovering genes in which RNA sequencing is especially impactful and identifying parameters when RNA evidence is not applicable can provide useful insights to aid

providers in risk assessment and test selection. The impact of RNA sequencing may continue to grow as its adoption becomes more widespread and its applications are validated.