Title: Characterization of *RPS20*-related colorectal cancer predisposition: a case series from a multigene panel testing cohort

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Introduction: Current established genes associated with adult-onset hereditary CRC include *APC, BMPR1A, MLH1, MSH2, MSH6, PMS2, EPCAM, MUTYH, PTEN, SMAD4, STK11, TP53, POLD1, POLE, NTHL1* (biallelic*), MSH3* (biallelic), *MBD4* (biallelic), and *AXIN2*, but these genes explain less than 20% of hereditary CRC. Despite more comprehensive sequencing technologies, few new genes associated with hereditary CRC have been uncovered. In 2014, Nieminen, *et al*. proposed a new gene associated with highly penetrant, early-onset CRC, *RPS20*, which encodes a ribosomal protein. Including this initial report, only 5 families have been published in the literature. Due to the rarity of published cases, this gene-disease relationship has remained uncharacterized with Limited gene-disease validity, such that no variants can be classified above a variant of uncertain significance (VUS) per the Clinical Genome Resource guidelines. We present a case series of 38 unrelated individuals with predicted loss-of-function variants (pLOF) in *RPS20* (premature truncations, initiation codon, canonical splice site variants), characterizing this gene-disease relationship for highly penetrant CRC predisposition.

Methods: Published reports of families and probands with *RPS20* variants were reviewed and recorded. Additionally, we retrospectively reviewed all hereditary cancer (HC) multigene panel test (MGPT) orders (up to 85 genes) with a pLOF in *RPS20* between March 2017 and July 2024. pLOF were confirmed heterozygous and included individuals referred for diverse cancer indications, reducing ascertainment bias from tumor-specific MGPT orders. Proband histories were obtained via test requisition forms and clinical documents submitted to our laboratory. Prevalence of tumors was compared to a wild-type (WT) MGPT cohort of 19,810 individuals with no pathogenic (P) or likely pathogenic (LP) variants identified in 85 HC genes using Fisher's exact test. Published and internal data were evaluated using an evidence-based, gene-disease validity (GDV) framework.

Results: Thirty-eight individuals were confirmed heterozygous for pLOF in *RPS20*, 31 (81.6%) of whom had at least one diagnosis of cancer, and 10 (2.6%) with multiple primary diagnoses. Of all cancer diagnoses (n=43), 34 (79.1%) were CRC, 5 (11.6%) breast, 1 (2.3%) anal, 1 (2.3%) appendiceal, 1 (2.3%) bladder, and 1 (2.3%) basal cell. Six (15.8%) individuals had 2 primary CRCs, and 1 (2.6%) had 3 primary CRCs. The median age of CRC diagnosis was 50.5 years (range 21-67). Nine individuals (23.7%) reported polyps, count ranging from 1 to greater than 50. Among the 5 patients with reported polyp pathology, 100% (5/5) reported adenomatous polyps. Of the 15 patients with reported mismatch repair (MMR) immunohistochemistry (IHC) tumor testing, 12 (80%) had normal MMR/IHC. All but one (37/38, 97.4%) individual were negative for additional LP/P via MGPT. One individual (female, CRC 51y) was heterozygous for an *ATM* pathogenic variant, a gene with limited evidence for CRC predisposition. Compared to the WT MGPT cohort, the prevalence of CRC in the *RPS20* pLOF cohort was significantly increased (3.9% versus 68.4%; OR 52.8; 95% CI [25.6-115.2]; p-value 7.7×10^{-28}). Scoring internal laboratory data with published case reports resulted in a GDV score of Moderate for *RPS20*-related CRC predisposition.

Conclusions: *RPS20* is associated with rare, highly penetrant MMR-proficient CRC predisposition caused by heterozygous pLOF. The median age of CRC diagnosis in *RPS20* pLOF heterozygotes is significantly lower than the general population age of diagnosis of 66y, and comparison to a WT MGPT cohort revealed a dramatically higher prevalence of CRC (53-fold) in individuals with *RPS20* pLOF. Notably, over 18% (7/38) of the *RPS20* cohort includes individuals with multiple primary CRC diagnoses. Polyps were reported in nearly a quarter (23.9%) of individuals and may be part of the *RPS20*-related cancer predisposition spectrum. Sequencing of this gene is warranted in individuals with a significant personal and/or family history of early-onset CRC and previously negative MGPT to guide clinical management.