Germline Pathogenic Variants in Homologous Recombination and DNA Repair Genes in an Asian Cohort of Young-Onset Colorectal Cancer

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Abstract

Background: Growing evidence suggests a role for cancer susceptibility genes such as BRCA2 and PALB2 in young-onset colorectal cancers. Using a cohort of young colorectal cancer patients, we sought to identify and provide functional evidence for germline pathogenic variants of DNA repair genes not typically associated with colorectal cancer.

Methods: We recruited 88 patients with young-onset colorectal cancers seen at a general oncology center. Whole-exome sequencing was performed to identify variants in DNA repair and colorectal cancer predisposition genes. Pathogenic BRCA2 and PALB2 variants were analyzed using immunoblot and immunofluorescence on patient-derived lymphoblastoid cells.

Results: In general, our cohort displayed characteristic features of young-onset colorectal cancers. Most patients had left-sided tumors and were diagnosed at late stages. Four patients had familial adenomatous polyposis, as well as pathogenic APC variants. We identified 12 pathogenic variants evenly distributed between DNA repair and colorectal cancer predisposition genes. Six patients had pathogenic variants in colorectal cancer genes: APC (n = 4) and MUTYH monoallelic (n = 2). Another six had pathogenic variants in DNA repair genes: ATM (n = 1), BRCA2 (n = 1), PALB2 (n = 1), NTHL1 (n = 1), and WRN (n = 2). Pathogenic variants BRCA2 c.9154C>T and PALB2 c.1059delA showed deficient homologous recombination repair, evident from the impaired RAD51 nuclear localization and foci formation.

Conclusion: A substantial portion of pathogenic variants in young-onset colorectal cancer was found in DNA repair genes not previously associated with colorectal cancer. This may have implications for the management of patients. Further studies are needed to ascertain the enrichment of pathogenic DNA repair gene variants in colorectal cancers.
in low- to moderate-penetrance genes. Only 10% are attributed to germline pathogenic variants in CRC predisposition genes including the mismatch repair genes (3%), APC (1%), MTHYH biallelic (<1%), and MTHYH monouallelic (2%) (7). The remaining 20% may be contributed by germline variants in other moderate- to high-penetrance genes not typically associated with CRCs (8–10).

Recent genomic studies on CRC have identified pathogenic germline variants among DNA repair genes not typically associated with CRCs. Pathogenic variants of BRCA2 and PALB2 have been consistently seen among CRC patients and a threefold increased risk of developing early-onset CRC has also been reported in BRCA2 mutation carriers (7,11–13). However, the mechanism of CRC carcinogenesis has predominantly been overactivation of the Wnt signaling pathway secondary to disruption of tumor suppressors such as APC (14,15). It may be premature to conclude the relevance of DNA repair genes in CRC susceptibility given the limited population and functional evidence (7,11,12). A study on germline mutations of DNA repair genes among young-onset CRCs identified seven germline pathogenic variants in BRCA2 and PALB2 but only one of the tumors showed a loss of heterozygosity (LOH) for BRCA2 (12).

Notably, DNA damage repair processes are not exclusive and often interact on a molecular level (16). For instance, the homologous recombination (HR) proteins BRCA1 and BARD1 interact physically with MSH2 and MSH6 to regulate downstream processes in DNA mismatch repair (MMR) (16). Therefore, it is possible that defects in HR may contribute to CRC pathogenesis (17). Functional studies such as tumor LOH and HR repair analyses, as well as population studies, will be useful in clarifying the relevance of defective HR pathway in CRC development (7). We sought to fill this gap by profiling the spectrum of pathogenic variants in DNA repair and CRC predisposition genes and evaluate the functional impact of identified variants within an Asian cohort with young-onset CRC. In addition, to investigate if PALB2 and BRCA2 germline variants would indeed result in functional impairment, we evaluated our identified variants functionally.

Materials and Methods

Study Design

We recruited patients diagnosed with CRC before age 50 years on follow-up status at our general medical oncology center from November 2014 to December 2016. We excluded patients with MMR-deficient tumors given our study aim to investigate CRC predisposition in DNA repair genes beyond the known CRC susceptibility genes. Although some individuals with MMR-deficient tumors may have germline pathogenic variants in these genes, this proportion would be reasonably small given the majority of MMR-deficient tumors were accounted for by somatic or germline MMR gene mutations (18). Healthy individuals with no prior history of cancer were obtained from a local database of 831 Asian volunteers (median age = 40 years). Clinicopathological data on age, sex, personal and family (first-degree relatives) cancer history, tumor histology, American Joint Committee on Cancer (AJCC) staging, site, and immunohistochemistry staining for MMR proteins were retrieved from electronic medical records.

Whole-Exome Sequencing

Patient-derived genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) and sequenced on an Illumina Hiseq 4000 (Illumina, Inc, San Diego, CA, USA). Reads were aligned to the human reference genome (hs37d5) as previously described and elaborated in Supplementary Methods (available online) (19). To prioritize candidate germline variants, we evaluated variants with minor allele frequency (MAF) below 0.01 for functional and genetic evidence of pathogenicity according to American College of Medical Genetics and Genomics (ACMG) criteria and curation tools from ClinGene. MAF was defined in reference to Exome Aggregation Consortium and 1000 Genomes databases and an in-house database of local control population, which provided a better reflection of polymorphisms in the Singaporean population, hence a more accurate estimate of allele frequency for evaluation of PM2 criterion (20,21). MAF of 0.03 was used for genes with autosomal recessive inheritance. Pathogenic variants were validated by Sanger sequencing using BigDye Terminator v3.1 (ABI, ThermoFisher Scientific Corporation, Waltham, MA, USA). Resulting chromatograms were analyzed using Mutation Surveyor (Softgenetics, State College, PA, USA). Tumor DNA, where available, was extracted to assess for LOH of pathogenic variants. Patient genomic DNA was also analyzed for copy number variations using digital multiplex ligation-dependent probe amplification (digitalMLPA) (Supplementary Materials, available online). Genetic testing was performed exclusively on affected patients; relatives were not included.

Selection of CRC Predisposition and DNA Repair Genes

In sum, 20 CRC predisposition and 44 DNA repair genes associated with cancer susceptibility were evaluated. CRC predisposition genes include APC, AXIN2, BMPR1A, BRF, CDH1, CHEK2, EPCAM, FLCN, GREM1, MLN1, MSH2, MSH6, MUTYH, PMS2, POLD1, POLE, PTEN, SMAD4, STK11 and TP53. DNA repair genes include ATM, ATR, BAP1, BARD1, BLM, BRCA1, BRCA2, BRIP1, CDKN2A, CDK4, DDB2, ERCC2, ERCC3, ERCC4, ERCC5, FANCA, FANC, FANCC, FANCd2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCN, GEN1, MITF, MRE11A, NBN, NTHL1, PALB2, RAD50, RAD51, RAD51C, RAD51D, RAD54L, RECQL4, RET, RNF13, SLX4, UBE2T, WRN, XPA, XPC, and XRCC2.

Statistical Analysis

Patient characteristics and sequencing results were summarized as mean (SD) or median (interquartile range) for continuous variables and proportions for categorical variables. Prevalence (95% confidence interval [CI]) of variants were estimated as a proportion of young-onset CRC patients. Fisher exact test, and one-way ANOVA were used to compare clinicopathological variables among carriers and noncarriers of pathogenic variants.

Cell Treatment

Patient-derived lymphoblastoid cells (LCLs) were treated with 10 µM etoposide for 1 hour, subsequently recovered by incubation in fresh medium for 1 hour or 6 hours, and then harvested for immunoblot and immunofluorescence analyses. Vehicle control contained an equivalent volume of dimethyl sulfoxide.

Immunoblot

Subcellular fractionation was performed using the nuclear/cyttoplasmic separation protocol previously described (22).


Fractionated proteins were electrophoresed on sodium-dodecyl-sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore, Sigma-Aldrich, St. Louis, MO, USA). Membranes were blocked, incubated overnight with primary antibody followed by secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (DakoCytomation, Glostrup, Denmark; P044701 and P044801, 1/10 000). Immunoreactivity was detected with enhanced chemiluminescent horseradish peroxidase substrate (Advansta, Menlo Park, CA, USA) and quantified using ImageJ (NIH, MD, USA) software.

**Immunofluorescence**

Cells were fixed 15 minutes using 4% paraformaldehyde, permeabilized 5 minutes using 0.3% Triton-X 100, and blocked 30 minutes with 10% goat serum in phosphate-buffered saline before incubation at 37°C for 30 minutes with primary antibodies. Slides were stained with AlexaFluor 488 or 594 conjugated secondary antibodies (ThermoFisher Scientific Corporation; A11008 and A11020, 1:1000) and mounted with Prolong Gold antifade reagent with 4’, 6-diamidino-2-phenylindole (Life Technologies, ThermoFisher Scientific Corporation). Images were acquired by confocal microscope (Zeiss LSM800). Cells containing five or more foci were scored positive for RAD51 foci formation.

**Results**

**Clinicopathological Characteristics of Patients**

Of the 88 young CRC patients, males and females were equally represented; the majority was Chinese (73.9%). Age at diagnosis averaged 41 years, ranging from 17 to 49 years. Most patients had left-sided colorectal cancer (80.7%) and were diagnosed at late stages (AJCC stage III and IV; 84.1%). Other than young age at diagnosis, high-risk phenotypic features necessitating clinical genetic testing were absent in most patients; more than half of the patients did not have first-degree relatives with cancer or treatment (24).

**Variant Pathogenicity in Colorectal Predisposition Genes**

Using whole-exome sequencing, we identified 316 germline variants fulfilling the MAF criteria described earlier (Figure 1A), of which 12 were classified pathogenic. No copy number variation data were summarized in Table 1.

**Variant Pathogenicity in DNA Repair Genes**

The remaining half of the pathogenic variants occurred in DNA repair genes, affecting six patients. Clinically, carriers of pathogenic variants in DNA repair genes were similar to noncarriers (Table 1). These variants included one truncating ATM variant, one missense BRCA2 variant, one missense NTHL1 variant, one truncating PALB2 variant, and two truncating WRN variants (Table 2). Notably, two of the six variants occurred in BRCA2 and PALB2. BRCA2 and PALB2 form a protein complex essential for RAD51 nuclear localization, which is critical for the repair process (31). As both BRCA2 and PALB2 pathogenic variants occurred in domains key to proper RAD51 localization, we hypothesized that these mutations would impair RAD51 nuclear localization and foci formation. To validate our hypothesis, we performed immunoblotting and immunofluorescence assays for BRCA2 c.9154C>T and PALB2 c.1059delA variants.

**Functional Studies**

To assess the functional impact of BRCA2 c.9154C>T and PALB2 c.1059delA, we induced double-stranded DNA breaks and measured the HR efficiency by quantifying the RAD51 nuclear localization and foci formation on patient-derived LCLs. Compared to healthy controls, BRCA2 and PALB2 mutation carriers showed impaired RAD51 nuclear localization and foci formation (Figures 2 and 3). To further support the tumorigenesis role of these variants, we assessed for tumor LOH but did not observe LOH of PALB2 (Supplementary Figure 1, available online). LOH data was unavailable for BRCA2 c.9154C>T due to insufficient tumor sample.

Among the 236 variants of uncertain significance (VUS), eight occurred in BRCA2 and PALB2. Of these, we were only able to obtain LCL of BRCA2 c.440A>G, which occurred in a site with no known function (Figure 1B). As the variant was enriched among patients (MAF < 0.005; OR = 2.34, 95% CI = 0.49 to 11.18) and located near the PALB2-binding domain responsible for PALB2-RAD51 interaction (Figure 1B), we evaluated this variant functionally but found no evidence of perturbed RAD51 foci formation or tumor LOH (Figures 2 and 3; Supplementary Figure 1, available online).

**Discussion**

To the best of our knowledge, this was the first study of pathogenic DNA repair gene variants in Asian patients with young-onset CRCs. Similar to previous studies, we found most patients presented advanced-stage diseases in their forties (2,11,23). Another characteristic feature was a predilection for the distal colon and rectum, which would manifest as early symptoms of changes in bowel habit and constipation (24). Unfortunately, because of the patients’ relatively young age, clinicians tended to dismiss these nonspecific symptoms, hence delaying diagnosis and treatment (24).

Exceptions to this trend were patients with high-risk phenotypic features such as personal or family history of hereditary CRC syndromes or pathogenic variants in CRC predisposition genes. Studies on young-onset CRCs, including ours, observed that these patients were often diagnosed at a younger age and earlier disease stages compared with their peers who lack those features (5,7,11,12). This pattern of diagnosis might be related to the close surveillance and prompt treatment received by at-risk patients, which often translated to improved survival.
| Characteristics                              | All patients (n = 88) | Patients without pathogenic variants (n = 76) | Patients with pathogenic CRC predisposition gene variants (n = 6) | Patients with pathogenic DNA repair gene variants (n = 6) | P     |
|--------------------------------------------|----------------------|-----------------------------------------------|------------------------------------------------------------------|-------------------------------------------------|-------|
| Sex                                        |                      |                                               |                                                                  |                                                 |       |
| Male                                       | 44 (50.0)            | 35 (46.1)                                     | 4 (66.7)                                                         | 5 (83.3)                                       | .149  |
| Female                                     | 44 (50.0)            | 41 (53.9)                                     | 2 (33.3)                                                         | 1 (16.7)                                       |       |
| Race                                       |                      |                                               |                                                                  |                                                 |       |
| Chinese                                    | 65 (73.9)            | 55 (72.4)                                     | 4 (66.7)                                                         | 6 (100.0)                                      | .017  |
| Malay                                      | 11 (12.5)            | 11 (14.5)                                     | 0 (0.0)                                                          | 0 (0.0)                                        |       |
| Indian                                     | 4 (4.5)              | 2 (2.6)                                       | 2 (33.3)                                                         | 0 (0.0)                                        |       |
| Others                                     | 8 (9.1)              | 8 (10.5)                                      | 0 (0.0)                                                          | 0 (0.0)                                        |       |
| Age of diagnosis, y                        |                      |                                               |                                                                  |                                                 |       |
| Mean ± SE                                  | 41.4 ± 8.0           | 41.8 ± 7.3                                    | 33.2 ± 6.4                                                       | 44.8 ± 1.9                                     | .019* |
| Range                                      | 17 to 49             | 17 to 49                                      | 18 to 49                                                         | 36 to 49                                       |       |
| CRCs in first-degree relatives             |                      |                                               |                                                                  |                                                 |       |
| Present                                    | 13 (18.6)            | 8 (13.8)                                      | 2 (33.3)                                                         | 3 (50.0)                                       | .059  |
| Absent                                     | 57 (81.4)            | 50 (86.2)                                     | 4 (66.7)                                                         | 3 (50.0)                                       |       |
| Missing                                    | 18                   | 18                                             | 0                                                                | 0                                              |       |
| Breast cancers in first-degree relatives    |                      |                                               |                                                                  |                                                 |       |
| Present                                    | 4 (5.7)              | 4 (6.9)                                       | 0 (0.0)                                                          | 0 (0.0)                                        | .645  |
| Absent                                     | 66 (94.3)            | 54 (93.1)                                     | 6 (100.0)                                                        | 6 (100.0)                                      |       |
| Missing                                    | 18                   | 18                                             | 0                                                                | 0                                              |       |
| Ovarian cancers in first-degree relatives   |                      |                                               |                                                                  |                                                 |       |
| Present                                    | 1 (1.4)              | 1 (1.7)                                       | 0 (0.0)                                                          | 0 (0.0)                                        | .900  |
| Absent                                     | 69 (98.6)            | 57 (98.3)                                     | 6 (100.0)                                                        | 6 (100.0)                                      |       |
| Missing                                    | 18                   | 18                                             | 0                                                                | 0                                              |       |
| Any cancers in first-degree relatives       |                      |                                               |                                                                  |                                                 |       |
| Present                                    | 32 (45.7)            | 23 (39.7)                                     | 4 (66.7)                                                         | 5 (83.3)                                       | .416  |
| Absent                                     | 38 (54.3)            | 35 (60.3)                                     | 2 (33.3)                                                         | 1 (16.7)                                       |       |
| Missing                                    | 18                   | 18                                             | 0                                                                | 0                                              |       |
| Location of CRC                            |                      |                                               |                                                                  |                                                 |       |
| Right sided                                | 17 (19.3)            | 16 (21.1)                                     | 0 (0.0)                                                          | 1 (16.7)                                       | .447  |
| Left sided                                 | 71 (80.7)            | 60 (78.9)                                     | 6 (100.0)                                                        | 5 (83.3)                                       |       |
| Histological subtype                       |                      |                                               |                                                                  |                                                 |       |
| Adenocarcinoma                             | 5 (5.7)              | 3 (3.9)                                       | 1 (16.7)                                                         | 1 (14.3)                                       | .387  |
| Mucinous adenocarcinoma                    | 8 (9.1)              | 7 (9.2)                                       | 0 (0.0)                                                          | 1 (14.3)                                       |       |
| Adenocarcinoma, NOS                        | 75 (85.2)            | 66 (86.8)                                     | 5 (83.3)                                                         | 4 (66.7)                                       |       |
| Tumor differentiation                      |                      |                                               |                                                                  |                                                 |       |
| Well                                       | 5 (5.7)              | 4 (5.3)                                       | 1 (16.7)                                                         | 0 (0.0)                                        | .724  |
| Moderate                                   | 65 (73.9)            | 55 (72.4)                                     | 5 (83.3)                                                         | 5 (83.3)                                       |       |
| Poor                                       | 6 (6.8)              | 6 (7.9)                                       | 0 (0.0)                                                          | 1 (16.7)                                       |       |
| Not specified                              | 12 (13.6)            | 11 (14.5)                                     | 0                                                                | 0                                              |       |
| AJCC disease stage                         |                      |                                               |                                                                  |                                                 |       |
| I                                          | 1 (1.1)              | 0 (0.0)                                       | 1 (16.7)                                                         | 0 (0.0)                                        | .022† |
| II                                         | 13 (14.8)            | 11 (14.5)                                     | 1 (16.7)                                                         | 1 (16.7)                                       |       |
| III                                        | 47 (53.4)            | 40 (52.6)                                     | 3 (50.0)                                                         | 4 (66.7)                                       |       |
| IV                                         | 27 (30.7)            | 25 (32.9)                                     | 1 (16.7)                                                         | 1 (16.7)                                       |       |
| MMR status [IHC]                           |                      |                                               |                                                                  |                                                 |       |
| MMR-deficient                              | 0 (0.0)              | 0 (0.0)                                       | 0 (0.0)                                                          | 0 (0.0)                                        | .272  |
| MMR-proficient                             | 69 (100.0)           | 60 (100.0)                                    | 3 (100.0)                                                        | 6 (100.0)                                      |       |
| Missing                                    | 19                   | 16                                             | 3                                                                | 0                                              |       |
| ECOG at diagnosis                          |                      |                                               |                                                                  |                                                 |       |
| 0                                          | 64 (73.6)            | 53 (70.7)                                     | 6 (100.0)                                                        | 5 (83.3)                                       | .585  |
| 1                                          | 21 (2.4)             | 20 (26.7)                                     | 0 (0.0)                                                          | 1 (16.7)                                       |       |
| 2                                          | 2 (2.3)              | 2 (2.7)                                       | 0 (0.0)                                                          | 0 (0.0)                                        |       |
| Missing                                    | 1                    | 1                                              | 0                                                                | 0                                              |       |
| CEA at diagnosis                           |                      |                                               |                                                                  |                                                 |       |
| 5 or less                                  | 44 (50.0)            | 36 (47.4)                                     | 4 (66.7)                                                         | 4 (66.7)                                       | .462  |
| More than 5                                 | 44 (50.0)            | 40 (52.6)                                     | 2 (33.3)                                                         | 2 (33.3)                                       |       |

*Post hoc analysis showed statistically significant differences when comparing patients with pathogenic CRC predisposition gene variants against those without any pathogenic variants (P = .026) and those with pathogenic DNA repair gene variants (P = .027). AJCC = American Joint Committee on Cancer; CEA = carcinoembryonic antigen test; CRC = colorectal cancer; ECOG = Eastern Cooperative Oncology Group Performance Status; IHC = immunohistochemistry; MMR = mismatch repair; NOS = not otherwise specified.

†Statistically significant difference was observed between patients with pathogenic CRC predisposition gene variants and those without any pathogenic variants (P = .004).
outcomes (25). These benefits could be extended to more patients, given that young-onset CRCs had a heritable component beyond CRC predisposition genes.

Previously, pathogenic variants identified among CRC patients predominantly involved CRC predisposition genes, partly because of use of limited gene panels (5,10,11). Broader use of pan-cancer panels resulted in increasing discovery of pathogenic variants in DNA repair genes and an enrichment of pathogenic \( \text{PALB2} \) variants was observed in a study on CRC patients (5,7,11,12). It is conceivable that HR deficiency contributed to CRC tumorigenesis, given the indispensable role of HR in repair of highly damaging double-stranded DNA breaks (26).

In accordance with the Knudson two-hit hypothesis, deficient repair process and eventual tumorigenesis resulted from the inactivation of a wild-type allele in an individual with a germline pathogenic variant (27). Although tumor LOH has not been reported in patients with \( \text{PALB2} \) and \( \text{BRCA2} \) pathogenic variants, these variants could still be relevant in CRC tumorigenesis. Here, we demonstrated that \( \text{BRCA2} \) and \( \text{PALB2} \) monoallelic pathogenic variants were associated with impaired RAD51 foci formation. Likewise, previous studies established haploinsufficiency for both \( \text{BRCA2} \) and \( \text{PALB2} \) where a monoallelic pathogenic variant sufficed to directly impair function or promote the inactivation of the wild-type allele (28,29). In addition, tumor LOH was absent in most \( \text{PALB2} \) germline mutation carriers and most \( \text{PALB2} \) heterozygous cancers displayed high HR deficiency scores (12,30). An impaired HR might predispose the patient to worsening genomic instability and eventual tumorigenesis (26). Clinically, patients with pathogenic variants in DNA repair genes did not share any characteristic phenotype suggestive of a distinct CRC subset, unlike those with pathogenic variants in the CRC predisposition genes who tended to be younger with more right-sided colon involvement and earlier disease stages (5,7,11,12). To ascertain whether germline pathogenic variants in HR genes were truly drivers of CRCs and whether these CRCs shared a distinct tumor phenotype, more

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**Figure 1.** A) Variants identified in this study. B) Locations of pathogenic DNA repair variants. Red represents frameshift mutations; blue represents missense mutations. \( \text{BRCA2} \) c.440A>G (p.Q147R) is a VUS included in the functional studies. CRC = colorectal cancer; VUS = variants of uncertain significance.
studies on tumor LOH and mutational signatures in various HR-
deficient CRCs will be needed. Besides HR, there were other prominent DNA repair path-
ways such as nonhomologous end joining and base excision repair (29,31,32). Nonhomologous end joining was the alternative repair process for double-stranded DNA breaks under regulation by checkpoint kinases such as \(\text{ATM}\) (32). Germline variants of \(\text{ATM}\) were associated with autosomal recessive ataxia telangiec-
tasia and autosomal dominant cancers, namely breast cancer. More recently, pathogenic variants in \(\text{ATM}\) were also found to be enriched in CRC patients (7,11,12). However, the precise CRC risks for \(\text{ATM}\) pathogenic variants were unclear and \(\text{ATM}\) was not considered a CRC predisposition gene by the National Comprehensive Cancer Network guidelines (12,33–35).

Apart from \(\text{ATM}\), we identified monoallelic variants in \(\text{NTHL1}\) and \(\text{WRN}\). The \(\text{NTHL1}\) gene was involved in base excision repair, and was recently validated as an autosomal recessive CRC susceptibility gene after \(\text{NTHL1}\) biallelic variants were found to cause CRCs (36–38). A homozygous nonsense variant in \(\text{NTHL1}\) (p.Gln90*) was detected among seven CRC patients from three unrelated families whereas compound heterozygous \(\text{NTHL1}\) variants were found in a patient with multiple primary tumors, including CRC (38). For \(\text{WRN}\), which encoded for a heli-case with exonuclease function, preliminary studies hinted at the possibility of \(\text{WRN}\)-BRCA1 interaction in HR pathway (39). Pathogenic variants in \(\text{WRN}\) were predominantly truncating and \(\text{WRN}\) somatic hypermethylation was also observed in various tumors, including colon tumors (40). However, the in-
creased risk of neoplasia was only seen in biallelic \(\text{WRN}\) mutations (41). Similar to \(\text{NTHL}\), the unclear role of \(\text{WRN}\) monoallelic variants in young-onset CRCs will require further evaluation.

### Table 2. Clinicopathological characteristics of pathogenic variants

| Pathogenic variant | Sex | Race   | Current age, y | Age at CRC diagnosis, y | Other personal history of cancers | Family history of cancers in first-degree relatives | MMR status |
|--------------------|-----|--------|----------------|-------------------------|-----------------------------------|------------------------------------------------|------------|
| APC c.1884_1885delTT | Male | Indian | 34            | 29                      | Familial adenomatous polyposis; multiple gastric and colonic polyps | Not recorded* | Not recorded |
| APC c.3921_3925delAAAG | Female | Indian | 23            | 18                      | Familial adenomatous polyposis; single gastric and multiple colonic polyps | Mother (adenomatous polyposis coli) | Not recorded |
| APC c.4615delIT | Male | Chinese | 39            | 32                      | Gardner syndrome, with synchronous desmoid tumor at 32yo; multiple gastric and colonic polyps | Mother (cervical cancer); siblings had negative findings on colonoscopy | Not recorded |
| APC c.3928A>T | Male | Chinese | 29            | 22                      | Familial adenomatous polyposis; multiple gastric and colonic polyps | Paternal cousin (unknown cancer); no family history of FAP | Proficient |
| MUTYH c.857G>A | Male | Chinese | Deceased at 50 | 49                      | None | Sister (uterine cancer, 60yo); brother (colon cancer, 63yo) | Not recorded |
| MUTYH c.934-2A>G | Female | Chinese | 53            | 49                      | None | Sister (uterine cancer, 60yo); brother (colon cancer, 63yo) | Proficient |
| ATM c.477_481delATCTC | Male | Chinese | 41            | 36                      | None | Father (Gastric cancer, 63yo) | Proficient |
| BRCA2 c.9154C>T | Male | Chinese | 52            | 46                      | None | Father (Gastric cancer, 63yo) | Proficient |
| NTHL1 c.793G>A | Male | Chinese | 56            | 48                      | None | Mother (colon cancer, 68yo); paternal uncle (colon cancer, 60yo); paternal aunt (breast cancer, 67yo); maternal uncle (melanoma, 40yo); maternal aunt (breast cancer) | Proficient |
| PALB2 c.1059delIA | Male | Chinese | 48            | 46                      | None | Father (lung cancer, 30yo); paternal uncle (colon cancer, 60yo); paternal aunt (breast cancer, 67yo); maternal uncle (melanoma, 40yo); maternal aunt (breast cancer); maternal cousin (ovarian cancer) | Proficient |
| WRN c.499_500delAA | Female | Chinese | 48            | 44                      | Krukenberg tumor, 44yo | Father (colon cancer, 75yo); paternal aunt (colon cancer, 60yo); maternal aunt (uterine cancer); maternal cousin (ovarian cancer) | Proficient |
| WRN c.499_500delAA | Male | Chinese | 55            | 49                      | None | Father (stomach cancer); brother (colon cancer) | Proficient |

*Not recorded in the patient’s electronic medical records. CRC = colorectal cancer.
|                        | Matthew et al. (7)  | Pearlman et al. (11) | Saud et al. (12) | Stoffel et al. (5) | Our study |
|------------------------|---------------------|----------------------|------------------|-------------------|-----------|
| **n**                  | 1058 (%)            | 450 (%)              | 680 (%)          | 430 (%)           | 88 (%)    |
| **Age of diagnosis**   | 55.7 ± 12.6 (y)     | 42.5                 | 68.8 ± 10.3      | 40.0              | 41.4 ± 0.8 |
| **Study setting**      | Gastrointestinal cancer institute | General oncology clinics | General oncology clinics | Cancer genetics clinics | General oncology clinic |
| **Genetic studies**    | Next-generation sequencing | Next-generation sequencing | Next-generation sequencing | Next-generation sequencing | Next-generation sequencing |
| **Functional studies** | None                | Tumor LOH            | Tumor LOH        | None              | None      |
| **CRCs in first-degree relatives** | 138 (13.0) | 33 (45.8) | 164 (25.0) | 111 (25.8) | 13 (18.6) |
| **Breast cancers in first-degree relatives** | 138 (13.0) | 8 (11.1) | 85 (19.0) | NA | 4 (5.7) |
| **Ovarian cancers in first-degree relatives** | 23 (2.2) | 2 (2.8) | 19 (4.3) | NA | 1 (1.4) |
| **Any cancers in first-degree relatives** | 870 (82.2) | 53 (11.8) | 395 (59.0) | 226 missing data | 33 (36.8) |
| **MMR-deficient tumors/MSI-H** | 83 (14.5); 486 missing data | 48 (10.7); no missing data | 92 (16.0); 113 missing data | 41 (0.1); 226 missing data | 0 (0.0); 19 missing data |
| **CRC predisposition genes tested** | APC, BMPR1A, CDH1, CHEK2, EPCAM, MLH1, MSH2, MSH6, MUTYH, PMS2, PTEN, SMAD4, STK11, TP53 | APC, BMPR1A, CDH1, CHEK2, EPCAM, MLH1, MSH2, MSH6, MUTYH, PMS2, PTEN, SMAD4, STK11, TP53 | APC, BMPR1A, CHEK2, MLH1, MSH2, MSH6, MUTYH, PMS2, POLD1, POLE, PTEN, SMAD4, STK11, TP53 | APC, AXIN2, BMPR1A, BRAF, CDH1, CHEK2, EPCAM, KRAS, MLH1, MSH2, MSH6, MUTYH, PMS2, POLD1, POLE, PTEN, SMAD4, STK11, TP53 | APC, AXIN2, BMPR1A, BRAF, CDH1, CHEK2, EPCAM, FLCN, GREM1, MLN1, MSH2, MSH6, MUTYH, PMS2, POLD1, POLE, PTEN, SMAD4, STK11, TP53 |
| **DNA repair genes tested** | ATM, BARD1, BRCA1, BRCA2, BRIP1, CDK4, CDKN2A, CHEK2, NBN, PALB2, RAD51C, RAD51D | ATM, BARD1, BRCA1, BRCA2, BRIP1, CDK4, CDKN2A, CHEK2, NBN, PALB2, RAD51C, RAD51D | ATM, ATR, BAP1, BARD1, BLM, BRCA1, BRCA2, BRIP1, DDB2, ERCC2, ERCC3, ERCC4, ERCC5, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCN, FANCQ, FANCR, GEN1, MRE11A, NBN, NTH11, PALB2, PCNA, POLH, RAD51, RAD51C, RAD51D, RAD54L, RECA4, SLX4, UBE2T, WRN, XPA, XPC, XRCC3 | ATM, ATR, BAP1, BARD1, BLM, BRCA1, BRCA2, BRIP1, DDB2, ERCC2, ERCC3, ERCC4, ERCC5, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCN, MRE11A, NBN, NTH11, PALB2, PCNA, POLH, RAD51, RAD51C, RAD51D, RAD54L, RECA4, SLX4, UBE2T, WRN, XPA, XPC, XRCC3 | ATM, ATR, BAP1, BARD1, BLM, BRCA1, BRCA2, BRIP1, CDK4, CDKN2A, CHEK2, NBN, PALB2, PCNA, POLH, RAD51, RAD51C, RAD51D, RAD54L, RECA4, RET, RFWD3, SLX4, UBE2T, WRN, XPA, XPC, XRCC3 |
| **DNA used**           | Germline DNA        | Germline DNA and tumor DNA | Germline DNA     | Germline DNA      | Germline DNA and tumor DNA | (continued) |
Table 3. (continued)

| Pathogenic variants in CRC predisposition genes | Matthew et al. (7) n = 1058 (%) | Pearlman et al. (11) n = 450 (%) | Saud et al. (12) n = 680 (%) | Stoffel et al. (5) n = 430 (%) | Our study n = 88 (%) |
|-----------------------------------------------|---------------------------------|---------------------------------|-----------------------------|--------------------------|-------------------|
| **Pathogenic variants in DNA repair genes**    | MLH1 (n=13), MSH2 (n=7), MSH6 (n=6), PMS2 (n=7), MUTYH biallelic (n=3), APC (n=19), MUTYH monoallelic (n=18), CHEK2 (n=2) | MLH1 (n=13), MSH2 (n=16), MSH6 (n=2), PMS2 (n=5), MUTYH biallelic (n=4), APC (n=10), MUTYH monoallelic (n=4), CHEK2 (n=4), TP53 (n=2) | MLH1 (n=24), MSH2 (n=25), MSH6 (n=5), PMS2 (n=2), APC (n=8), MUTYH monoallelic (n=6), CHEK2 (n=1), SMAD4 (n=2) | APC (n=4), MUTYH monoallelic (n=2) |
| **Features of patients with pathogenic CRC predisposition gene variants** | Younger age† | Younger age | Younger age | Right-sided colon involvement† | Younger age† |
| | Right-sided colon involvement† | Right-sided colon involvement | Family history of breast cancers† | MSI tumors† | Earlier stage of diagnosis† |
| | Earlier stage of diagnosis† | Earlier stage of diagnosis | MSI tumors | Family history of CRCs† | Earlier stage of diagnosis† |
| | Personal and family history of multiple CRCs† | Personal and family history of multiple CRCs† | None | None | None |

*Compared to noncarriers of germline pathogenic variants. CRC = colorectal cancer; digitalMLPA = digital multiplex ligation-dependent probe amplification; MMR = mismatch repair; MSI-H = microsatellite instability-high.
†Statistical significance (P < .05) was observed.
Table 4. Pathogenicity classification using ACMG criteria

| Variant          | Population data | Predictive data | Functional data | Segregation data | De novo data | Allelic data | Reputable database* | References |
|------------------|-----------------|-----------------|-----------------|------------------|--------------|--------------|--------------------|------------|
| APC c.1884_1885del | PM2             | PVS1            | NA              | NA               | NA           | NA           | NA                 | NA         |
| APC c.3921_3925del | PM2             | PVS1            | PM1             | NA               | NA           | NA           | NA                 | PP5        |
| APC c.615delT     | PM2             | PVS1            | NA              | NA               | NA           | NA           | NA                 | NA         |
| APC c.3928A>T     | PM2             | PVS1            | PM1             | NA               | NA           | NA           | NA                 | NA         |
| MUTYH c.857G>A    | BS1             | PVS1            | PS3             | NA               | NA           | NA           | NA                 | PP5        |
| ATM c.477_481delATCTC | PM2         | PVS1            | NA              | NA               | NA           | NA           | NA                 | PP5        |
| BRCA2 c.9154C>T   | PM2             | PP3             | PS3             | NA               | NA           | NA           | NA                 | PP5        |
| NTNL1 c.793G>A    | PM2             | PP3             | PM1             | NA               | NA           | NA           | NA                 | NA         |
| PALB2 c.1059delA  | PM2             | PVS1            | NA              | NA               | NA           | NA           | NA                 | PP5        |
| WRN c.499_500delAA | PM2            | PVS1            | PM1             | NA               | NA           | NA           | NA                 | NA         |
| VUS              | PM2             | NA              | NA              | NA               | NA           | NA           | NA                 | NA         |

*Variants were assessed for pathogenicity using ClinVar archives, Mastermind search engine by Genomenon, and genetic databases such as the Leiden Open Variation Database (LOVD), BRCA Exchange, InSiGHT, and ARUP MEN2 database. VUS = variants of uncertain significance.

Figure 2. A) Nuclear localization of RAD51 expressed as a ratio of nuclear (N) to cytoplasmic (C) RAD51 levels. Cells with BRCA2 c.9154C>T, PALB2 c.1059delA, and BRCA2 c.8945_8946delAA showed impaired RAD51 nuclear localization at the first hour posttreatment, whereas those with BRCA2 c.440A>G displayed normal RAD51 nuclear localization. Triplicates were performed for patients with pathogenic variants. Three healthy controls were used and duplicates were done per control. Independent t test was used to compare RAD51 nuclear localization between variants and healthy controls. A single asterisk (*) refers to P < .05, a double (**) to P < .005. B) Representative blot showing changes in nuclear and cytoplasmic RAD51 levels at 1 hour and 6 hours following etoposide treatment. In the healthy controls, nuclear RAD51 was higher than the cytoplasmic RAD51 following treatment. In contrast, the nuclear RAD51 remained similar to the cytoplasmic RAD51 in BRCA2 c.9154C>T, PALB2 c.1059delA, and BRCA2 c.8945_8946delAA. DMSO = dimethyl sulfoxide.
The cumulative risk of young-onset CRC associated with MUTYH monoallelic variants was modest and statistically insignificant (0.8%, 95% CI = 0.5% to 1.3%), unlike those of biallelic MUTYH variants (24.8%, 95% CI = 7.7% to 57.1%) (42). Moreover, the frequency of MUTYH monoallelic variants in CRC patients (1.6% to 2.2%) was similar to that of controls (1.7%) (7,11,12,43). Currently, CRC screening guidelines do not require carriers of MUTYH monoallelic variants to undergo more intensive screening than the general population (44).

In addition to identifying pathogenic variants, we functionally evaluated a VUS in BRCA2 (c.440A>G). This variant was previously reported in a Chinese family with multiple breast cancers where it was found in all three sisters with breast cancers and absent in the father and another unaffected sister (45). However, we did not observe any functional evidence for pathogenicity consistent with the low in silico prediction score for pathogenicity (REVEL score = 0.338). Hence we concluded that this variant was unlikely to be pathogenic.

However, our study has several limitations. We had a small sample size of 88 patients. Our study was thus underpowered to perform statistical inference of the odds ratio, which was required as part of the ACMG curation criteria (20). Nonetheless, we identified similar clinical and genetic features as the larger studies. In addition, our cohort had similar characteristics of young-onset CRCs as the earlier studies. Another limitation was that we were unable to perform tumor LOH analysis for the MUTYH pathogenic variants, which would have been useful in confirming their pathogenicity status.

To reliably interpret germline variants, both genetic and functional evidence are required. Currently, there is a lack of
functional validation of pathogenic variants in the DNA repair genes. Most were limited to tumor LOH studies. Although presence of biallelic inactivation offered evidence for the variant as a driver mutation, monoallelic variants could still have an impact on tumorigenesis. Direct assessment of protein function would be essential to determine the pathogenicity of a variant in question, especially for genes such as BRCA2 and PALB2, which exhibited haploinsufficiency and did not require LOH to exert deleterious functional effects. Complementing tumor LOH studies, our patient-based RAD51 assays could directly evaluate the function of the variant protein.

A substantial portion of patients with young-onset CRC had biallelic germline pathogenic variants. Excluding those with hereditary CRC syndromes, most patients with pathogenic variants did not have high-risk phenotypic features. Hence, germline genetic testing should preferably be performed for all young-onset CRC patients. Screening of germline variants in HR genes such as BRCA2 and PALB2 might improve the yield of germline genetic testing. These genes contributed a considerable portion of the pathogenic variants identified among young-onset CRCs and many had established risk management guidelines (46). Furthermore, pathogenic variants in these genes do exert functional consequences and are potentially tumorigenic. Although these genes have yet to be acknowledged as CRC predisposition genes, the evidence is growing. Larger population studies and genome-wide association studies will provide the much-needed conclusion on their relevance in CRC tumorigenesis.

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The authors declare no relevant conflict of interest.

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