

## DATA REPORT

A deleterious *RNF43* germline mutation in a severely affected serrated polyposis kindred

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We report a germline nonsense mutation within the extracellular domain of the RING finger ubiquitin ligase *RNF43*, segregating with a severe form of serrated polyposis within a kindred. The finding provides evidence that inherited *RNF43* mutations define a familial cancer syndrome.

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The serrated polyposis syndrome comprises multiple epithelial polyps in the colon and rectum of serrated histology. WHO clinical criteria<sup>1</sup> are the presence of >20 serrated polyps throughout the colon, or >5 proximal to the rectum. Serrated polyps, particularly large sessile polyps in the proximal colon, frequently exhibit the oncogenic V600E mutation together with hypermethylation of the mismatch repair protein *MLH1* and are responsible for 15–20% of sporadic colorectal cancer (CRC).<sup>2</sup>

The serrated polyposis syndrome is associated with CRC risk. Serrated polyposis shows familial clustering,<sup>3</sup> and first and second-degree relatives of index cases with serrated polyposis without CRC are more likely to have been diagnosed with CRC or pancreatic cancer.<sup>4</sup> The risk of CRC in relatives is higher in those cases diagnosed below the age of 50 years.<sup>4</sup> A small number of serrated polyposis patients harbor dominant germline mutations in mismatch repair proteins or biallelic *MUTYH* mutations, however, when these patients are excluded the familial risk of CRC remains<sup>4</sup> and the genetic basis for familial serrated polyposis has not been established. The appearance of serrated polyposis in consanguineous kindreds and in monozygotic twins<sup>5</sup> has led to the hypothesis that serrated polyposis may be due in part to recessive or codominant mutations.<sup>5</sup>

As serrated polyposis is a relatively newly described condition, its natural history is not known and the lifetime risk of CRC in serrated polyposis is also not known. Significantly, it is also not known whether those first and second-degree relatives of serrated polyposis cases, who had a history of CRC or pancreatic cancer, themselves had serrated polyposis.<sup>4</sup> If so, then a dominant mode of inheritance in at least a subset of serrated polyposis is likely. If not, however, serrated polyposis could be the result of several codominant alleles.

We identified a severely affected kindred with serrated polyposis. The proband developed microsatellite instability (MSI)-CRC age 23 years arising from a serrated polyp, in the setting of multiple (>50) large serrated polyps throughout the colon; one sibling has multiple serrated polyps and another a single large adenoma. Their mother developed pancreatic cancer and died before this study at age 50 years. On the paternal side,

an informative pedigree had no history of multiple polyposis or CRC in the father's generation. Full sequencing of the *MUTYH* gene in the proband did not reveal any abnormality.

We performed exon capture and deep sequencing in this kindred to identify strong and potentially interacting cancer alleles.

## EXOME SEQUENCING

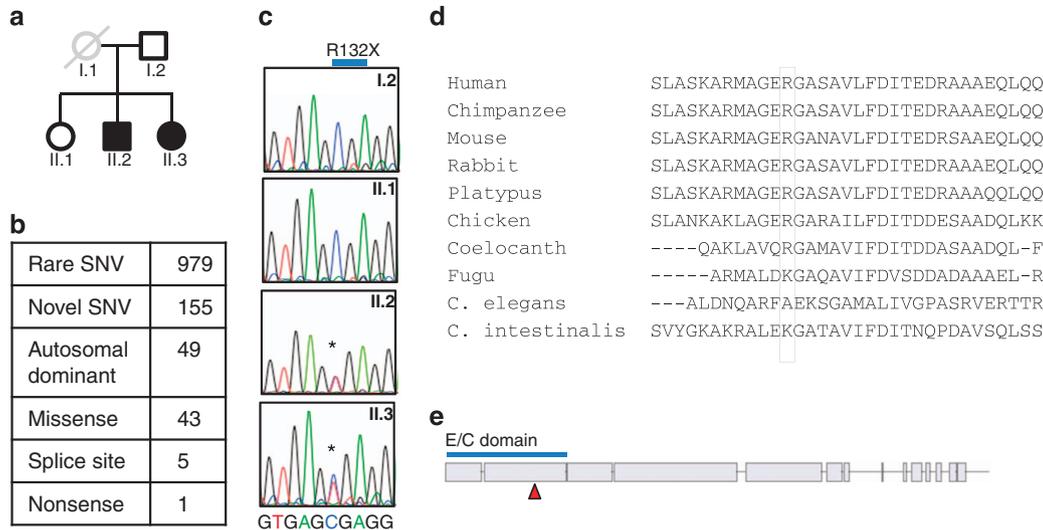
DNA library preparation and exome enrichment was performed at the Australian Phenomics Facility, Australian National University. Input DNA was extracted from saliva, analyzed for integrity then fragmented by mechanical shearing (Covaris AFA). The Agilent XT2 Human, all exon, V5.0 kit and referenced reagents (Agilent, Santa Clara, CA, USA) were utilized for DNA library preparation and exome enrichment. Libraries were indexed and pooled in batches of six before capture. Enriched libraries were sequenced as paired end reads (100 bp runs) on an Illumina HiSeq 2000 at the Australian Cancer Research Foundation (ACRF) Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University.

## BIOINFORMATICS

Sequence reads were mapped to the GRCh37 assembly of the reference human genome using the default parameters of the Burrows–Wheeler Aligner.<sup>6</sup> Untrimmed reads were aligned allowing a maximum of two seed mismatches with repetitively aligned reads discarded. Sequence variants were identified with SAMtools<sup>7</sup> and classified as novel, rare (mean allele frequency/MAF ≤ 0.02), or common (MAF > 0.02). Variants were overlapped to ENSEMBL v75 exons and splice site coordinates and annotated using the Ensembl Variant Effect Predictor (VEP)<sup>8</sup> to obtain PolyPhen2<sup>9</sup> and SIFT<sup>10</sup> scores for estimating the effect of amino acid substitutions on protein structure and function. Deleterious SNVs in cancer genes were assessed against the COSMIC database and assigned a value. Variant analysis of sequenced pedigrees

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**Figure 1.** (a) Pedigree with closed symbols, affected; open symbols, unaffected; gray symbol, deceased. (b) Summary of combined results from whole exome capture and sequencing of genomic DNA from subjects I.2, II.1, II.2 and II.3. Mutations were deemed to be rare (MAF < 0.02) or novel in reference to dbSNP. The number of missense, splice site and nonsense single-nucleotide variants (SNV) are shown assuming II.2 and II.3 are affected, and I.2 and II.1 are unaffected. (c) Sanger sequencing of genomic DNA from extant members of the kindred. (d) Conservation of region encompassing arginine converted to stop codon by nonsense mutation. (e) Summary of RNF43 gene structure. Mutated residue is indicated (red triangle) located within the extracellular domain.

(pVAAS)<sup>11</sup> was performed treating subject 002 as ‘unaffected’ or ‘affected’.

#### VALIDATION OF CANDIDATE DELETERIOUS MUTATIONS

Genotyping for the specific mutations in the probands and relatives were performed by exon-specific PCR and Sanger sequencing.

Subjects gave written informed consent for the study, which received approval from the ACT Health Research Ethics Committee.

#### RESULTS

The pedigree of the affected kindred is shown in Figure 1a. The proband II.2, a male nonsmoker of 23, underwent emergency right hemicolectomy for an obstructing CRC and multiple synchronous polyps were palpated at operation. The primary tumor was staged T4N0MX. Immunohistochemistry for the mismatch repair genes was normal. The somatic KRAS codon 12 and 13 mutation panel (COSMIC ID: 516, 517, 518, 520, 521, 522 and 532) was negative. Sequencing of the *MUTYH* gene in the germline was normal. A female sibling II.3, age 27 years, underwent screening colonoscopy where >20 large serrated polyps were identified. After endoscopic failure to achieve endoscopic clearance of polyps the subject underwent elective subtotal colectomy; >60 polyps were present in the resection specimen. A third sibling II.1, age 21 years, had a single adenoma of the rectum; a second colonoscopy at 1 year was normal.

We performed whole-exome capture and sequencing (median depth for four exomes was 99×). Variant analysis of sequenced pedigrees was performed treating subject II.1 as ‘unaffected’ or ‘affected’. Rare and novel variant analysis identified 49 variants that conformed to an autosomal dominant inheritance pattern. Of these, 9 were novel variants and 40 were rare (MAF < 0.02), and only one was a nonsense variant, which was a novel (R132X) mutation in *RNF43* (Figure 1b). This allele was found in subjects II.2 and II.3. Separately, 42 rare or novel variants were identified irrespective of inheritance pattern. Of these, 20 nonsense mutations were identified of which only the previously identified missense *RNF43* mutation was segregated with the serrated

polyposis phenotype in the pedigree. We confirmed the *RNF43* nonsense mutation by Sanger sequencing (Figure 1c).

The mutant allele detected in subjects II.2 and II.3 was present on the minus strand, chromosome 17q22 coordinates 56431037 to 56494931 and represent a C>T transversion at cDNA position 2350 within exon 2 (g.54014C>T). The residue is highly conserved in mammals and birds (Figure 1d), and is located within a well-conserved region that encodes the extracellular protein domain. As a result of this premature stop codon, mutant mRNA is predicted to undergo nonsense mediated decay.

*RNF43* encodes a transmembrane RING finger ubiquitin ligase. Amongst its substrates are Wnt agonist frizzled receptors, which are targeted to the lysosome for degradation;<sup>12</sup> a mutation in the RING finger domain that inactivated E3 ligase activity increased Wnt signaling.<sup>13</sup>

Complete Sanger sequencing of *RNF43* in each extant member of the pedigree revealed one additional coding variant g.56656C>T in II.3. This missense substitution encodes P231L, and is not predicted to be damaging. Twenty-three instances of homozygosity for this variant have been reported in the TGP database. No somatic mutations replicating this variant have been reported in cancer samples.

We did not determine expression of either the mutant allele or the paternal allele in neoplastic tissue in subjects II.2 or II.3. We expect little or no expression of the mutant allele owing to nonsense mediated decay. As a candidate tumor suppressor gene, *RNF43* may be subject to allelic loss or somatic inactivation of the wild-type allele, which is a question for further scrutiny.

*RNF43* germline mutations (encoding R113X) were amongst several germ line mutations reported recently in 2 of 20 unrelated sporadic serrated polyposis cases.<sup>14</sup> Recently, Giannakis *et al.*<sup>15</sup> described somatic mutations in *RNF43* occurring in 18.9% of 185 CRC cases, 17.6% of an independent cohort of 222 CRC cases and 18.1% of 248 endometrial cancer. The majority of *RNF43* somatic mutations reported were truncating events (including three instances of R132X), and were strongly associated with MSI-positive cancers and negatively associated with *APC* mutations, leading Giannakis *et al.* to propose that mismatch repair deficiency leads to a permissive environment for the acquisition of *RNF43* mutations. Taken together with the prior report of Gala *et al.*, our data indicate that *RNF43* truncations may instead be pathogenic in

the serrated polyposis-cancer sequence, which is independent of the canonical APC mutant adenomatous polyp and in which MSI, when it occurs, arises owing to somatic methylation of the *MLH1* gene.<sup>16</sup> Furthermore, our report of evidence of heterozygous *RNF43* mutations segregating with serrated polyposis within a kindred raises the possibility that this represents a new familial cancer syndrome.

## HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at <http://dx.doi.org/10.6084/m9.figshare.hgv.582>.

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## AUTHOR CONTRIBUTIONS

DT and MC designed the research. DT, MC, BW, YZ, LM, DA, MF and WL performed the research. DR contributed research participants and clinical information. DT, MC, DA, MF and CG were responsible for bioinformatics analysis. DT and MC wrote the manuscript.

## COMPETING INTERESTS

The authors declare no conflict of interest.

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