Germline mutations in a DNA repair pathway are associated with familial colorectal cancer

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Introduction

Colorectal cancer (CRC) is a common disease with a high mortality rate in the world. Germline predisposition and environmental factors affect CRC susceptibility. Importantly, the inherited germline contribution is known to influence about 12%–35% of all cases (1, 2). However, only 5%–7% of CRC cases are caused by germline mutations in genes that are responsible for Mendelian cancer syndromes. Lynch syndrome and familial adenomatous polyposis (FAP) are the most frequent forms of Mendelian CRC syndromes. Classic hereditary CRC syndromes are mainly due to germline mutations in genes that are responsible for Mendelian cancer syndromes. Lynch syndrome and familial adenomatous polyposis (FAP) are the most frequent forms of Mendelian CRC syndromes. Lynch syndrome and familial adenomatous polyposis (FAP) are the most frequent forms of Mendelian CRC syndromes.

In addition to hereditary forms, around 30% of CRC cases also present familial aggregation — but with an unknown inherited cause. The hypothesis of rare high-penetrance mutations in genes yet to be discovered is a very likely explanation for the underlying predisposition in a portion of these familial CRC cases. Therefore, past efforts have been made in these familial CRC cases, and next-generation sequencing technologies added a new unbiased approach to facilitate the identification of new genes responsible for predisposition to CRC. New candidate genes related to CRC have been found, such as POLQ, POLE (encoding DNA polymerases), NTHL1 (encoding a base-excision repair protein), MSH3, GREM1, RNF43, RSP20, MLH3, FAF1, and MCM8 (5–14). However, a large part of the heritability of colorectal adenomatous polyposis and CRC remains unexplained and is widely postulated to be enshrined in unidentified, rare variants.

With the aim of identifying new hereditary CRC genes, we performed whole-exome sequencing (WES) in patients with familial CRC. Our final goal is to facilitate genetic counsel and to be able to correctly address prevention strategies in these families.
Results

Clinical characteristics and germline sequencing results. We examined 24 individuals from 21 families with CRC or advanced colorectal adenoma (CRA) by WES (Supplemental Tables 1 and 2; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.148931DS1). All individuals had strong disease aggregation compatible with an autosomal-dominant pattern of inheritance. To exclude known Mendelian cancer syndromes, all individuals were screened for mutations in APC, MUTYH, and mismatch repair genes (MSH2, MSH6, PMS2, MLH1) before WES analysis. All individuals were tested without mutations in known hereditary CRC, including FAP or Lynch syndrome.

WES was performed in all individuals with a mean coverage of 86× (Supplemental Table 3). Germline WES data analysis was selected for only vary rare variants (<0.1%) producing a loss of function (LOF) or variants located on genes with a function compatible with cancer development (7, 8, 10).

After filtering the variants, we detected LOF mutations in 6 different genes (RMI1, PALB2, FANCI, AMER1, CTNNB1, SGK2; Figure 1 and Table 1). Among this, we found 3 mutations related to the Fanconi anemia (FA) DNA repair pathway (RMI1, PALB2, FANCI; Table 1). We also found 3 mutations in POLE in 2 individuals. All variants were verified by Sanger sequencing (Supplemental Figure 1). Additional segregation for these variants was performed when available. Unfortunately, most of family members were not accessible (Figure 1).

In addition, we found 1 potentially pathogenic CHEK2 variant in the pedigree K (Figure 2A) that contained 2 individuals (II-1 and III-1) that had been sequenced. In this family, the proband (individual III-1) was first diagnosed with CRA at 50 years old and had recurrently multiple CRAs and 7 years later, with the biggest adenoma being 1.5 × 1.5 cm and tubular. The proband’s father (individual II-1) was diagnosed with sigmoid colon adenocarcinoma at 73 years old due to ileus and underwent a colectomy. He was followed by colonoscopy every 6 or 12 months. At least 33 polyps were detected in his colon, with the biggest polyp being 2.5 × 2.3 cm, and they were predominantly advanced adenomas. The proband’s brother (individual III-2) was first diagnosed with multiple CRAs at 49 years old. The proband’s grandfather was diagnosed with gastric cancer at 70 years old and died several months after surgery. All the known members of this pedigree were not diagnosed with breast cancer.

We searched the sequences for genes that harbored damaging variants. After performing variant filtration and quality control, we filtered annotated variants and identified 3 heterozygous variants in 3 genes (Supplemental Table 4; Supplemental Table 5, A and B; Figure 2B; and Supplemental Table 6 for analysis flow chart). Two of these variants were missense mutations in CHD8 and MOB1B, but the mutations were not found in the key domain of the protein. The third variant contained the highest Combined Annotation-Dependent Depletion (CADD) score (CADD score of 35) and was the only LOF variant. This CHEK2 (GenBank: NM_001126112:c.742C>T, p.Gln27*, also named as p.Q27*; Figure 2C). Sanger sequencing further confirmed the variant in individuals II-1 and III-1 (Figure 2D), as well as another affected family member (III-2; Figure 2D). Segregation analysis revealed the presence of the same germline CHEK2 variant in affected family members (II-1, III-1, III-2) — where 1 individual exhibited CRC and multiple CRAs (individual II-1), and 2 individuals exhibited multiple CRAs (individual III-1 and III-2; Figure 2A). The 1000 Genomes Project, Exome Aggregation Consortium (ExAC), and the Genome Aggregation Database (gnomAD) do not contain this variant, confirming the identified mutation as a rare event (ftp-trace.ncbi.nih.gov/1000genomes; https://gnomad.broadinstitute.org/).

Hence, we consider this mutation (CHEK2 p.Q27*) as a good candidate for CRA/CRC.

Tumor analysis. Furthermore, we examined 7 tumors (6 adenomas and 1 carcinoma) from 3 carriers of the CHEK2 p.Q27* variant using WES analysis (Supplemental Tables 7 and 8) to explore somatic mutations in tumor tissues. All tumors were screened for KRAS and BRAF driver mutations, and a total of 83 cancer-related genes were screened, including APC, CTNNB1, PIK3CA, and FBXW7 (Supplemental Table 9 and refs. 15, 16). All tumors were microsatellite stable (MSS; Table 2 and Supplemental Table 8). As shown in Table 2, somatic mutations in tumor tissues were mainly found in APC, TP53, and FBXW7. For APC, all mutations were LOF variants (nonsense and frameshift). There was also a missense mutation in TP53 (NM_001126112:c.742C>T, p.R248W) in the carcinoma tissues, suggesting that TP53 may be involved in the colorectal tumorigenesis in this case. The TP53 mutation was located in the DNA-binding domain, which is an important structural domain. However, mutations were not found in other pathogenic genes, including CTNNB1, KRAS, and BRAF.

Loss of heterozygosity (LOH) involving the germline WT allele was found in the cancer tissue of II-1 (Supplemental Table 8). However, IHC staining of Chk2 in the adenoma tissues of 3 CHEK2 variant carriers including...
individual II-1 revealed nuclear expression of the protein (Figure 3). It is noteworthy that in II-1’s cancer tissue, Chk2 expressed in adjacent normal cells but not in cancer cells, indicating somatic inactivation of the WT allele in cancer cells. This is consistent with the \( \text{CHEK2} \) LOH in cancer tissue detected by WES (Supplemental Table 8).

**CRISPR/Cas9 CHEK2 KO modeling.** As shown in pedigree K, we found that the \( \text{CHEK2} \) variant (p.Q27*) led to a premature stop at amino acid 27 and resulted in the loss of normal protein structure and function in Chk2. Using CRISPR/Cas9 technology, we generated \( \text{CHEK2} \) knock-out \( \text{(CHEK2}^{\text{KO}} \text{)} \) SW480 cells to reproduce the LOF mutant and further investigate the physiological role of Chk2 (Figure 4A). According to bioinformatic CRISPR prediction tools, a single-guide RNA (sgRNA) targeting the tenth exon was selected. The genotype

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**Figure 1. Pedigrees of study participants with strong family histories of CRC and the identifiable mutations.** (A–I) Squares indicate male family members, and circles represent female members. A slash through a symbol indicates that the family member has died. Filled symbols indicate those affected by colorectal cancer or advanced colorectal adenomas. (+), mutation carrier; (−), nonmutation carrier. The proband is indicated by an arrow. The clinical details, including the features of the additional relatives and the number of tumors in those families, are shown in Supplemental Table 13.
of \textit{CHEK2}^{	ext{KO}}$ clones was determined by Sanger sequencing (Figure 4B). The complete loss of Chk2 production in \textit{CHEK2}^{	ext{KO}}$ cells was confirmed by Western blot analysis (Figure 4C). We found that there was no influence on cell proliferation after \textit{CHEK2}^{	ext{KO}}$ (Figure 4D). This indicated that this CRISPR cell model can be used to explore the functional characterization of \textit{CHEK2}.

\textbf{Functional characterization of germline variants.} \textit{CHEK2}^{	ext{KO}}$ cells show a defect in G2 cell cycle arrest after DNA damage. Given that Chk2 is activated in response to DNA damage and may regulate cell cycle arrest, we treated \textit{CHEK2}^{	ext{WT}}$ and \textit{CHEK2}^{	ext{KO}}$ SW480 cells with nocodazole, a microtubule-disrupting agent that traps cells in mitosis. When cells were treated with nocodazole at different concentrations, the expression of phospho-\gammaH2AX was elevated when compared with the untreated cells, and DNA damage was induced (Supplemental Figure 2A). When cells were treated with nocodazole for 6 hours, the cell cycle was arrested and about 50\% of \textit{CHEK2}^{	ext{WT}}$ and \textit{CHEK2}^{	ext{KO}}$ cells were arrested in G2 phase (Figure 5, A and B). After 12 hours, more \textit{CHEK2}^{	ext{KO}}$ cells entered G1 and S phases relative to \textit{CHEK2}^{	ext{WT}}$ cells. These results indicated that there was a defect in G2 cell cycle arrest in \textit{CHEK2}^{	ext{KO}}$ cells after DNA damage.

\textit{CHEK2}^{	ext{KO}}$ cells were resistant to DNA damage–induced apoptosis and influence p53 phosphorylation during DNA damage. If damaged DNA cannot be repaired, cells can initiate apoptosis, which may also be regulated through the Chk2 kinase. To investigate the role of Chk2 in apoptosis, Adriamycin (which can induce double-stranded DNA breaks) was used to treat \textit{CHEK2}^{	ext{WT}}$ and \textit{CHEK2}^{	ext{KO}}$ cells. When \textit{CHEK2}^{	ext{WT}}$ cells were treated

### Table 1. Description of the candidate variants

| Gene   | Mutation         | SNP ID       | Protein effect             | CADD_PHRED | ClinVar accession no. | gnomAD_exome | Dormain/region                  | Function                                                                 |
|--------|------------------|--------------|-----------------------------|------------|-----------------------|--------------|---------------------------------|--------------------------------------------------------------------------|
| RMI1   | c.1281_1285del   | rs764792608  | Frameshift deletion         | 4.18E-06   | SCV001754808          |              |                                 | Fanconi anemia pathway, double-strand break repair via homologous repair |
| PALB2  | c.172_175del     | rs180177143  | Frameshift deletion         | 3.66E-05   | SCV001754809          |              |                                 | Fanconi anemia pathway, double-strand break repair via homologous repair |
| FANCI  | c.2960C>T        | rs138432305  | Missense                    | 2.84E-05   | SCV001754757          |              | FANCI solenoid 3 domain         | Fanconi anemia pathway, interstrand cross-link repair                   |
| BRF1   | c.1954G>A        | rs202049411  | Missense                    | 8.98E-05   | SCV001754761          |              |                                 | DNA-templated transcription, positive regulation of transcription by RNA polymerase III |
| CHEK2  | c.79C>T          | rs202049411  | Stopgain                    | 35         | SCV001754535          |              |                                 | DNA damage checkpoint                                                  |
| SGK2   | c.560G>A         | rs35187177   | Missense                    | 34         | SCV001754807          | 5.83E-05     |                                 | PI3K-Akt signaling pathway                                              |
| CTNNB1 | c.1444C>G        | rs0482E      | Missense                    | 23.8       | SCV001754805          |              | Armadillo-like helical           | Wnt signaling pathway                                                   |
| AMER1  | c.3145C>T        | rs1049*      | Stopgain                    | 36         | SCV001754806          | 5.96E-06     |                                 | p53 destruction complex assembly                                       |
| POLE   | c.1187A>G        | rs0496G      | Missense                    | 27.8       | SCV001754759          |              |                                 | DNA-directed DNA polymerase, family B, exonuclease domain               |
| POLE   | c.2925G>A        | rs142563997  | Missense                    | 35         | SCV001754762          | 3.25E-05     |                                 | DNA-directed DNA polymerase, family B, multifunctional domain           |
| POLE   | c.3146C>T        | rs780299O12  | missense                    | 20.4       | SCV001754763          | 3.27E-05     |                                 | Ribonuclease H-like domain                                              |

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Table 1. Description of the candidate variants
with Adriamycin (6 μM), around 69% and 57% of cells were viable after 24 and 48 hours, respectively (Figure 5, C and D, and Supplemental Figure 2B). With the same concentration of Adriamycin, around 80% and 71% of CHEK2 KO cells were viable after 24 and 48 hours, respectively (Figure 5, C and D, and Supplemental Figure 2B). There was a significantly lower percentage of apoptotic cells in Adriamycin-treated CHEK2 KO cell samples compared with Adriamycin-treated CHEK2 WT cell samples (Figure 5, C and D). The crystal violet

Figure 2. Gene discovery and characterization. (A) Pedigree K. Squares indicate male family members, and circles represent female members. A slash through a symbol indicates that the family member has died. Filled symbols indicate a clinically affected family member. The proband is indicated by an arrow. WES analysis was performed on 2 individuals, which are marked by the letter S. The CHEK2 p.Q27* mutant germline allele that was detected by Sanger sequencing is shown below each individual. “V/N” indicates a heterozygous variant carrier, and “N/N” indicates a noncarrier. Age of tumor diagnosis is shown beneath each symbol. (B) The filter-based computational algorithm that is used to narrow candidate variants for pedigree K. Single-nucleotide variant, SNV; minor allele frequency, MAF. (C) The functional domains of Chk2 and the predicted truncated Chk2 protein that would result from the variant. SQ/TQ indicates the SQ/TQ motif, which is the consensus site for phosphoinositide-kinase-related kinases (PIKKs). FHA indicates the forhead-associated domain. NLS indicates the nuclear localization signal. (D) Sanger sequencing–based validation of the germline CHEK2 p.Q27* mutation in individuals of this pedigree. The red arrowhead and black box indicate the location of the heterozygous substitution of C to T in the mutated locus of CHEK2.
assay also confirmed that Adriamycin-treated CHEK2 KO cells had higher cell viability than Adriamycin-treated CHEK2 WT cells. As the concentration of Adriamycin increased, the difference in cell survival between CHEK2 WT and CHEK2 KO cells became apparent (Figure 5, E and F). CHEK2 KO cells were also resistant to apoptosis induced by other agents that induce DNA damage, such as nocodazole (Figure 5E and Supplemental Figure 2, C and D). This result indicates that CHEK2 KO cells were resistant to DNA damage-induced apoptosis.

It has been reported that Chk2 regulates cell cycle arrest and apoptosis during the DNA damage response through p53. Chk2 can directly phosphorylate p53 on serine20 to disrupt its association with Mdm2, thus promoting its stability (17–19). To explore the function of Chk2 in p53 regulation, the protein level of p53 after DNA damage was assessed. As shown in Figure 5G, CHEK2 WT cells expressed high protein levels of phosphorylated p53 on serine 20 when treated with Adriamycin. In contrast, CHEK2 KO cells had reduced levels of pan-p53 and phosphorylated p53 compared with CHEK2 WT cells (Figure 5G). This result suggests that Chk2 may influence p53 phosphorylation during DNA damage.

Screening of the candidate gene variants in an independent cohort. In order to investigate the frequency of CHEK2 in sporadic CRC/CRA and normal populations, we genotyped another cohort to screen for the mutation encoding the CHEK2 p.Q27* variant. The cohort included 352 individuals of Chinese ancestry with colorectal tumors (including 230 individuals with CRC and 122 individuals with CRA; Supplemental Table 10). For comparison, we genotyped 100 control individuals, who were of Chinese ancestry but had not been diagnosed with polyposis or CRC (Supplemental Table 10). We found no additional cases or no controls that were heterozygotes for the mutation encoding the CHEK2 p.Q27* variant, further confirming CHEK2 p.Q27* was a rare event.

We screened the entire CHEK2 coding sequence in the germline DNA from 126 sporadic CRC patients by Sanger sequencing. An additional rare missense variant (NM_001005735:c.766 C>G, p. P256A) was detected in these sporadic cases (Supplemental Figure 1). This identified missense variant was not found in a large population dataset (gnomAD; https://gnomad.broadinstitute.org/) and was predicted to be potentially pathogenic in silico tools (e.g., SIFT prediction: deleterious) (http://provean.jcvi.org/index.php). However, segregation for the detected genetic variant was not available in the family.

**Discussion**

In this study, we identified potential CRC predisposition variants in genes (RMI1, PALB2, FANCI, and CHEK2) that regulate the DNA damage response, including the FA DNA repair pathway and the cell cycle.

It is widely held that genomic instability is a prerequisite for cancer formation. Colorectal epithelial cells are recurrently exposed to endogenous and exogenous mutagens and having high turnover rates (20). Therefore, DNA damage repair (DDR) mechanisms are fundamental to maintain the genomic integrity of the cell. The malfunctioning of DDR is strongly associated with carcinogenesis agents. Checkpoint mechanisms serve a major regulatory function in governing the DDR and ensure the coordination of DNA repair proteins, which detect and repair DNA damage to protect cells from genome instability.

FA is an inherited genomic instability disorder that contains bone marrow failure, growth abnormalities, and cancer predisposition. FA patients have chromosome fragility and hypersensitivity to drugs that induce DNA interstrand crosslinks (ICLs; ref. 21). The FA repair pathway is thought to coordinate a complex mechanism that contains elements of 3 classic DNA repair pathways in response to genotoxic insults. These 3 pathways include homologous recombination, nucleotide excision repair, and mutagenic translation synthesis (22). When the FA pathway is impaired, cells are hypersensitive to DNA damage and are unable to
successfully repair damaged DNA and cause genome instability. Mutations in the FA proteins lead to a high tumor incidence. Previous studies have indicated that malfunctioned FA genes and proteins have been found to be associated with a variety of cancers (23–27).

Previous studies have found FANCD2/FANCI-associated nuclease 1 gene (FAN1) mutations in the inherited susceptibility to CRC (28). In our study, by exome sequencing, we identified 3 potential CRC predisposition variants (RMI1, PALB2, FANCI) that were involved in the FA repair pathway.

RecQ-mediated genome instability protein 1 (RMI1) is an essential component of the RMI complex that plays an important role in the processing of homologous recombination. The RMI1 mutation (NM_024945: c.1281_1285del, p.I427fs) (ClinVar accession SCV001754808) is predicted to cause a premature stop and loss of protein function. It is likely to contribute to genomic instability. It has been reported that the RMI1 gene polymorphisms were associated with the risk of cancer (29, 30), but further studies are needed to investigate.

Partner and localizer of BRCA2 (PALB2, also termed FA complementation group N [FANCN]) plays a critical role in homologous recombination repair (HRR) through its ability to recruit BRCA2 and RAD51 to DNA breaks. Mutations in PALB2 have been reported in breast cancer, Fanconi anemia subtype FA-D1, and pancreatic cancer (25, 26, 31–33). Previous studies also identify the association between PALB2 mutations and early-onset CRC (34). In our study, the PALB2 mutation (NM_024675: c.172_175del, p.L58fs) (ClinVar accession SCV001754809) is predicted to abolish protein function, thus causing the LOF in the HRR. It is likely to cause the impaired FA repair pathway and contribute to tumorigenesis.

Figure 3. Chk2 IHC staining in the colorectal tumors developed by mutation carriers or WT controls. Representative photos of Chk2 IHC staining. Left, CHEK2 mutation carriers II-1, III-1, and III-2. Right, CHEK2 WT controls. Note the cancer cells with no nuclear staining, whereas the adjacent normal cells show strong staining in family member II-1. Scale bars: 50 μm or 100 μm, as indicated.
FA Complementation Group I (FANCI) plays an essential role in the repair of DNA double-strand breaks by homologous recombination. It takes part in the repair of DNA ICLs with FANCD2. It has been reported that FANCI mutations possibly involved in breast cancer and ovarian cancer susceptibility (27, 35). The FANCI mutation (NM_001113378: c.2960C>T, p.T987M) (ClinVar accession SCV001754757) is located inside the FANCI solenoid 3 domain. In silico pathogenicity tools predicted it as a possible pathogenic mutation. Because the unique nuclear protein complex that ubiquitinates FANCD2 and FANCI leads to formation of DNA repair structures, it is postulated that they may affect cancer risk in a specific manner.

FA repair pathway plays an important role in maintaining the genome stability; cell cycle regulations also are critical for DDR. In our study, we also identified a rare genetic variant with plausible pathogenicity in the CHEK2 gene in 3 individuals from 1 family with CRC. A functional characterization of mutation was performed in CRISPR/Cas9 cellular model to further confirm the pathogenicity and involvement in germline predisposition to CRC.

The serine/threonine protein kinase Chk2 encoded by CHEK2 is activated in response to DNA damage and subsequently regulates downstream effector proteins, including p53, BRCA1, and BRCA2, which are critical for DNA repair, cell cycle regulation, and cellular apoptosis (36, 37). Given the critical role of mitosis in cell survival, defects in cell cycle regulation may lead to abnormal cell division during DNA damage. Thus, germline mutation in CHEK2 may cause genomic instability and lead to cancer predisposition. Germline CHEK2 variants were first reported in families with Li-Fraumeni syndrome that lack TP53 mutations (38). Later, numerous studies reported that CHEK2 is a multiorgan cancer susceptibility gene, such as breast (39), ovarian (40), prostate (41), and renal cancer (42). Recently, a multicenter case-control analysis using WES provided evidence for germline CHEK2 LOF variants as new moderate-penetrance variants in testicular germ cell tumors (43). Although it has been reported that CHEK2 I157T associates with an increased risk of CRC (44), no causal germline mutation of CHEK2 in CRC has been identified. In our study, a new germline CHEK2 LOF mutation was found in a CRC family, which is unavailable in the gnomAD database. The identified LOF mutation in CHEK2 (CHEK2_p.Q27*) is predicted to cause a premature stop codon in the SQ/TQ motif, and in silico pathogenicity tools showed this mutation has the highest CADD score. We
also identified another rare genetic variant (NM_001005735:c.766 C>G, p. P256A) in CHEK2 in 126 sporadic CRCs. These mutations in CHEK2 may represent a genetic cause of intestinal neoplasia and cancer.

It is also known that Chk2 is activated during DNA damage and subsequently inhibits CDC25C phosphatase, preventing cells from entering mitosis, and stabilizes p53, resulting in a cell cycle arrest in G1. In our study, by performing CHEK2 gene editing in a cellular model, we were able to demonstrate its plausible effect on cell cycle arrest and maintain genomic integrity. Consistent with previous reports, CHEK2KO cells were unable to effectively maintain cell cycle arrest in the G2 phase with nocodazole treatment and were resistant to apoptosis after DNA damage. Lower levels of pan-p53 and phosphorylated p53 were detected in CHEK2KO cells compared with WT cells when double-strand DNA breaks...
were induced. These results are consistent with the view that Chk2 plays an important role in cell cycle regulation and genomic integrity maintenance.

Loss of cell cycle checkpoint capacity caused by CHEK2 mutant genotypes may lead to specific somatic mutations in affected colon tissues. In our study, we found APC somatic mutations in adenomas of CHEK2 mutant carriers. APC somatic mutations have been shown to precede other germline gene mutation carriers in CRC or adenomas (7, 8, 12). CHEK2 LOH was found in the cancer tissue of II-1, and IHC staining showed Chk2 expressed in adjacent normal cells but not in cancer cells, indicating second-hit somatic inactivation of the WT allele in cancer cells. In addition, in this pedigree, we also observed a preferential development of CRC with TP53 mutations in germline CHEK2 mutant carriers, where CHEK2 second-hit inactivation may precede TP53 mutation. In previous studies, somatic mutations in KRAS, BRAF, and PIK3CA have been found in CRC or adenomas from other germline gene mutation carriers. But in our study, somatic mutations were not found in these genes, suggesting that the tumors did not follow the pathway of colorectal tumorigenesis induced by somatic mutations in KRAS, BRAF, and PIK3CA. It has been reported that Chk2 can stabilize p53 during DNA damage. A possible mechanism is that the LOF Chk2 could not effectively stabilize p53.

Besides the genes related to the FA DNA repair pathway or the cell cycle, we also found another 2 genes (CTNNB1 and AMER1; CTNNB1:c.1444C>G; p.Q482E, AMER1:c.3145C>T; p.R1049*; ClinVar accessions SCV001754805 and SCV001754806) related to the Wnt signaling pathway. AMER1 is located on chromosome X, and the mutation carrier was a male patient. Therefore, the mutation in AMER1 was a homozygous variant. It is well known that the Wnt signaling pathway is strongly associated with colorectal carcinogenesis. Although somatic mutations in CTNNB1 occur frequently in colon cancer, germline mutations have been less implicated in hereditary CRC. This finding may indicate that germline mutations in CTNNB1 or AMER1 may be the candidate genes for CRC. Additionally, we also found a missense mutation in SGK2 that took part in the PI3K-Akt signaling pathway. We also identified rare variants in POLE and BRF1, which have been reported as the pathogenic genes (7, 45). Among this, 1 patient carrying the POLE variant was an early-onset CRC. Another patient carried compound heterozygous mutations in POLE. This confirmed that POLE takes part in the colorectal tumorigenesis.

In summary, our results highlight some candidate genes for CRC germline predisposition, which involved in DNA repair and the cell cycle. Our findings implicate germline CHEK2 mutations in the inherited susceptibility to CRC, as well as the defective cell cycle arrest and apoptosis, as the plausible underlying mechanism. Our results further support the relationship between DNA repair and cancer predisposition.

**Methods**

**Patients.** We selected 24 individuals from 21 families. All of these individuals had strong CRC aggregation, but other known germline alterations of hereditary cancer syndromes (FAP and Lynch syndrome) tested negative. APC and MUTYH were tested by Sanger sequencing to identify FAP. Lynch syndrome was excluded by IHC to test the expression of MSH2, MSH6, PMS2, and MLH1. These individuals fulfilled the following criteria: they had 2 or more relatives with CRC or CRA, and 2 or more consecutive affected generations, and all individuals screened negative for FAP and Lynch syndrome. Family members were included for segregation analysis of genetic variants.

In order to investigate the frequency of candidate mutations in sporadic CRC/CRA and normal populations, 100 healthy controls who were of Chinese ancestry but had not been diagnosed with polyposis or CRC and 352 individuals with colorectal tumor (CRC/CRA) were recruited from Renji Hospital, School of Medicine, Shanghai Jiao Tong University, for further variant genotyping.

**DNA extractions and WES.** Peripheral-blood genomic DNA was extracted by QIAamp DNA Blood Kit (Qiagen). WES was performed in individuals, similar to previous reports with some modifications (46). In brief, whole-exome capture and library preparation were performed using the Twist Fast Hybridization target enrichment system. The captured library was sequenced on the Illumina HiSeq platform (Illumina Nova 6000) according to the manufacturer’s protocol. Subsequently, reads were trimmed and mapped to the human hg19 genome reference assembly with Burrows-Wheeler Alignment (BWA) and sorted by Picard-tools. Single-nucleotide variants (SNVs) and indel variants were called with Genome Analysis Toolkit (GATK). The variants were further annotated by ANNOVAR with 1000 Genomes Project, gnomAD, ExAC, SIFT, PolyPhen2 (http://genetics.bwh.harvard.edu.ph2/dokuwiki/overview), MutationTaster (http://www.mutationtaster.org/), CLINVAR, CADD (http://cadd.gs.washington.edu/score), COSMIC (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/), Generic mutation, GO terms (http://www.geneontology.org/), KEGG pathway (http://www.genome.jp/kegg/pathway.html).
Bioinformatic analysis. To identify the candidate genes, we further analyzed the results using standard filters steps: (a) First, we excluded the variants that did not pass the quality filter. (b) Then, we excluded variants that were not protein-coding or splicing sites. Synonymous variants were also excluded. (c) We excluded variants in 1000 genomes or gnomAD or ExAC at frequency ≥ 0.001 (7, 8, 10). (d) We screened the deleterious variants by ≥ 3 in silico prediction tools for missense variants or frameshift variants. (e) We then presented the functional or bibliographical terms filter. (f) We screened the known CRC susceptibility genes including APC, MSH2, and PTEN (Supplemental Table 11). Individuals were selected if they carried the deleterious variants among these known genes. The other individuals did not carry the known CRC germline mutation genes for next step screen. (g) Variants were screened by manual reviews using the filtering standards: rare nonsynonymous and possibly damaging variants in DDR genes or participating in cell apoptosis, autophagy, cell cycle, cell growth, cell proliferation, angiogenesis, inflammatory response, cell differentiation, cell adhesion, and chromatin modification function. (h) We filtered the prioritize variants that fulfilled previous criteria — with interesting gene function and interactions — and were located in protein domains. (i) The filtered candidate genes were listed for further analysis. The screening flow chart is shown in Supplemental Figure 3.

Somatic mutation screening with WES. DNA extraction from formalin-fixed paraffin-embedded tissue was performed with the QIAamp DNA FFPE Tissue Kit (Qiagen) according to manufacturer’s instructions. The methods of WES and annotations were performed as described above. KRAS, BRAF, and FBXW7 mutations and a total of 83 cancer-related genes were screened, including APC, CTNNB1, and PIK3CA (Supplemental Table 9).

Sanger sequencing of the candidate genes. The candidate pathogenic variants were verified by Sanger sequencing. One hundred healthy controls and 352 CRC/CRAs were also tested for the candidate variant using Sanger sequencing. The entire CHEK2 coding sequence was also performed by Sanger sequencing. Primer sequences are given in Supplemental Table 12.

IHC. Immunostains for Chk2 protein expression were performed on 4 μm sections from colon tumor and normal mucosa from family K and CRC control or healthy control. After deparaffinization, citrate buffer was used to retrieve antigen. Endogenous peroxidase was inactivated with 3% H2O2 dilution for 15 minutes. Tissues were blocked with 10% goat serum for 1 hour at room temperature. The Chk2 primary antibody (ab109413, diluted at 1:100, Abcam) was incubated overnight at 4°C. The goat anti-rabbit/mouse secondary antibody (Thermo Fisher Scientific, D-3004) was incubated for 1 hour at room temperature and subsequently revealed with DAB substrate (Dako) for 3 minutes. Slides were finally stained in hematoxylin. Immunostains for MSH2 (Thermo Fisher Scientific, diluted at 1:150, 33-7900), MSH6 (Abcam, diluted at 1:150, ab92471), PMS2 (Abcam, diluted at 1:100, ab110638), and MLH1 (Abcam, diluted at 1:100, ab92312) proteins expressions were performed as described above.

Functional characterization of genetic variants. The SW480 human CRC cell line was purchased from American Type Culture Collection (ATCC) and was cultured in 1640 medium supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin solution (MilliporeSigma). Cells were maintained in a humidified incubator adjusted with 5% CO2 at 37°C.

Establishment of CRISPR-KO cells. In order to generate CHEK2Δ/Δ mutant cells, the CRISPR-Cas9 system was used in accordance with manufacturer’s protocol. The CRISPR designing tool (http://www.rge nome.net/cas-designer/) was used to design the single guide RNA (sgRNA). The sequences were used as list in Supplemental Table 12. The sgRNA was cloned into the pSpCas9(BB)-2A-GFP (PX458) vector and transiently transfected into the SW480 CRC cell line using FuGENE HD transfection reagent (Promega). Forty-eight hours later, transfected cells were plated onto 96-well plates for single cell cloning. After 2 weeks, genomic DNA was extracted from each clone and subjected to PCR amplification. The positive clones were screened by Sanger sequencing. The expression of Chk2 of the positive clones was verified by Western blot.

Protein extraction, Western blot, and antibodies. Whole-cell protein extracts were prepared with RIPA buffer supplemented with complete Protease Inhibitor Cocktail (Roche Life Sciences) and quantified using BCA protein assay kit (Thermo Fisher Scientific). Equal aliquot of protein lysate was run on a Tris protein gel electrophoresis and transferred onto a PVDF membrane (MilliporeSigma), according to manufacturer’s protocols. Proteins were blocked in 5% BSA for 1 hour and blotted with the indicated primary antibodies, which were diluted with 5% BSA at 4°C overnight. Secondary antibodies were labeled and detected using ECL Kit (Pierce Biotech) by ChemiDoc Touch Imaging System (Bio-Rad) as described previously (47).

The antibodies were used as follows: anti-Chk2 (Abcam, diluted at 1:5000, ab109413); anti–phospho-γH2AX (phospho Ser139; Cell Signaling Technology [CST], diluted at 1:1000, 9718); anti-p53 (Abcam,
diluted at 1:1000, ab32389); anti-p53 (phospho Ser20; Abcam, diluted at 1:1000, ab157454); and anti–β-actin (KangChen, diluted at 1:3000, KC-5A08).

**Cell proliferation.** The proliferation ability of cells was measured using the Cell Counting Kit-8 (CCK8) kit. Cells were inoculated into 96-well plates at a density of 2000 cells per well in sextuplicate. In total, 10 μL of CCK8 aqueous reagent and 90 μL 1640 medium was added to each well after 24, 48, 72, or 96 hours, respectively. After incubation at 37°C for 2 hours, the absorbance was read at 450 nm with an Epoch Microplate Spectrophotometer. All experiments were repeated 3 times.

**Crystal violet assay.** Cells were inoculated into 24-well plates at a density of 2 × 10^4 cells per well. After being treated with nocodazole (MedChenExpress, 31430-18-9) or Adriamycin (Selleck Chemicals, S1208) for 48 hours, cells were fixed with 4% formaldehyde for 20 minutes and dyed with 0.05% crystal violet for 20 minutes. All experiments were repeated 3 times. The ImageJ (NIH) software was used to calculate the normalized cell population in each well.

**DNA damage.** DNA damage was accessed in the presence of nocodazole with a concentration range from 0.8 μM to 2 μM or Adriamycin with a concentration range from 0.5 μM to 6 μM for a certain amount of time such as 24 hours or 48 hours. Cell lysates were collected and subjected to SDS-PAGE for Western blot analysis using primary antibodies.

**Apoptosis and cell cycle assays.** Cell apoptosis and cell cycle were measured after being treated with nocodazole or Adriamycin at the indicated time. Apoptotic cells were detected by labeling the samples with FITC–annexin V and propidium iodide (PI) (556547, BD Biosciences) in accordance with the manufacturer’s protocol. For cell cycle analysis, cells were washed in phosphate-buffered saline and vortexed while adding 75% ethanol drop-wise to fix them. Cells were incubated at 4°C overnight. After that, cells were centrifuged at 400 g at 4°C for 5 minutes and resuspended in PBS containing 400 μL PI (550825, BD Biosciences). Cells were incubated at 37°C in the dark for 20 minutes before flow cytometry analysis. Samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences); the FlowJo vision 10 software and the Modfit LT software were used to define apoptotic cells or number of cells in each cell cycle.

**Accession numbers.** The accession numbers for CNVs reported in this paper are ClinVar: SCV001754535, SCV001754757, SCV001754759, SCV001754761, SCV001754762, SCV001754763, SCV001754805, SCV001754806, SCV001754807, SCV001754808, and SCV001754809.

**Statistics.** Data from at least 3 independent experiments performed are presented as the mean ± SEM. Measurement of data between 2 groups was performed using nonparametric Mann–Whitney U test. Statistical tests were 2-tailed, and a P value less than 0.05 was considered statistically significant. SPSS statistical software was used for analyses. Graphs and associated statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software).

**Study approval.** This study was approved by the institutional ethics committee (KY2019-007) of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, as well as by the Chinese National Review Committee for Genetics Studies. A written informed consent was signed by each participant.

**Author contributions**
PX contributed experimental design and conducted experiments, data analyses, and manuscript writing. DS contributed experimental design and data analyses, as well as manuscript writing. YG and YJ performed experiments. GZ, MZ, JC, ZW, and QL provided colon cancer specimens and clinical and pathological information. JH and HC discussed the data. PX, DS, and JYF conceived of the study and wrote the manuscript. YXC and JYF designed or/and supervised this project and revised the manuscript. The order of the co–first authors was based on the contributions to the work.

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