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Leiden**  
The Netherlands

## **Characterization of candidate genes in unexplained polyposis and colorectal cancer**

Abayzeed Elsayed Osman, F.

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# Chapter 6

## Monoallelic *NTHL1* loss-of-function variants and risk of polyposis and colorectal cancer

Fadwa A. Elsayed\*, Judith E. Grolleman\*, Abiramy Ragunathan\*,  
*NTHL1* study group, Daniel D. Buchanan#, Tom van Wezel#,  
Richarda M. de Voer#

\*Authors share co-first authorship

#Authors share co-senior authorship

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## Introduction

The endonuclease III-like protein 1, encoded by *NTHL1*, is a bifunctional glycosylase involved in base-excision repair (BER) that recognizes and removes oxidized pyrimidines<sup>1</sup>. Similar to biallelic loss-of-function (LoF) variants in *MUTYH*<sup>2</sup>, biallelic LoF variants in *NTHL1* predispose to colorectal polyps and colorectal cancer (CRC)<sup>3</sup>. Recently, a multitumor phenotype was observed in individuals diagnosed with *NTHL1* deficiency<sup>4</sup>. Carriers of monoallelic pathogenic variants in *MUTYH* have an increased, albeit small, risk of CRC<sup>5</sup>. Thus far, it is unknown if monoallelic *NTHL1* LoF variants also increase the risk of polyposis and/or CRC. This information is especially important for carriers of the most common LoF variant in *NTHL1* (p.(Gln90\*); NM\_002528.5), which is heterozygous in approximately 0.28% of the general population<sup>6</sup>. Identification of monoallelic *NTHL1* LoF variants currently presents a clinical conundrum regarding how best to counsel carriers with respect to their cancer risk because of the lack of published evidence. Here, we show that monoallelic LoF variants in *NTHL1* are not enriched in individuals with polyposis and/or CRC compared to the general population. Furthermore, 13 colorectal tumors from *NTHL1* LoF carriers did not show a somatic second hit, and we did not find evidence of a main contribution of mutational signature SBS30, the signature associated with *NTHL1* deficiency, suggesting that monoallelic loss of *NTHL1* does not substantially contribute to colorectal tumor development.

## Methods

A total of 5,942 individuals with unexplained polyposis, familial CRC, or sporadic CRC at young age or suspected of having Lynch syndrome with CRC or multiple adenomas were included in this study and defined as case patients (individual studies and their ascertainment are described in Supplementary Methods and Supplementary Table 1). Three independent data sets were used as controls, including (1) the non-Finnish European subpopulation of the genome aggregation database (gnomAD; n = 64,328)<sup>6</sup>, (2) a Dutch cohort of individuals without a suspicion of hereditary cancer who underwent whole-exome sequencing (WES) (Dutch WES; n = 2,329)<sup>7</sup>, and (3) a population-based and cancer-unaffected cohort from the Colon Cancer Family Registry Cohort (CCFRC; n = 1,207) (Supplementary Methods and Supplementary Table 1).

Pathogenic *NTHL1* LoF variants were identified in case patients by sequencing the exonic regions of *NTHL1* (n = 3,439) or by genotyping of 2 LoF variants in

*NTHL1* (c.268C>T, p.(Gln90\*); n = 2503 and c.806G>A, p.(Trp269\*); n = 261) (Supplementary Table 1). For control individuals, all pathogenic LoF variants were retrieved from gnomAD and the Dutch WES-cohort<sup>6,7</sup>, and for the CCFRC control individuals, the exonic regions of *NTHL1* were sequenced (Supplementary Table 1). Odds ratios between case patients and control groups were calculated and a Fisher exact test was performed to assess the significance of difference in carrier rates. Cosegregation analysis was performed by using Sanger sequencing. Two adenomas and 11 primary CRCs from *NTHL1* LoF variant carriers were subjected to WES, and subsequently, mutational signature analysis was performed (Supplementary Methods and Supplementary Table 2). For signature analysis comparison, we included 3 CRCs from individuals with a biallelic *NTHL1* LoF variant.

## Results

Monoallelic *NTHL1* LoF variants were identified in 11 of 3,439 case patients (0.32%) and in 5 of 1,207 (0.41%) of CCFRC control individuals, indicating no significant difference ( $P = .784$ ) (Figure 1A, Supplementary Table 1). Genotyping of the *NTHL1* p.(Gln90\*) variant in another 2,503 case patients identified 7 additional carriers (0.28%). The overall frequency of *NTHL1* p.(Gln90\*) in case patients was not different from the frequency in the gnomAD (17/5,942 vs 250/64,328;  $P = .914$ ), CCFRC (17/5,942 vs 3/1,207;  $P = .556$ ) or Dutch WES control individuals (17/5,942; vs 17/2,329;  $P = .998$ ) (Figure 1A and Supplementary Table 1).

Via cosegregation analysis, we identified 3 additional *NTHL1* p.(Gln90\*) carriers. The phenotype of all carriers identified in this study is described in Supplementary Table 2. Thirteen colorectal tumors from *NTHL1* LoF carriers underwent WES (details in Supplementary Table 2). The *NTHL1* wild-type allele was unaffected by somatic mutations or loss of heterozygosity in all tumors tested. In contrast to *NTHL1*-deficient tumors, in none of the tumors of the carriers was mutational signature SBS30 the main signature, because it was only present in 1 tumor, where it had a minor contribution (Figure 1B and Supplementary Table 2)<sup>4</sup>. These observations indicate that biallelic inactivation of *NTHL1* through a somatic second hit was not evident and that monoallelic inactivation of *NTHL1* was insufficient to result in the accumulation of somatic mutations that are characteristic of an *NTHL1*-deficiency phenotype.

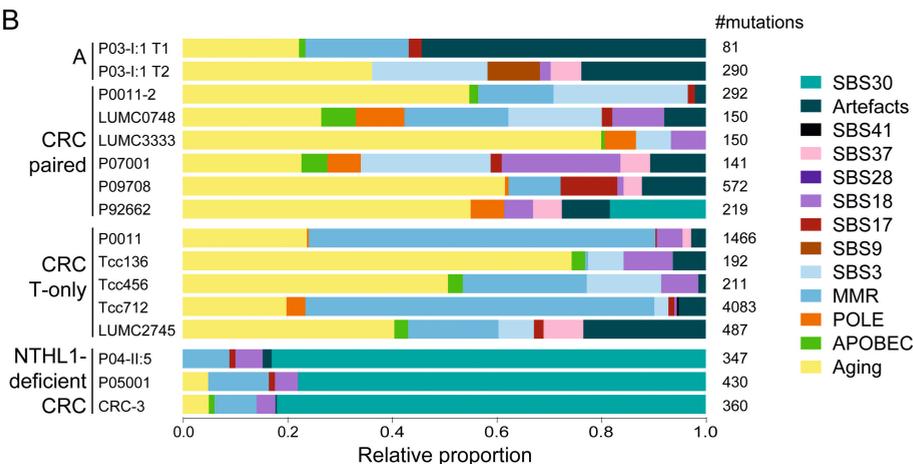
A

|  | Monoallelic <i>NTHL1</i> LoF variant carriers ( <i>n</i> = 11/3,439) |           |         |
|--|--|-----------|---------|
|  | OR   | 95% CI    | P-value |
| gnomAD non-Finnish European ( <i>n</i> = 311/64,328)               | 0.66   | 0.36-1.21 | 0.939   |
| Colon Cancer Family Registry Cohort controls ( <i>n</i> = 5/1,207) | 0.77   | 0.27-2.22 | 0.784   |
| Dutch WES controls ( <i>n</i> = 17/2,329)                          | 0.44   | 0.20-0.93 | 0.991   |

|  | Monoallelic <i>NTHL1</i> p.(Gln90*) carriers ( <i>n</i> = 17/5,942) |           |         |
|--|---|-----------|---------|
|  | OR  | 95% CI    | P-value |
| gnomAD non-Finnish European ( <i>n</i> = 250/64,328)               | 0.74  | 0.40-1.20 | 0.914   |
| Colon Cancer Family Registry Cohort controls ( <i>n</i> = 3/1,207) | 1.15  | 0.34-3.94 | 0.556   |
| Dutch WES controls ( <i>n</i> = 17/2,329)                          | 0.39  | 0.20-0.77 | 0.998   |

B



**Figure 1.** Enrichment and mutational signature analysis of *NTHL1* LoF variants in individuals with polyposis and/or CRC (case patients).

(A) Frequencies of germline monoallelic *NTHL1* LoF variants and monoallelic *NTHL1* p.(Gln90\*) variants in individuals with polyposis and/or CRC (case patients) compared with control populations. (B) Mutational signature analysis of tumors from carriers with a monoallelic *NTHL1* LoF variant. Mutational signatures with shared etiologies were grouped for display purposes, which are the signatures associated with aging (SBS1, SBS5 and SBS40), DNA mismatch repair deficiency (SBS6, SBS15, SBS20, SBS21, SBS26 and SBS44), Polymerase Epsilon (POLE) exonuclease domain deficiency (SBS10a and SBS10b), Apolipoprotein B mRNA editing enzyme (APOBEC) activity (SBS2 and SBS13), and artifact signatures (SBS45, SBS51, SBS52, SBS54 and SBS58). Data availability: paired: tumor and normal or tumor data were available; T-only: only data from 1 tumor tissue were available. A, adenomatous polyp; CI, confidence interval; OR, odds ratio.

## Discussion

In this study, the largest investigating monoallelic LoF variants in *NTHL1* to date to our knowledge, we observed no evidence of an association between carriers and the risk of polyposis and/or CRC. In our case patients, the prevalence of

pathogenic *NTHL1* LoF variant alleles is comparable to that of the general population. However, we cannot rule out that a small risk for CRC, similar to what is observed for *MUTYH* carriers, still exists.

Colorectal tumors from monoallelic *NTHL1* LoF variant carriers did not show evidence of a somatic second hit in *NTHL1* nor of defective base-excision repair, which is typically associated with biallelic *NTHL1* inactivation. Only 1 tumor showed a minor SBS30 contribution to the mutation profile, but this contribution was far less significant compared to *NTHL1*-deficient CRC and is likely the result of multiple testing correction. Our data suggest that inactivation of the *NTHL1* wild-type allele is a rare event in colorectal tumors, which is in agreement with the observation that loss of heterozygosity of chromosome arm 16p is not frequently observed in CRC<sup>8</sup>. We were unable to discriminate between individuals with polyposis or CRC due to the historical nature of the case collections. Therefore, differences in frequencies of monoallelic *NTHL1* LoF variants between control individuals and these 2 phenotypes were not made separately. However, because we identified *NTHL1* LoF variants in individuals with polyposis or CRC, we do not consider a major difference between these 2 phenotypes. Because *NTHL1* deficiency may also predispose to extracolonic tumors, the risk for these tumor types in monoallelic *NTHL1* carriers still needs further assessment.

In conclusion, the evidence to date does not support an increased risk of polyposis and/or CRC for carriers of monoallelic *NTHL1* LoF variants, and consequently, no additional surveillance is currently warranted beyond population screening for CRC, unless family history characteristics point to a reason for colonoscopy.

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## Supplementary methods

### Study cohorts

We included 5,942 patients with unexplained polyposis, familial CRC, or sporadic CRC at a young age or suspected of having Lynch syndrome with CRC or multiple adenomas (Supplementary Table 1) from the Netherlands (n = 3,158); United Kingdom (n = 275); Poland (n = 144); Germany (n = 104); Spain (n = 35); North Macedonia (n = 273); and North America, Canada, and Australia (CCFRC; n = 1,953)<sup>1-3</sup>. All participants provided written informed consent. Local medical ethical committees approved this study (Radboudumc [Commissie mensgebonden onderzoek (CMO)-light, 2015/2172 and 2015/1748], Leiden University Medical Center (LUMC) [P01-019], and Ontario Cancer Research Ethics Board, University of Melbourne Human Research Ethics Committee, and Fred Hutchinson Cancer Research Center Institutional review board).

A total of 1,207 cancer-unaaffected control individuals were available from the population-based recruitment arms of the CCFRC<sup>2,3</sup>. From the Netherlands, 2,329 WES control individuals with a >90-fold median coverage without a suspicion of hereditary cancer were available<sup>4</sup>. The European non-Finnish population of gnomAD was used to determine overall frequencies of LoF variants<sup>5</sup>.

### Targeted resequencing

#### *Hi-Plex*

Leukocyte DNA from 1,953 CRC-affected case patients and 1,207 control individuals was used to screen the coding regions of *NTHL1* by using multiplex polymerase chain reaction (PCR)-based targeted sequencing and variant calling approach (HiPlex2 and Hiplexpipe, [hiplex.org](http://hiplex.org), [github.com/khalidm/hiplexpipe](https://github.com/khalidm/hiplexpipe))<sup>6</sup>. Germline variants in *NTHL1* (NM\_002528.5) were prioritized according to quality—the sequence depth of >30 reads and variant frequency of >30%.

#### *Molecular Inversion Probe-Based sequencing*

Leukocyte DNA from 1,486 polyposis and/or CRC cases was screened for all coding regions and intron-exon boundaries of *NTHL1* (NM\_002528.5) by using molecular inversion probe MIPsequencing, combined with a panel of base excision repair genes, as described previously<sup>1</sup>. Reads were mapped with Burrows-Wheeler Aligner (BWA), and variant calling was performed with UnifiedGenotyper<sup>7</sup>. Somatic variants in *NTHL1* were prioritized according to

quality: sequence depth of > 40 reads, > 20 variant reads, variant frequency of > 25% and quality by depth scores > 8,000.

Variants from HiPlex and MIP screenings were further selected based on predicted LoF of *NTHL1*. We selected all nonsense, frameshift canonical splice sites and included only coding and noncoding splice site region variants with a predicted change of > 20%, based on Alamut (Interactive BioSoftware, Rouen, France) (MaxEnt, NNSplice, and Human Splicesite Finder [HSF]).

### **KASPar assay**

Leukocyte DNA (n = 1,260) or germline DNA extracted from formalin-fixed, paraffin embedded (FFPE) surgical specimens (n = 982) was genotyped for *NTHL1* p.(Gln90\*) by using KBioscience Competitive Allele-Specific PCR (KASPar) assay <sup>1</sup>.

### **Allele-Specific Polymerase Chain Reaction**

Leukocyte DNA from 261 individuals with sporadic or familial CRC was subjected to an allele-specific PCR (AS-PCR) specific for *NTHL1* p.(Gln90\*) and p.(Trp269\*); primers are available upon request.

### **Sanger sequencing**

Sanger sequencing was used for variant validation and to sequence the entire open reading frame of *NTHL1* in confirmed heterozygous cases. In addition, when available, family members were sequenced by using Sanger sequencing for cosegregation purposes.

### **Statistical analysis**

A one-sided Fisher exact test was performed to determine differences in the frequency of monoallelic *NTHL1* germline LoF variants in carriers with polyposis and/or CRC compared to control individuals. We calculated the *P* value, odds ratio, and the 95% confidence interval using R (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>). Three control data sets were used in this comparison.

First, we retrieved all LoF variants (nonsense, frameshift canonical splice sites, and coding or noncoding splice site regions with > 20% splice site change) in canonical transcripts of *NTHL1* listed in the non-Finnish European subpopulation of the genome aggregation database (gnomAD) <sup>5</sup>. All variants were checked manually in gnomAD for their quality. Second, LoF variants in *NTHL1* identified in the Dutch WES cohort (n = 2,329 individuals without a suspicion of hereditary

cancer) were extracted in a similar way as described earlier<sup>4</sup>. Third, LoF variants in *NTHL1* identified in the CCFRC control group of 1,207 individuals, sequenced in this study, were used.

### Whole-Exome sequencing

Exome captures (Supplementary Table 2) were performed according to the manufacturer by using either Agilent Clinical Research Exome (CRE) V2 (Agilent, Santa Clara, CA) in combination with sequencing on a NovaSeq 6000 (Illumina, San Diego, CA), Agilent SureSelect XT<sup>HS</sup> Human All Exon V6 enrichment Kit in combination with sequencing on a NextSeq 500, or xGEN Exome Research Panel (Integrated DNA Technology [IDT]), Coralville, IA) in combination with sequencing on a NovaSeq 6000.

Novaseq 6000 sequencing reads were trimmed by using Trimmomaticv0.36 and aligned to hs37d5 by using BWA-MEM, followed by merging and PCR duplicate removal with Sambamba (version 0.5.8)<sup>8,9</sup>. Variant calling was performed by using Strelka (version 2.017) and Freebayes for paired samples; only variants called by both callers were reported<sup>10,11</sup>. For LUMC2745, no paired sample was available, and variant calling was performed with Mutect2 (GATK version 4.1.0.0; GATK, Broadinstitute, Cambridge, MA). Trimmed NextSeq 500 sequencing reads were aligned to GRCh37 by using BWA-MEM, and duplicates were flagged by using Picard Tools, version 1.90. Variants were called with Mutect2 (GATK version 4.1.0.0), with or without matched germline samples; variant filtering was performed as described<sup>1</sup>, with minor modifications. Variants in dbSNPv132 (minus catalogue of somatic mutations in cancer [COSMIC]), microsatellites, homopolymers, simple repeats and variants called outside of the respective exome capture target were removed. Somatic variants with a variant allele frequency of < 10%, < 20x coverage in both normal and tumor, and fewer than 4 reads supporting the variant were removed. For tumor-only analysis, variants shared by more than 1 individual and variants with a variant allele frequency of > 80% were removed to reduce germline leakage.

### Mutational signature analysis

Mutation spectra were generated by using In-depth characterization and analysis of mutational signatures (ICAMS), version 2.1.2 ([github.com/steverozen/ICAMS](https://github.com/steverozen/ICAMS)), and mutational signature analysis was performed by using mSigAct v2.0.0.9018<sup>12</sup>. Tissue-specific CRC signature universes were inferred from the Pan-cancer analysis of whole genomes (PCAWG) signature assignments<sup>13</sup>. The signature universe was extended with SBS30 and potential artefact

signatures SBS45, SBS51, SBS52, SBS54, and SBS58, which were present in a subset of the samples of this cohort. Signatures were normalized to the trinucleotide abundance of the respective exome capture panel used. Per mutation spectrum, mutational signature assignment was performed by using `mSigAct::SparseAssignActivity`, with  $p = .5$  to reduce sparsity. The presence of SBS30 was then determined using `mSigAct::SignaturePresenceTest` using the signatures determined by `mSigAct::SparseAssignActivity` plus SBS30 as well as the aging-associated signatures SBS1, SBS5, and SBS40 (Supplementary Table 2). Multiple testing correction was done according to Benjamini-Hochberg.

**Supplementary Table 1.** Characteristics of case and control cohorts and identified case patients and control individuals with monoallelic *NTHL1* LoF variants in this study

| Approach  | Sequencing method and cohorts   | Samples, n | Selection <sup>a</sup> criteria  | Genes tested   | Monoallelic <i>NTHL1</i> p.(Gln90*), n | Other monoallelic <i>NTHL1</i> LoF variants, n | Total monoallelic <i>NTHL1</i> LoF variants, n |  |
|---|---|------------|--|--|--|--|--|--|
| <i>NTHL1</i> -targeted resequencing (n = 3,439 cases) | <b>Hi-Plex multiplex PCR-based sequence screening of <i>NTHL1</i> exons (control individuals)</b> |            |  |  |  |  |  |  |
|   | Colon Cancer Family Registry  | 1,207      | Population-based healthy individuals with no history of polyposis and/or CRC | NA   | 3                                      | 2  | 5  |  |
|   | <b>Hi-Plex multiplex PCR based sequence screening of <i>NTHL1</i> exons (case patients)</b>       |            |  |  |  |  |  |  |
|   | Colon Cancer Family Registry  | 1,953      | Population-based CRC   | <i>APC</i> , <i>MUTYH</i> , <i>POLE</i> , <i>POLD1</i> , <i>MMR</i> <sup>ab</sup>                            | 4                                      | 1  | 5  |  |
|   | <b>MIP-based sequence screening of <i>NTHL1</i> (case patients)</b>                               |            |  |  |  |  |  |  |
|   | ParliBED (the Netherlands)  | 600        | Polyposis, CRC, or CRC and additional tumor                                  | No disease-causing mutation found after routine diagnostics  | 0                                      | 0  | 0  |  |
|   | Oxford (United Kingdom)   | 275        | Polyposis  | <i>APC</i> , <i>MUTYH</i>  | 4                                      | 0  | 4  |  |
|   | Leiden (the Netherlands)  | 150        | Polyposis or familial CRC  | <i>APC</i> , <i>MUTYH</i>  | 0                                      | 0  | 0  |  |
|   | Nijmegen (the Netherlands)  | 147        | Polyposis or familial CRC  | <i>APC</i> , <i>MUTYH</i>  | 0                                      | 0  | 0  |  |
|   | Szczecin (Poland)   | 144        | Familial CRC   | <i>POLE</i> , <i>POLD1</i> , <i>MMR</i> <sup>ab</sup>  | 1                                      | 0  | 1  |  |
|   | Dresden (Germany)   | 104        | Polyposis or familial CRC  | <i>APC</i> , <i>MUTYH</i>  | 0                                      | 0  | 0  |  |
|   | Santiago de Compostela (Spain)  | 35         | Polyposis or familial CRC  | <i>APC</i> , <i>MUTYH</i> (in part), <i>POLE</i> , <i>POLD1</i> , <i>BMPRIA</i> , <i>SMAD4</i> , <i>PTEN</i> | 0                                      | 0  | 0  |  |
|   | Groningen (the Netherlands)   | 19         | Polyposis or familial CRC  | <i>APC</i> , <i>MUTYH</i>  | 0                                      | 0  | 0  |  |
|   | Skopje (North Macedonia)  | 12         | Polyposis, recessive inheritance   | <i>MMR</i> <sup>ab</sup> , <i>APC</i> , <i>TP53</i> , <i>MUTYH</i> , <i>POLE</i> , <i>POLD1</i>              | 1                                      | 0  | 1  |  |

Supplementary Table 1. (continued)

| Approach                                  | Sequencing method and cohorts   | Samples, n | Selection <sup>a</sup> criteria                                     | Genes tested  | Monoallelic <i>NTHL1</i> p.(Gln90*), n | Other monoallelic <i>NTHL1</i> LoF variants, n | Total monoallelic <i>NTHL1</i> LoF variants, n |
|---|---|------------|---|---|--|--|--|
| <i>NTHL1</i> genotyping (n = 2,503 cases) | <b><i>NTHL1</i> p.(Gln90*) genotyping by KASPar assay (case patients)</b> |            |   |   |  |  |  |
|   | Leiden (the Netherlands)  | 1,894      | Polyposis or familial CRC, with or without suspected Lynch syndrome | <i>APC</i> , <i>MUTYH</i> , <i>POLE</i> , <i>POLD1</i> , MMR <sup>a,b</sup> | 3                                      | NA   | 3  |
|   | Nijmegen (the Netherlands)  | 348        | Polyposis or familial CRC   | <i>APC</i> , <i>MUTYH</i> , <i>POLE</i> , <i>POLD1</i> , MMR <sup>a,b</sup> | 1                                      | NA   | 1  |
|   | Skopje (North Macedonia)  | 200        | Sporadic CRC  | None  | 2                                      | 0  | 2  |
|   | Skopje (North Macedonia)  | 61         | Polyposis or familial CRC   | TruSight Hereditary Cancer Panel (Illumina)                                 | 1                                      | 0  | 1  |

NA, not applicable; ParelIBED, The Dutch Parelisnoer Institute Biobank Hereditary Colorectal Cancer<sup>14</sup>.

<sup>a</sup> Polyposis is defined as the cumulative occurrence of at least 10 polyps. Familial CRC is defined as the proband having a CRC  $\leq 50$  years of age and at least one first degree relative with CRC  $\leq 60$  years of age. Sporadic CRC is defined as patients with CRC without a family history, irrespective of age. <sup>b</sup>MMR\* genes: *MLH1*, *MSH2*, *MSH6* and *PMS2*.

**Supplementary Table 2.** Phenotypic description and details on the tumors subjected to WES of identified carriers of a monoallelic *NTHL1* LoF variant

| Number | Patient ID           | Identification method | Amino acid change | Sex | Polyps | Malignancies <sup>a</sup>    | Tumor type for WGS | Matched normal available | Exome enrichment kit | Sequencing platform | Median coverage (tumor(s)) | Number of somatic variant calls | P-value SBS30        |
|--------|----------------------|-----------------------|-------------------|-----|--------|------------------------------|--------------------|--------------------------|----------------------|---------------------|----------------------------|---------------------------------|----------------------|
| 1      | P09708               | Hi-Plex               | p.(Gln287*)       | M   |        | Cecum (73), CRC (73)         | CRC                | Yes, blood               | Agilent CRE V2       | Novaseq 6000        | 221                        | 572                             | 0.976                |
| 2      | P92662               | Hi-Plex               | p.(Gln90*)        | M   |        | CRC (53)                     | CRC                | Yes, blood               | Agilent CRE V2       | Novaseq 6000        | 189                        | 219                             | 1.61×10 <sup>3</sup> |
| 3      | P07001               | Hi-Plex               | p.(Gln90*)        | M   |        | CRC (43)                     | CRC                | Yes, blood               | Agilent CRE V2       | Novaseq 6000        | 116                        | 141                             | 0.331                |
| 4      | P58832               | Hi-Plex               | p.(Gln90*)        | F   |        | CRC (46), UC (29)            | --                 | --                       | --                   | --                  | --                         | --                              | --                   |
| 5      | P00387               | Hi-Plex               | p.(Gln90*)        | F   |        | Cecum (42), UC (23), LC (53) | --                 | --                       | --                   | --                  | --                         | --                              | --                   |
| 6      | P0011 <sup>a</sup>   | MIP screen            | p.(Gln90*)        | M   |        | CRC (56), LIC (unk)          | CRC                | No <sup>b</sup>          | Agilent V6           | NextSeq500          | 133                        | 1466                            | 0.976                |
| 7      | P0011-2 <sup>a</sup> | Co-segregation        | p.(Gln90*)        | F   |        | CRC (55)                     | CRC                | Yes, FFPE                | Agilent V6           | NextSeq500          | 86                         | 292                             | 0.953                |
| 8      | P0804                | MIP screen            | p.(Gln90*)        | F   |        | CRC (50)                     | CRC <sup>c</sup>   | Yes, FFPE                | Agilent V6           | NextSeq500          | --                         | --                              | --                   |
| 9      | P0468 <sup>b</sup>   | MIP screen            | p.(Gln90*)        | M   | A (43) |                              | --                 | --                       | --                   | --                  | --                         | --                              | --                   |
| 10     | P0567 <sup>b</sup>   | Co-segregation        | p.(Gln90*)        | F   | A (55) |                              | --                 | --                       | --                   | --                  | --                         | --                              | --                   |

Supplementary Table 2. (continued)

| Number | Patient ID           | Identification method | Amino acid change | Sex | Polyps    | Malignancies <sup>a</sup>              | Tumor type for WGS | Matched normal available | Exome enrichment kit | Sequencing platform | Median coverage tumor(s) <sup>b</sup> | Number of somatic variant calls | P-value SBS30    |
|--------|----------------------|-----------------------|-------------------|-----|-----------|--|--------------------|--------------------------|----------------------|---------------------|---------------------------------------|---------------------------------|------------------|
| 11     | P0567-2 <sup>b</sup> | Co-segregation        | p.(Gln90*)        | F   | A (61)    |  |                    |                          |                      |                     |                                       |                                 |                  |
| 12     | P0523                | MIP screen            | p.(Gln90*)        | M   | A (59)    | CRC (58)                               |                    |                          |                      |                     |                                       |                                 |                  |
| 13     | P0568                | MIP screen            | p.(Gln90*)        | M   | A (unk)   |  |                    |                          |                      |                     |                                       |                                 |                  |
| 14     | P0602                | MIP screen            | p.(Gln90*)        | F   | A (unk)   |  |                    |                          |                      |                     |                                       |                                 |                  |
| 15     | K134                 | KASPar assay          | p.(Gln90*)        | F   | A (48-56) | CRC (49)                               |                    |                          |                      |                     |                                       |                                 |                  |
| 16     | LUMC3333             | KASPar assay          | p.(Gln90*)        | M   |           | CRC (<69), Cecum (69)                  | CRC                | Yes, FPPE                | IDT xGEN             | Novaseq 6000        | 131                                   | 150                             | 0.888            |
| 17     | LUMC2745             | KASPar assay          | p.(Gln90*)        | M   |           | CRC (72); CRC, SCC (61)                | CRC                | No                       | IDT xGEN             | Novaseq 6000        | 99                                    | 487                             | 0.053            |
| 18     | LUMC0748             | KASPar assay          | p.(Gln90*)        | F   |           | CRC (56), OVC (56), CRC (56), CRC (68) | CRC                | Yes, FPPE                | IDT xGEN             | Novaseq 6000        | 84                                    | 150                             | 1                |
| 19     | Tcc136               | AS-PCR                | p.(Gln90*)        | M   |           | CRC (75)                               | CRC <sup>c</sup>   | No                       | Agilent V6           | NextSeq500          | 195                                   | 192                             | 0.331            |
| 20     | Tcc456               | AS-PCR                | p.(Gln90*)        | M   |           | PC, CRC (72)                           | CRC                | No                       | Agilent V6           | NextSeq500          | 140                                   | 211                             | 0.052            |
| 21     | Tcc712               | AS-PCR                | p.(Gln90*)        | F   | 7A (71)   | EC (66), CRC (71)                      | CRC <sup>c</sup>   | No                       | Agilent V6           | NextSeq500          | 180                                   | 4083                            | 1                |
| 22     | P03-1:1              | c                     | p.(Gln90*)        | M   | A, HP     |  | A                  | No                       | IDT xGEN             | Novaseq 6000        | T1=64<br>T2=39                        | T1=81<br>T2=290                 | T1=1<br>T2=0.088 |

Supplementary Table 2. (continued)

| Number | Patient ID | Identification method | Amino acid change             | Sex             | Polyps               | Malignancies <sup>e</sup>  | Tumor type for WGS         | Matched normal available | Exome enrichment kit   | Sequencing platform    | Median coverage <sup>f</sup> tumor(s) <sup>g</sup> | Number of somatic variant calls | P-value SBS30 <sup>h</sup> |
|--------|------------|-----------------------|-------------------------------|-----------------|----------------------|----------------------------|----------------------------|--------------------------|------------------------|------------------------|--|---------------------------------|----------------------------|
| --     | P04-II:5   | c                     | p.Gln90*/<br>p.Ile245Asnfs*28 | F --            | --                   | --                         | NTHL1-<br>deficient<br>CRC | Yes, FEPE                | IDT xGEN               | Novaseq<br>6000        | 162  | 347                             | 3.11x10 <sup>-45</sup>     |
| --     | P05001     | Hi-Plex               | p.(Gln90*/<br>p.(Ala79fs)     | F A, HP<br>(61) | CRC (61), BC<br>(63) | NTHL1-<br>deficient<br>CRC | Yes, blood                 | Agilent CRE<br>V2        | Novaseq<br>6000        | 108                    | 430  | 430                             | 1.82x10 <sup>-39</sup>     |
| --     | CRC-3      | d                     | p.(Gln90*/<br>p.(Gln90*)      | M --            | --                   | --                         | NTHL1-<br>deficient<br>CRC | Chapter 5 <sup>1</sup>   | Chapter 5 <sup>1</sup> | Chapter 5 <sup>1</sup> | Chapter 5 <sup>1</sup>                             | 360                             | 3.08x10 <sup>-38</sup>     |

A: colorectal adenomatous polyps; BCC: basal cell carcinoma; CRC: colorectal cancer; EC: endometrial cancer; HP: hyperplastic polyps; LC: lung cancer; LIC: liver cancer; OvC: ovarian cancer; PC: prostate cancer; SCC: squamous cell carcinoma; UC: uterus cancer; unk: age unknown; --: not applicable.

<sup>a</sup>Sibling.

<sup>b</sup>Sibling.

<sup>c</sup>Identified by Grolleman et al., 2019<sup>1</sup>.

<sup>d</sup>Tumor data from Grolleman et al., 2019<sup>1</sup>.

<sup>e</sup>Numbers in parenthesis indicate age at diagnosis.

<sup>f</sup>Tumor P0804 was excluded from further analysis due to insufficient data quality.

<sup>g</sup>Fresh-frozen tumor material.

<sup>h</sup>The normal sample of the sibling was used for somatic variant extraction.

<sup>i</sup>Median read coverage (units=reads).

<sup>j</sup>Multiple testing correction was done according to Benjamini-Hochberg.

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