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***RNF43* is mutated less frequently in Lynch Syndrome compared with sporadic microsatellite unstable colorectal cancers**

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Abstract

The WNT signaling pathway is commonly altered during colorectal cancer development. The E3 ubiquitin ligase, *RNF43*, negatively regulates the WNT signal through increased ubiquitination and subsequent degradation of the Frizzled receptor. *RNF43* has recently been reported to harbor frequent truncating frameshift mutations in sporadic microsatellite unstable (MSI) colorectal cancers. This study assesses the relative frequency of *RNF43* mutations in hereditary colorectal cancers arising in the setting of Lynch syndrome. The entire coding region of *RNF43* was Sanger sequenced in 24 colorectal cancers from 23 patients who either (i) carried a germline mutation in one of the DNA mismatch repair genes (*MLH1*, *MSH6*, *MSH2*, *PMS2*), or (ii) showed immunohistochemical loss of expression of one or more of the DNA mismatch repair proteins, was *BRAF* wild type at V600E, were under 60 years of age at diagnosis, and demonstrated no promoter region methylation for *MLH1* in tumor DNA. A validation cohort of 44 colorectal cancers from mismatch repair germline mutation carriers from the Australasian Colorectal Cancer Family Registry (ACCFR) were sequenced for the most common truncating mutation hotspots (X117 and X659). *RNF43* mutations were found in 9 of 24 (37.5%) Lynch syndrome colorectal

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

cancers. The majority of mutations were frameshift deletions in the G659 G7 repeat tract (29%); 2 cancers (2/24, 8%) from the one patient harbored frameshift mutations at codon R117 (C6 repeat tract) within exon 3. In the ACCFR validation cohort, *RNF43* hotspot mutations were identified in 19/44 (43.2%) of samples, which was not significantly different to the initial series. The proportion of mutant *RNF43* in Lynch syndrome related colorectal cancers is significantly lower than the previously reported mutation rate found in sporadic MSI colorectal cancers. These findings identify further genetic differences between sporadic and hereditary colorectal cancers. This may be because Lynch Syndrome cancers commonly arise in colorectal adenomas already bearing the *APC* mutation, whereas sporadic microsatellite unstable colorectal cancers arise from serrated polyps typically lacking *APC* mutation, decreasing the selection pressure on other WNT signaling related loci in Lynch syndrome.

Keywords

RNF43; Lynch syndrome; MSI; Microsatellite instability; Colorectal cancer; HNPCC

Introduction

The WNT signaling pathway is frequently altered during colorectal carcinogenesis [1]. The E3 ubiquitin ligase, *RNF43*, acts as a negative regulator of the WNT pathway by inducing ubiquitin-mediated endocytosis of cell surface Frizzled receptors [2]. Recently, *RNF43* has been proposed as a key mutational target of microsatellite unstable (MSI) sporadic colorectal cancers [1, 3, 4].

Microsatellite instability refers to the high frequency of mutations within microsatellite (repetitive) regions of the genome [5], which develops as a result of impairment of a DNA mismatch repair gene [6]. Mutations driven by MSI occur most frequently in non-coding genomic regions but may also target coding repeat tracts in key tumor suppressor genes. Such mutations in key tumor suppressor genes including *TGFRBII* [7] contribute to tumor progression.

Lynch syndrome is the most common cause of hereditary colorectal cancer resulting from germline mutations in one of the DNA mismatch repair genes, predominantly in *MLH1* or *MSH2*, but also in the binding partners *MSH6* and *PMS2* [8].

Recent studies have shown that microsatellite instability arising in a sporadic setting is significantly associated with somatic mutation of the *RNF43* gene [3, 4, 9]. *RNF43* is a ubiquitin ligase that dampens the WNT signal. It achieves this through ubiquitination, inducing the endocytosis and subsequent degradation of the Frizzled receptor on the cell surface [2]. Inhibition of *RNF43* has been implicated in the increase in expression of Frizzled receptors on the cell membrane and resultant increased WNT signal. In sporadic MSI colorectal cancer, frameshift events have been shown to occur in repeat tracks of *RNF43*, primarily a single nucleotide deletion in the G7 repeat tract occurring in codon 659 within exon 9 [3]. We have previously identified a high rate of G7 repeat tract mutations (87%) in sporadic MSI colorectal cancers [3] compared with only 4% in microsatellite stable colorectal cancers [3].

In this study, we assessed the frequency of somatic mutational events within *RNF43* in two cohorts of Lynch syndrome colorectal cancers and compared this with previously reported *RNF43* somatic mutation frequency from sporadic MSI colorectal cancers.

Methods

Patient samples

The discovery cohort was comprised of 24 Lynch syndrome related colorectal cancer samples collected from patients at the time of surgical resection at the Royal Brisbane and Women's Hospital, Australia. Tumor samples were snap-frozen in liquid nitrogen and DNA was extracted using salt precipitation [10]. The validation cohort was comprised of 44 colorectal cancers from germline mismatch repair gene mutation carriers recruited to the Australasian Colorectal Cancer Family Registry (ACCFR). Details of ACCFR recruitment, tumor collection and mutation testing have been described in detail previously (Newcomb et al. 2007 CEBP) and are available at <http://coloncfr.org>. The study protocol was approved by the Human Research Ethics Committees of the Royal Brisbane and Women's Hospital, QIMR Berghofer Medical Research Institute and the University of Melbourne.

Sample selection

Cancers were included in this study if they met the following criteria: (i) carriers of a germline mutation in one of the DNA mismatch repair genes (*MLH1*, *MSH6*, *MSH2*, *PMS2*), or (ii) showed immunohistochemical loss of expression of one or more of the four DNA mismatch repair proteins, was *BRAF* wild type at V600E, patient age <60 years at colorectal cancer diagnosis, demonstrated no promoter region methylation for *MLH1* in tumor DNA, and were determined to have MSI. This was assessed using the National Cancer Institute's 5 marker panel [5] where MSI was classified if at least one mononucleotide and at least one other marker was positive [11].

Sequence analysis

Tumor DNA was amplified by PCR (See Table 1 for primer sequences). The primers were designed using Primer3 BLAST in conjunction with sequence data obtained from the CHIP SNPPER [12] online tool. Separate primers were designed for the ACCFR validation cohort to flank only the exon 3 and 9 hotspot (Table 1). PCR conditions are available on request. The PCR products were then treated with the ExoSapIT (Affymetrix, OH, USA) reagent as per the manufacturer's protocol and sequenced on an ABI3100 Capillary sequencer (Applied Biosystems, USA) (Table 1).

Software and statistical analysis

Statistical analysis was performed using GraphPad Prism 7 with a student's *t* test used for categorical data analysis. P values of <0.05 were considered statistically significant.

Results

Clinical and molecular features of Cohort

The discovery cohort (Brisbane cohort) consisted of 10 female and 14 male colorectal cancer-affected patients. The mean age of colorectal cancer diagnosis for patients was 48.40 ± 11.52 years. DNA mismatch repair immunohistochemistry results were available for 14 tumors. Loss of immunohistochemical staining was most frequently observed for the *MLH1* (6/14, 43%) and *MSH2* (5/14, 36%) proteins. Fifteen samples had a documented germline mismatch repair mutation. No tumors had a *BRAF*^{V600E} mutation, whilst all tumors were microsatellite unstable (Table 2). The ACCFR validation cohort was comprised of colorectal cancers from 18 female and 26 male participants confirmed to carry a germline DNA mismatch repair gene mutation. The mean age of colorectal cancer diagnosis was 50.1 ± 12.26 years. DNA mismatch repair immunohistochemistry results were available for all patients in the cohort. The most common germline defects were in the *MLH1* (18/44, 41%), and *MSH2* (17/44, 39%) genes (Table 3). All patients in the validation cohort were wild-type at *BRAF*^{V600E}.

Lynch syndrome colorectal cancers harbor moderate rates of *RNF43* frameshift mutations

In the discovery cohort, we identified 9 (37.5%) *RNF43* somatic mutations, all of which were frameshift events. Seven mutations (7/24, 29.1%) occurred at the hotspot G659 within exon 9 involving frameshift deletions of the G7 repeat tract. (Table 2; Fig. 1). The remaining two mutations (8.3%) occurred at R117 within exon 3 involving a frameshift within a C6 repeat tract. This occurred in two synchronous cancers from the same patient. In order to rule out a germline mutation we sequenced matched normal mucosal DNA, where we did not observe the R117 frameshift mutation. No sample contained more than one mutation. We sought to confirm the mutation rate of these two hotspot truncating events at X659 and X117 in a validation cohort (ACCFR Cohort) comprised of 44 colorectal cancers with confirmed germline mismatch repair gene mutations. Overall, *RNF43* was mutated in 19/44 samples (43.2%) where X659 hotspot mutations were identified in 16 tumors (16/44, 36.3%), and X117 mutations in 3 samples (3/44, 6.8%) (Table 3). No instances of concurrent X117 and X659 mutations in either cohort were observed. We found no statistically significant relationship between clinical data and mutation status of *RNF43*. Similarly, we found no evidence of an association between the specific mismatch repair gene deficiency and *RNF43* mutational frequency.

Lynch syndrome colorectal cancers have significantly lower *RNF43* mutation rates than sporadic colorectal cancers

We compared results from this study to previously obtained *RNF43* mutational data from sporadic colorectal cancer patients [3, 9]. Bond et al. reported *RNF43* somatic mutations in 47/54 (87.0%) sporadic MSI colorectal cancers; 43 (80%) of mutations identified were the X659 hotspot mutation and 6 (11%) were the X117 mutation [3], while Yan et al. reported truncating *RNF43* mutations in 17/20 (85%) sporadic MSI colorectal cancers in the initial cohort, and 30/39 (77%) sporadic MSI colorectal cancers in the TCGA validation cohort. (Fig. 2) [9]. We compared the rate of *RNF43* hotspot truncating mutations in our Lynch syndrome colorectal cancer cohorts to both Yan et al. and Bond et al. The combined hotspot

mutation rate in the initial and validation cohort was significantly lower in the Lynch syndrome related colorectal cancers compared with both Bond et al. and Yan et al. (Initial cohort: $p < 0.0001$ versus Bond et al. and $p < 0.001$ versus both Yan et al. cohorts; Validation cohort: $p < 0.0001$ versus Bond et al. and $p = 0.0024$, $p = 0.034$ for Yan et al. Hong Kong cohort, and TCGA cohorts, respectively). Therefore, hereditary Lynch syndrome MSI colorectal cancers harbor significantly fewer *RNF43* mutations than sporadic MSI-H colorectal cancers.

Discussion

Microsatellite instability (MSI) is a key characteristic of both hereditary and sporadic mismatch repair deficient colorectal cancers. Due to previous reports of sporadic MSI cancers harboring frequent *RNF43* frameshift mutations, we wanted to determine if hereditary Lynch syndrome related MSI cancers were similar in this respect. Previous literature has identified *RNF43* as mutated between 70 and 80% of sporadic MSI-H colorectal cancers [3, 4], our findings show that *RNF43* mutations occurred in around 40% of the Lynch syndrome related colorectal cancers, which was significantly lower than for sporadic MSI-H colorectal cancer. Our results revealed that despite the shared MSI phenotype, Lynch syndrome and sporadic MSI cancers may possess different mutational target genes. This is in line with previous research that showed a variety of factors determine which genes are targeted by MSI colorectal cancers [13]. Pinheiro et al. noted that hereditary MSI cancers mutated gene sets with a different frequency dependent on the location of the tumor. It may be that selective pressures on tumors deriving from germline or sporadic origins may drive particular targeted mutational profiles.

Data from this study is concordant with known molecular characteristics of MSI colorectal cancers. Wild type *RNF43* reduces membrane surface expression of Frizzled receptors and therefore acts as a negative regulator of downstream WNT signaling. Lynch syndrome related colorectal cancer have previously been shown to activate the WNT pathway to a greater degree than sporadic MSI carcinomas [14], typically via downstream pathogenic *CNN1B1* mutations, and to a lesser extent, *APC* mutation [15–17]. Loss of APC mutations occur in large proportions of conventional adenomas [14, 17], which are the precursor lesions to malignant Lynch syndrome colorectal cancers. In contrast, sporadic MSI colorectal cancer typically arise from the serrated polyps, which harbor high proportions of oncogenic *BRAF* mutations, namely *BRAF*^{V600E} and lower frequencies of *APC* mutations. Native APC regulates the WNT signaling pathway through degradation of β -catenin by forming a co-degradation complex with GSK3 and AXIN [18]. Pathogenic *APC* mutations prevent the complex from degrading β -catenin efficiently which then accumulates in the cell nucleus. Loss of APC as seen in Lynch syndrome cancers could reduce deleterious selection pressures at the *RNF43* locus and this is further supported by the mutational exclusivity shown between *RNF43* and *APC* [4].

Yan and colleagues noted in organoid studies that induction of the X659 frameshift mutation resulted in a phenotype of decreased dependence on R-spondin-1 and increased WNT dependence [9]. This is in line with the previous prediction of the deleterious nature of *RNF43* mutations noted by Giannakins et al. [4]. The current study adds further evidence

supporting this hypothesis. The selective nature of the mutations seen between the hereditary MSI and sporadic MSI cohorts indicates the high likelihood of a biological advantage to *RNF43* mutations in sporadic MSI colorectal cancer.

Inhibition of the WNT signaling pathway has proved challenging in cancer with mutated APC, however upstream inhibition of porcupine has shown some promise in a subsection of colorectal cancers. LGK974 is a small molecular inhibitor that targets Porcupine and results in a decreased WNT ligand secretion. LGK974 inhibits Porcupine and as a result reduces the level of WNT ligand secretion [19]. Reduction of WNT ligand secretion reduces Frizzled-WNT binding capacity and therefore results in a decrease in canonical WNT signaling. Inhibition of WNT ligand secretion can increase differentiation and decrease proliferation in WNT dependent cell lines [19]. Porcupine inhibitors have been shown to be exclusively effective against *RNF43* mutant cell lines [3, 20]. Cancers with deficient *RNF43* are unable to ubiquitinate and subsequently degrade surface Frizzled receptors thereby allowing the WNT pathway to continuously signal. As the majority of sporadic MSI colorectal cancers harbor the mutant *RNF43* genotype required for sensitivity, LGK974 may be an appropriate targeted therapy for this cancer type and is currently undergoing stage I/II safety and efficacy studies [21, 22]. However, we propose that hereditary Lynch syndrome MSI colorectal cancers should not be assumed to be sensitive to LGK974 or other porcupine inhibitors based solely on their MSI status, given they display a significantly lower rate of *RNF43* mutations than sporadic MSI colorectal carcinomas.

Here, we report that *RNF43* is less frequently mutated in hereditary versus sporadic MSI colorectal cancers. It is possible that differences in mutation preference is a result of selective pressures acting differentially on loci in the setting of sporadic versus Lynch syndrome colorectal cancer. Further, our study indicated that hereditary MSI colorectal cancers face significantly less selective pressure for *RNF43* inactivation, possibly due to pre-existing downstream mutation of other WNT pathway members, as previously reported in the literature [17]. Lastly, Porcupine inhibition may play a reduced role in Lynch syndrome colorectal cancer treatment as they have a lower mutation rate and hence are less likely to be sensitive to this therapeutic approach.

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Fig. 1.

Example of *RNF43* Sanger sequencing at codon G659. *Left* wild type *RNF43* codon G659 (G7 repeat tract) exon 9 (reverse sequence); *Right* mutant *RNF43* showing a frame shift at G659 from a one base pair deletion of G7 tract (reverse sequence). Sequence data figured from screenshot of finchTV sequence analysis software

Fig. 2. Combined mutation rate (%) at hotspots X117 and X659 from Lynch syndrome cohorts and previously reported sporadic microsatellite unstable colorectal cancers [3, 9]. *Asterisk* Indicates significance below 0.05 versus all sporadic cohorts

Table 1

Primers used for polymerase chain reaction and sanger sequencing (5'–3')

Exon	Forward primer sequence 5'–3'	Reverse primer sequence 5'–3'
2	GAGAAAGGAAGGGCCAAAAA	GAGAAAGGAAGGGCCAAAAA
3	CCAGCATCCTGAAGTGACAA	GGAAACATGGGGACAAGAGA
4	TTAGACCCAGTGACCCCTTG	TCAGCGTCATTACCCAGAT
5	CATCACTGAGGATCGAGCTG	TGTAGGTGAGGCCCAAAGAC
6	TAATCCCCTTGACAGTTCC	ACAGACGCTGGGGTATTTTG
7	TGAGTGACTGGGAACGGAGT	CAGGACACATGGGAAACAGA
8	TGATGCCAAAGCTCATACCA	GTGGCAGTTCTGCTTTCTCC
9A	CCAGAGCACAGGCAGCTTAG	GGGTGCTGTGAGGTGGATTG
9B	CTGGTCCCTTCTGCCATC	GGTCACACTAGGCTGCATGT
9C	ACTCTGTGGTCAACTGCACG	CACTGTGGGTTAGAGAGCCG
9D	CCTATTCCTCGGACACAGCC	AACTCCATTCAGAAGGCCCC
9E	CTGCTACCAGAAACCCAGG	GATACGCTGTCCCGATGGTT
R117	AGGTATATGGCTCTCACAGCTCACT	GGTGAAGCTCCGGGTGTGTGTA
X659	CCAGTACCAGCAGTCTGTTCAACTT	TGGGGACCAAGGATATGCCACACT

Table 2

Clinicopathological data from the Brisbane cohort

Sample	Age	Gender	Site	IHC	Germline mutation	MSI	MLH1 methylation	RNF43 mutation
1	40.6	Male	Rectum	NA ¹	Confirmed	High	Negative	<i>Wild-type</i>
2	44.3	Female	Rectum	NA ¹	Confirmed	High	Negative	X659
3	59	Female	Proximal	NA ¹	Confirmed	High	Negative	X117
4	59	Female	Proximal	NA ¹	Confirmed	High	Negative	X117
5	50.6	Male	NA ^a	MSH2	Confirmed	High	Negative	<i>Wild-type</i>
6	59.3	Male	Rectum	MLH1	NA ^a	High	Negative	X659
7	65.1	Female	Proximal	MSH6	NA ^a	High	Negative	X659
8	54.4	Male	Distal	MLH1	NA ^a	High	Negative	<i>Wild-type</i>
9	31.7	Female	Proximal colon	MSH2	Confirmed	High	Negative	<i>Wild-type</i>
10	34.8	Female	NA ^a	NA ¹	Confirmed	High	Negative	<i>Wild-type</i>
11	33.3	Male	Proximal	MLH1	Confirmed	High	Negative	<i>Wild-type</i>
12	39.9	Male	Proximal	MLH1	Confirmed	High	Negative	X659
13	50.0	Male	Proximal	MSH2	NA ^a	High	Negative	<i>Wild-type</i>
14	51.9	Male	Proximal	MLH1	NA ^a	High	NA ^a	<i>Wild-type</i>
15	43.8	Male	Distal	MLH1	Confirmed	High	Negative	<i>Wild-type</i>
16	63.2	Male	NA ^a	PMS2	Confirmed	High	Negative	<i>Wild-type</i>
17	62.1	Female	Proximal	MSH2	Confirmed	High	Negative	X659
18	25.0	Female	NA ^a	PMS2	Confirmed	High	Negative	<i>Wild-type</i>
19	58.5	Male	Proximal	NA	NA ^a	High	NA ^a	<i>Wild-type</i>
20	55.5	Male	NA ^a	NA ¹	NA ^a	High	Negative	<i>Wild-type</i>
21	60.3	Female	NA ^a	MSH2	Confirmed	High	Negative	<i>Wild-type</i>
22	38.8	Male	Rectum	NA ¹	NA ^a	High	Negative	<i>Wild-type</i>
23	43.8	Male	Rectum	NA ¹	NA ^a	High	Negative	X659
24	36.1	Female	Proximal	NA ¹	Confirmed	High	Negative	X659

^aNA data was not available at time of publication

Table 3

Clinicopathological data from the Australasian colorectal cancer family register cohort

Sample ID	Age	Gender	IHC summary	MSI	Germline defect	RNF43 exon 3 (C6)	RNF43 exon 9 (G7)	RNF43 mutation
1	72.0	Female	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
2	32.2	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
3	34.3	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	X659	Positive
4	49.1	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
5	80.1	Female	PMS2	High	PMS2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
6	52.3	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
7	60.9	Female	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
8	64.1	Male	MSH6	High	MSH6	<i>Wild-type</i>	X659	Positive
9	30.9	Female	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
10	55.1	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	X659	Positive
11	53.5	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
12	32.3	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
13	51.9	Female	MSH2/MSH6	High	MSH2	X117	<i>Wild-type</i>	Positive
14	28.7	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
15	32.9	Female	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	X659	Positive
16	37.2	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
17	52.0	Female	MSH6	High	MSH6	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
18	45.6	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
19	59.5	Male	PMS2	High	PMS2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
20	61.8	Female	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
21	40.0	Female	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	X659	Positive
22	54.8	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
23	50.3	Female	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
24	33.3	Female	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
25	56.1	Female	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
26	46.0	Male	MSH2/MSH6	NA	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
27	47.3	Female	MLH1/PMS2	NA	MLH1	X117	<i>Wild-type</i>	Positive
28	49.7	Male	PMS2	NA	PMS2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
29	33.2	Female	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
30	59.2	Female	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
31	64.5	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	X659	Positive
32	52.6	Male	MLH1/PMS2	NA	MLH1	<i>Wild-type</i>	X659	Positive
33	34.4	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
34	63.8	Male	MSH6	NA	MSH6	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
35	50.0	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
36	43.8	Male	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
37	49.7	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
38	45.1	Male	MLH1/PMS2	High	MLH1	<i>Wild-type</i>	X659	Positive
39	49.0	Female	MSH2/MSH6	High	MSH2	<i>Wild-type</i>	X659	Positive

Sample ID	Age	Gender	IHC summary	MSI	Germline defect	RNF43 exon 3 (C6)	RNF43 exon 9 (G7)	RNF43 mutation
40	58.4	Female	MLH1/PMS2	High	MLH1	X117	<i>Wild-type</i>	Positive
41	51.8	Male	MSH6	NA	MSH6	<i>Wild-type</i>	X659	Positive
42	55.8	Female	PMS2	NA	PMS2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
43	52.1	Male	PMS2	NA	PMS2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>
44	75.6	Male	MSH2/MSH6	NA	MSH2	<i>Wild-type</i>	<i>Wild-type</i>	<i>Wild-type</i>