Prevalence of cancer susceptibility variants in patients with multiple Lynch syndrome related cancers

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Along with early-onset cancers, multiple primary cancers (MPCs) are likely resulting from increased genetic susceptibility; however, the associated predisposition genes or prevalence of the pathogenic variants genes in MPC patients are often unknown. We screened 71 patients with MPC of the stomach, colorectal, and endometrium, sequencing 65 cancer predisposition genes. A subset of 19 patients with early-onset MPC of stomach and colorectum were further evaluated for variants in cancer related genes using both normal and tumor whole exome sequencing. Among 71 patients with MPCs, variants classified to be pathogenic were observed in 15 (21.1%) patients and affected Lynch Syndrome (LS) genes: **MLH1** (n = 10), **MSH6** (n = 2), **PMS2** (n = 2), and **MSH2** (n = 1). All carriers had tumors with high microsatellite instability and 13 of them (86.7%) were early-onset, consistent with LS. In 19 patients with early-onset MPCs, loss of function (LoF) variants in **RECQL5** were more prevalent in non-LS MPC than in matched sporadic cancer patients (OR = 31.6, 2.73–1700.6, \( p = 0.001 \)). Additionally, there were high-confidence LoF variants at **FANCG** and **CASP8** in two patients accompanied by somatic loss of heterozygosity in tumor, respectively. The results suggest that genetic screening should be considered for synchronous cancers and metachronous MPCs of the LS tumor spectrum, particularly in early-onset. Susceptibility variants in non-LS genes for MPC patients may exist, but evidence for their role is more elusive than for LS patients.

The identification of inherited DNA variants in cancer predisposition genes is clinically important for both cancer prevention and treatment as it can reduce cancer morbidity and mortality in individuals carrying pathogenic variants1. The prevalence of pathogenic variants in cancer predisposition genes is lower than 1% of the healthy population and 3–8% in patients diagnosed with cancer2-4. Pathogenic variants in cancer predisposition genes are therefore rare. On the other hand, wide-spread surveillance screening may result in false positive findings or findings of uncertain significance, yielding to increased anxiety and unnecessary or ineffective clinical procedures. Thus, continued research supporting more effective and precise genetic screening guidelines is needed1.

Multiple primary cancers (MPCs), referring to two or more histologically distinct cancers diagnosed in one individual, is gradually recognized as an important medical problem with a reported frequency of 2–17% in the cancer population5,6. Along with early-onset cancer, MPC have been regarded as high-risk of heritability7 and those patients therefore represent excellent candidates for genetic screening. However, limited data is available for the prevalence and type of pathogenic variants in the cancer predisposition genes of these patients.
Gastric cancer (GC) and colorectal cancer (CRC) are the two most common cancers in Korea, with 5-year survival up to 70% and their combination is the most common instance of MPCs, representing ~ 2% of Korean GC or CRC cases. About 15% of each of the cancer types can be characterized by elevated microsatellite instability (MSI) caused by mismatch repair (MMR) deficiency. Together with Endometrial cancer (EC)—another cancer characterized by MSI caused by MMR and CRC are the main cancer types associated with Lynch Syndrome (LS), caused by pathogenic variants in MMR genes, resulting in up to an 80% lifetime risk of cancers. In particular, 2–5% of CRC and EC, as well as ~ 15% of early-onset CRC are thought to be associated with LS. MPCs with cancer types in the spectrum of LS therefore represent some of the strongest candidates for genetic screening, as the prevalence of LS among MPC is unknown.

Here we present the results of the genetic screening of 71 patients with MPC of the stomach, colorectal and endometrium. We evaluate the distribution and prevalence of pathogenic variants in 65 cancer predisposition genes, including LS susceptibility genes, as a function of age and clinicopathological features. We further characterize inherited coding variants focusing on DNA repair and cancer related genes in 19 early-onset MPC (eoMPC) patients, proposing candidate MPC susceptibility genes based on the analysis of loss of function (LoF) variants in candidate tumor suppressor genes, their relative prevalence and association with the mutational landscape of the associated tumors.

### Results

**Identification of Lynch Syndrome patients by multigene targeting panel.** We investigated the prevalence of LoF variants in cancer predisposition genes using a cohort of 71 patients with MPCs. Fifty-four (76.1%) patients were diagnosed with GC and CRC (23–42.6% synchronous) while 14 diagnosed with EC and CRC (N = 13) or GC (N = 1). Three patients had 3 or more types of cancers. Thirty (42.3%) patients were diagnosed with MPC before age 55 and classified as eoMPCs.

| Case ID | Cancer type | Age at second diagnosis | Gene | Nucleotide change | Amino acid change | Mutation type | ACMG criteria | Class† | MSI status |
|---------|-------------|-------------------------|------|-------------------|-------------------|--------------|---------------|--------|------------|
| dou_002 | CRC/GC      | < 55                    | MLH1 | c.1721 T > C      | p.Leu574Pro       | Missense     | PM2, PP1, PP2, PP3, PP5 | LP     | MSI-H/MSI-H |
| dou_003 | GC/CRC      | < 55                    | MLH1 | c.1758dupC        | p.Met878HisfsTer6 | Frameshift_intron | VS1, PM2       | LP     | MSI-H/MSI-H |
| dou_005 | CRC/GC      | < 55                    | MLH1 | c.208-1G > A      | NA                | Splice_acceptor_variant | VS1, PM2, PP5 | P      | MSI-H/MSI-H |
| dou_006 | GC/CRC      | < 55                    | MLH1 | c.2041G > A       | p.Ala681Thr       | Missense     | PM2, PM5, PP3, PP5 | LP     | MSI-H/MSI-H |

**Table 1.** Summary of clinicopathologic characteristics of patients with multiple Lynch related primary cancers with pathogenic/likely-pathogenic germline variants by targeted panel. *The order of MSI/MMR status is matched to cancer type. †LP: likely pathogenic, P: Pathogenic. ‡The age of the patients is described as a range to preserve the anonymity of the patients as recommended by institutional review board. ACMG, the American College of Medical Genetics and Genomics.
There was one patient with multi-locus inherited neoplasia alleles syndrome (MINAS) harboring germline pathogenic variants in both MLH1 and BRCA1 gene\(^2^1\). One of recurrent P/LP variants of MLH1 was c.1758dupC, a common variant in Korean LS patients\(^2^2\). There was a P/LP variant in MSH2 (c.942 + 3A > T) that was reported as a frequent de novo mutation and founder variant in Newfoundland\(^2^3,2^4\), in a patient diagnosed with four types of cancer (GC, CRC, EC, and lung cancer). Of the remaining 56 of patients, 42 had VUS altering 43 genes, leaving the contribution of these genes to MPC undetermined.

Characteristics of LS patients. All 15 LS patients had at least one MSI-H tumor in contrast to 11/56 non-LS patients. LS patients were also more likely to have two or more first-degree relatives (9/10, OR = 17.67, 2.12–836.73, \(p = 0.001\), Supplemental file 1, Tables S1 and S2). The association of the variants with tumor characteristics were further investigated. The loss of expression of the protein corresponding to the gene with P/LP variants was confirmed in all 19 MSI-H tumors investigated from all LS patients (Supplemental file 1, Table S3)\(^2^5\). The LS patients were more likely to be diagnosed with eoMPC (13/15, OR = 14.31, 2.79–144.22, \(p < 0.001\), Fig. 1) but equally likely to present with synchronous tumors (6/15, OR = 0.89, 0.23–3.26, \(p > 0.999\)). This suggests that consistent with Lynch syndrome, LS MPC patients are diagnosed earlier. However, the variants are unlikely to influence the relative timing of the two cancer diagnosis. More importantly, these results showed that the majority of MPC patients do not have pathogenic variants known to cause LS. Even restricting to 13 patients with eoMPCs and strong family history of cancer (two or more first-degree relatives over two successive generations), cancer predisposition genes were not altered in nearly half of the patients (6/13, Table S2 in the Supplemental file 1). The likely genetic cause of MPC in these patients remains undetermined.

Identification of candidate cancer predisposition genes in early-onset MPC. To identify additional variants and genes underlying a possible cancer susceptibility in non-LS patients, we sequenced the whole exome of 19 eoMPC patients with gastric and colorectal cancer which is the most common combination of MPCs in Korea\(^9,1^0\). Seven of the patients were LS patients, as determined above, and used as controls for the cancer predisposition genes discovery process. We identified 111,842 high-confidence variants across all patients, of which 3,211 were rare (MAF < 0.01) in East-Asian population and predicted to be damaging, affecting 2,675 genes. We investigated a set of 382 genes (referred to as cancer genes) involved in DNA repair, cancer susceptibility and progression (Supplemental file 1, Table S4)\(^2^6\). Of those, 66 were altered by 82 variants, including 7 in the MLH1 gene of the LS patients, therefore confirming the panel sequencing results and validating the initial WES variant calling and filtering approach.

For a given candidate cancer predisposition genes, we expect that the prevalence of LoF alterations observed in eoMPC cohort would be higher than in a matching sporadic cancer cohort. Despite some LS phenotypes being reported\(^3^5\), TCGA gastric and colorectal cancer patients are mainly from sporadic cancers. We selected 70 of these patients with East-Asian ancestry to closely match the Korean ancestry of eoMPC cohort and reduce spurious findings resulting from population differences. In these patients, we identified a total of 87 LoF variants.
FANCG germline variants: were altered via mutations and 15 through LOH. Among these, 2 genes were affected by high-confidence LoF patients, we identified 17 genes in 10 patients also affected by bi-allelic alterations in one or more tumors. Two non-LS patients. Expanding the analysis to other candidate genes affected by LoF germline variants in non-LS RECQL5 in the main candidate susceptibility gene in non-LS patients, were not high-confidence LoF variant genes, at least for high-confidence LoF variants in known predisposition genes. In contrast, the variants this observation confirms the validity of the investigation of bi-allelic alteration to identify cancer predisposition.

| Gene   | N mutated cases | Non-Lynch (n = 12) | Lynch (n = 7) | Total (n = 19) | TCGA_EAS (n = 70) | p-value* (TCGA) | p-value* (TCGA) |
|--------|-----------------|--------------------|--------------|----------------|-------------------|----------------|----------------|
| MLH1   | 0               | 7                  | 7            | 2              |                   | >0.999         | <0.0001 |
| RECQL5 | 4               | 0                  | 4            | 1              |                   | 0.0013         | >0.999 |
| EMB2   | 2               | 0                  | 2            | 1              |                   | 0.0547         | >0.999 |
| EP100  | 2               | 0                  | 2            | 1              |                   | 0.0547         | >0.999 |
| MUTYH  | 2               | 0                  | 2            | 1              |                   | 0.0547         | >0.999 |
| MSH3   | 2               | 0                  | 2            | 2              |                   | 0.1002         | >0.999 |
| COL7A1 | 2               | 0                  | 2            | 4              |                   | 0.2108         | >0.999 |
| PITCHI | 2               | 0                  | 2            | 7              |                   | 0.6134         | >0.999 |

Table 2. Genes affected by Loss of function variants in two or more patients with eoMPC at stomach and colon. p < 0.01 values are in bold. *Fisher's exact p-value for the frequency of each gene between non-lync and TCGA. +Fisher's exact p-value for the frequency of each gene between Lynch and TCGA.

Bi-allelic alterations in corresponding tumors as a guide for cancer predisposition genes identification. According to Knudson's two-hit model, the wild-type allele of tumor suppressor genes is frequently lost or mutated in the tumor. Such combination of inherited deleterious variants with somatic loss—referred to as bi-allelic alteration—can help prioritize candidate cancer predisposition genes. We sequenced the exome of 37 tumors from all 19 patients with eoMPC (Supplemental file 1, Table S7) and identified 35 somatic bi-allelic alterations by LoF mutations (N = 5), loss of heterozygosity (N = 27) or both (N = 3) focusing on candidate germline LoF genes.

The MLH1 gene was somatically altered in 13/15 tumors from LS patients and at least one tumor from each of the 7 LS patients (Table 3). Importantly, 5/7 MLH1 LoF variants in LS patients were predicted to be high-confidence LoF variants, indicating that most, but not all, known pathogenic variants can be identified through this approach. RECQL5 was the only gene significantly more altered in non-LS patients than in TCGA patients (4/12 vs 1/70, OR: 3.67, 2.73-1700.6, p = 0.0013, Table 2). In particular two of these patients had the same RECQL5 variant (p.R441Q which is not seen in the TCGA patients (p = 0.02, Table S5 in the Supplemental file 1) The variant is rare, with a global minor allelic fraction of 3.6 × 10–7. Interestingly 89 of the 101 observed minor alleles (out of 278,974 total alleles observed) in the gnomAD dataset belong to the East-Asian population, suggesting a possible Korean-specific effect.

Discussion
As expected, the present result confirmed that individuals with MPC, particularly early onset, have a higher likelihood of LS: MMR related P/LP germline variants were observed in 21% of MPC (15/71) and in 43% of eoMPC (13/30) patients, a proportion comparable to ~ 15% of early-onset CRC. The elevated extracolonic cancer risk following colorectal cancer in Lynch syndrome was reported (~ 5 and 40 times of gastric and endometrial cancer risk compared to general population, respectively), and this study supports those results. Therefore, due to the high prevalence of germline mutations individuals with MPC in the LS tumor spectrum should undergo germline testing, including when MSI status is unknown.

Because MSI-H was considered as a hallmark of LS, universal screening of all CRC and EC has been recommended. In addition, MSI screening for gastric cancers in regions where gastric cancer is highly prevalent like Korea is a way to identify individuals and families who can benefit from germline testing of LS genes,
Table 3. Summary of candidate loss-of-function germline candidate genes and the status of loss of heterozygosity of corresponding tumors. (1) High confidence LoF prediction in bold. *Significantly recurrent in eoMPC. **Bi-allelic alteration was observed in 2 tumors. ***Bi-allelic alteration was observed in 3 tumors.

| Patient group | Case ID | Genes with germline LoF variants (1) | N tumors (N sequenced) | Tumor MSI status (GC/CRC) | Bi-allelic alterations in at least one tumor (1) |
|---------------|---------|-------------------------------------|------------------------|---------------------------|-----------------------------------------------|
| Lynch group   | dou_002 | MLH1*, FANC1, RAD54L, RFC3, TSC2, FANCA, ARID1B, POLN | 2 (1)                  | MSI-H/MSI-H               | MLH1                                          |
|               | dou_003 | MLH1*, POL, SMO                        | 3 (3)                  | MSI-H/MSI-H/MSI-H         | MLH1**, SMO**                                 |
|               | dou_005 | MLH1*, FANC2, UNG, POLN, RFC3, ARID1A, FANC1 | 2 (2)                  | MSS/MSI-H                | UNG, POLN, MLH1***, ARID1A**                  |
|               | dou_006 | MLH1, BRCA1, CBL, RAD50, UVSSA         | 2 (2)                  | MSI-H/MSI-H               | MLH1, RAD50                                   |
|               | dou_011 | MLH1*, ARID2, ERCC2, EGFR, TMEM127, RHBD2 | 2 (2)                  | MSI-H/MSI-H               | ARID2, RHBD2, MLH1**                           |
|               | dou_016 | MLH1*, DCLRE1C, ERCC6, IAK3            | 3 (3)                  | MSI-H/MSI-H/MSI-H         | MLH1***                                        |
|               | dou_017 | MLH1*, GN5                             | 2 (2)                  | MSI-H/MSI-H               | MLH1**                                        |
| Non-Lynch group| dou_001 | MUTYH, PBRM1, PTC1, ABL1               | 2 (2)                  | MSS/MSI2                  | MUTYH, PBRM1                                  |
|               | dou_004 | CDH1, FANC1, FANCG, FANCM, RAD50, MSH1 | 2 (2)                  | MSS/MSI2                  | CDH1, FANC1, FANCG, FANCM                      |
|               | dou_007 | BRCA2, MUTYH, EME2, MBD4, RECLQ5*, WRN, COL7A1 | 2 (2)                  | MSS/NA                    |                                               |
|               | dou_008 | AXIN1, COL7A1, RECLQ5*, MSH4, PTC1, NEIL3, KLF4 | 2 (2)                  | MSS/MSI2                  | COL7A1                                        |
|               | dou_009 | NOTCH2, REV3L, RECLQ5*, SMARCA4, EP300, DOCK8 | 2 (2)                  | MSI-H/MSI-H/MSI-H         | SMARCA4                                       |
|               | dou_010 | CASP8, EME2, NOTCH1                    | 2 (2)                  | MSS/MSI2                  | CASP8                                         |
|               | dou_012 | POLM, EME1                             | 2 (2)                  | MSS/MSI2                  | EME1                                          |
|               | dou_013 | FLCN, CIT, NFAF1CH2, XAB2              | 2 (2)                  | MSS/MSI2                  | FLCN                                          |
|               | dou_014 | EP300, XPC, RECLQ5*                    | 2 (1)                  | MSS/NA                    |                                               |
|               | dou_015 | RAD52, CHEK2                           | 2 (2)                  | MSS/MSI2                  | RAD52                                         |
|               | dou_016 | PARG, TET2, NF2, UVSSA, BCCR          | 2 (2)                  | MSS/MSI2                  | PARG, TET2, UVSSA, NF2                        |
|               | dou_019 | MSH2, ERCC6, ALKBH2                    | 2 (2)                  | MSS/MSI2                  | ERCC6                                         |

so that surveillance and risk reducing interventions can be undertaken along with cascade testing of family members. At the same time, MSI testing is valuable for other types of solid cancers for other purpose: MSI-H is a biomarker for response to immune checkpoint inhibitors that is a breakthrough for treating advanced solid cancers regardless of its origin. As MSI-H is predictive of LS across a broad tumor spectrum, screening of tumor MSI status in all patients with an initial cancer, especially for LS spectrum tumors and early-onset cancer, and consecutive germline testing for patients with MSI-H tumor will help refine the diagnosis, giving an opportunity to diagnose LS and avoid or detect the second cancer as early as possible.

Genetic analysis of families with high occurrence of cancer using cancer predisposition genes panels or exome sequencing has become standard to identify the underlying cancer susceptibility variants. But penetrance, interaction with environmental factors, and size of the pedigree may affect power of such linkage studies. Evaluating both germline and somatic alterations is another reasonable approach to find cancer predisposition genes. The present results showed that characterizing recurrent LoF genes, truncating variants and loss of heterozygosity are useful to prioritize candidate cancer predisposition genes; any combination of them unambiguously identified germline MLH1, a known cancer predisposition genes in LS patients, in multiple patients clinically diagnosed with LS. It suggests that MRC is a strong phenotype to find cancer predisposition genes, therefore this approach would be worthy to be expanded to other combinations of MRCs. However, the types of MRCs would be different by geographical regions or ancestry: region-specific environmental factors can contribute to the oncogenesis and lead to different cancer types within the LS spectrum. For instance, MRCs including GC could be a hallmark of LS in Korea but not in the United States where GC incidence is much lower. This could explain why GC risk in LS may have been underestimated. Therefore, geographical and/or ancestry specific cancer epidemiology needs to be accounted for in the genetic screening guidelines.

In non-LS patients with eoMPC, however, evidence of germline susceptibility was more elusive. RECLQ5 was a frequently recurrent gene and variant (p.R441Q) compared to East Asian sporadic cancer population (TCGA). Despite careful matching, some differences between the eoMPC and matched TCGA cohorts remained and could have confounded this observation. Notably, eoMPC patients were all younger than 55 and exclusively of Korean descent, while the TCGA patients included in the analysis were not selected for age and included non-Korean Asians. Importantly, the allele frequency of the RECLQ5 variant was 0.4% of EAS population in gnomAD, and there was no evidence of somatic LOH in the corresponding tumors, suggesting that additional analysis and experiment are required to establish its pathogenicity. CASP8 and FANC were high-confidence LoF germline gene with bi-allelic alteration in CRC and GC, respectively. CASP8 encodes a member of caspase family and play a role in apoptosis, and some of its polymorphism have been reported as susceptibility to various cancers including CRC. FANC is a gene encoding Fanconi anemia (FA) group G protein, and part of the FA DNA
damage repair pathway and in which germline pathogenic variants often predispose to cancers\textsuperscript{44–46}. Thus, the CASP8 and FANCG variants identified in these two MPC cases could underlie their disease susceptibility though additional functional studies would be necessary to demonstrate their pathogenicity\textsuperscript{47–50}.

Despite evaluating all inherited coding variants in cancer genes and somatic changes in the corresponding tumors, we did not find a clear inherited predisposition that caused multiple cancers in over half of patients with eoMPC, even with strong family cancer history. Not only cancer predisposition genes, but also environmental factors such as smoking and alcohol also increase the risk of cancers\textsuperscript{51–53}.

Our study has inherent limitations. The present results did not cover epigenetic changes affecting candidate cancer predisposition genes in tumors, and methylation of the MLH1 promoter in particular is known to be a mechanism for somatic loss of function in ~20% of CRC\textsuperscript{54}. Furthermore, we restricted the analysis to 382 well studied cancer genes, more likely to impact cancer susceptibility, therefore leaving the possibility to miss unexpected cancer predisposition genes. The complete analysis of whole exomes to identify rare disease susceptibility variants would indeed be intractable for the cohort under study. Careful analysis of the pedigree and genetic comparison of family members, a typical standard in such genetic susceptibility studies, was not possible due to the absence of family history information in many cases and to the retrospective nature of the study. However, this study is to our knowledge the largest study of patients with MPCs, evaluated using the same multigene targeting panel, and where both germline and multiple tumors DNA were investigated in the highest risk patients. eoMPC of colorectal, endometrial, and gastric cancer are considerably enriched for LS patients, supporting the genetic screens of related family members as well as enhanced monitoring for younger patients after their first diagnosis. Routine tumor screening of MSI for patients with initial LS related cancers and consecutive germline testing for patients with MSI-H is worthy to diagnose LS and early detection or avoid second cancer. While susceptibility variants in non-LS genes for MPC patients may exist, evidence for their role is more elusive than for LS patients and would require deeper genetic investigation and complementary functional studies.

Methods

Population. We performed a retrospective population-based study targeting patients who were treated for two or more cancers in the stomach, colorectum, or endometrium at Severance Hospital, Yonsei University College of Medicine between January 2001 and December 2016. All methods were carried out in accordance with relevant guidelines and regulations. This study was approved by Institutional Review Board (IRB) of Severance hospital of the Yonsei University Health System and by the IRB of the University of California, San Diego (4-2017-0434, 191,543). The IRB of Severance hospital of the Yonsei University Health System waived the requirement for patient informed consent as the study is retrospective by design. The patients were selected using following criteria: (1) two or more cancers were pathologically confirmed, (2) multiple tumors were histologically different, not a metastatic or recurrent tumor from one cancer, (3) normal tissues were available and histologically confirmed for sequencing. DNA was obtained from formalin fixed paraffin embedded (FFPE) normal and tumor tissues. Early-onset MPC (eoMPC) was defined by the diagnosis of a second cancer at age 55 or younger. The clinico-pathologic characteristics of the patients and tumors including age, sex, family history of cancer, MSI or MMR status of tumors were evaluated. For evaluating MSI status, two mononucleotide repeat markers (BAT25 and BAT26) and three dinucleotide repeat markers (D5S346, D2S123, and D17S250) were used by polymerase chain reaction\textsuperscript{55}, and MSIsensor was used with cut-off of MSI score > 3.5 in Whole Exome Sequencing (WES) data\textsuperscript{56}. To evaluate MMR protein expression, immunohistochemistry (IHC) was conducted in four MMR genes; MLH1 (ready-to-use, clone M1, Roche, Indianapolis, IN, USA), MSH2 (ready-to-use, clone G219-1129, Roche), MSH6 (1:100, clone 44, Cell Marque, Rocklin, CA, USA), and PMS2 (1:40, clone MRQ28, Cell Marque). An MMR-deficient (dMMR) tumor was defined as a tumor showing loss of expression of any of the four MMR proteins. If a tumor was classified as any one of MSI-high (MSI-H) or deficiency MMR (dMMR) it was considered as MSI-H. The concurrency of tumors was classified as synchronous tumors when the interval between tumors was less than 1 year, otherwise considered as metachronous tumors.

Germline multigene targeting panel. Germline DNA of patients with MPCs was evaluated using a customized targeted capture sequencing panel (OncoRisk, Celemics, Seoul, Korea) covering all coding sequences and intron–exon boundaries of the coding exons of known 65 cancer predisposition genes (Supplemental file 1, Table S8)\textsuperscript{57}. Both structural variants and nucleotide variants were evaluated. The germline variants were classified into pathogenic, likely pathogenic, variant of uncertain significance (VUS) and reported following the guidelines of the American College of Medical Genetics and Genomics 2017\textsuperscript{58}. Only pathogenic or likely pathogenic (P/LP) germline variants in cancer predisposition genes were considered in the analysis. Read-depth based detection of structural variants was conducted using the ExomeDepth software. Chromosomal copy number variations (CNVs) detected by ExomeDepth were further crosschecked using our custom pipeline; this retrieved base-level depth-of-coverage for each bam file using SAMtools software and normalized the depths against those of other samples in the same batch. There were no significant pathogenic CNV detected in the studied samples.

Whole exome sequencing. Data generation. WES of normal and tumors was conducted for patients with eoMPCs in the stomach and colon. Genomic DNA was extracted from the confirmed normal and tumor tissues of FFPE. SureSelect sequencing libraries were prepared according to the manufacturer’s instructions (Agilent SureSelect All Exon V6 kit, Santa Clara, CA, USA), and HiSeq2500 sequencing system (Illumina\textsuperscript{”}, San Diego, CA, USA) performed sequencing with read lengths of 2 × 100 bp. The statistics and quality metrics of normal and tumors were described in Supplemental file 1, Tables S7 and S9.
Data analysis. The reads were aligned to hg19 reference genome by Burrows-Wheeler Aligner software (BWA 0.7.17) and duplicated reads were removed by Picard (MarkDuplicates) through bc2bio-nextgen (<v1.2.3>) For germline and somatic analysis, variants were called by Genome Analysis Toolkit Haplotype joint caller (GATK v.4.1.0) and annotated by mutect260,61, respectively. Variants were annotated and filtered using ANNOVAR62, CADD scores (CADD13 and CADDindel)63, population allele frequency in ExAC v3.064, and membership to ClinVar65. To discover novel cancer predisposition genes, we focused on high-confidence (TLOD > 12, FS < 10, SEQQ ≥ 60, MQ ≥ 60, STRANDQ > 40, DP > 10), rare (minor allele fraction was < 0.01 in ExAC of Eastern Asian population), and damaging (exonic/splicing, non-synonymous variants with > 20 of CADD score) variants in 382 genes of 11 cancer-relevant pathways26, and they were defined as LoF germline variants. High-confidence population), and damaging (exonic/splicing, non-synonymous variants with > 20 of CADD score) variants in 10,389 adult cancers.

To assess loss of heterozygosity (LOH) of a given heterozygous germline variants were estimated using hapLOHseq69. Bi-allelic alteration of corresponding tumors. When there was any one of somatic damaging mutation or AI event in germline variants of tumors were obtained by GATK haplotype caller and filtering out variants were not observed in germline variants in normal sample. Phase of genotypes were estimated with a companion phasing utility of LoF variants were predicted by Loss-Of-Function Transcript Effect Estimator (LOFTEE) plugin for Ensembl VEP (>99) that targeting stop-gained, splice site disrupting, frameshift variants66,67. Copy number alterations of tumors compared to normal was estimated using CNVkit (V0.8)68. The gene level copy number ratio was calculated as the weighted mean of all bins covered by the whole segment overlapping the gene. Genes had ≥3 segments of copy number changes were included, and log2 copy number < −1 was defined as deletion. Somatic allele imbalance (AI) in tumors of a given heterozygous germline variants were estimated using hapLOHseq69. Germline variants of tumors were obtained by GATK haplotype caller and filtering out variants were not observed in germline variants in normal sample. Phase of genotypes were estimated with a companion phasing utility of hapLOHseq. Event prevalence was set 0.1, and AI was defined when posterior probability of being in AI was over 90%. To assess loss of heterozygosity (LOH) of a given heterozygous germline variants in tumors as a secondary hit mechanism of inactivation of cancer predisposition genes26,28, we evaluated germline and somatic bi-allelic alteration of corresponding tumors. When there was any one of somatic damaging mutation or AI event in candidate germline cancer predisposition genes, it was considered as bi-allelic alteration.

To compare the frequency of recurrent cancer predisposition genes in patients of eoMPCs to that of the cancer genome atlas (TCGA) cohort, germline data of East-Asian patients (≥80% of admixture)60 with stomach cancer or colorectal cancer in TCGA cohort was used26,71. Variants were filtered for rare and damaged variants similar to LoF germline variants in MPCs cohort. For recurrent variants and genes in eoMPCs cohort, the frequency was compared between eoMPCs cohort and selected TCGA cohort.

Statistical analysis. Continuous variables were compared by two-sided Mann–Whitney test and categorical variables were compared by two-sided Fisher's exact test. P<0.05 was considered as statistically significant and R version 3.6.1(R Foundation for Statistical Computing, Vienna, Austria) was used for generating figures and statistical analysis.

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References
1. Rahman, N. Realizing the promise of cancer predisposition genes. Nature 505, 302–308. https://doi.org/10.1038/nature12981 (2014).
2. Huang, K. L. et al. Pathogenic germline variants in 10,389 adult cancers. Cell 173, 355–370. https://doi.org/10.1016/j.cell.2018.03.039 (2018).
3. Evans, J. P., Powell, B. C. & Berg, J. S. Finding the rare pathogenic variants in a human genome. JAMA 317, 1904–1905. https://doi.org/10.1001/jama.2017.0432 (2017).
4. Mersch, J. et al. Prevalence of variant reclassification following hereditary cancer genetic testing. JAMA 320, 1266–1274. https://doi.org/10.1001/jama.2018.13152 (2018).
5. Cybulski, C., Nazarali, S. & Narod, S. A. Multiple primary cancers as a guide to heritability. Int J Cancer 135, 1756–1763. https://doi.org/10.1002/ijc.28988 (2014).
6. Vogt, A. et al. Multiple primary tumours: challenges and approaches, a review. ESMO Open 2, e000172. https://doi.org/10.1136/esmoopen-2017-000172 (2017).
7. Whitworth, J. et al. A clinical and genetic analysis of multiple primary cancer referrals to genetics services. Eur. J. Hum. Genet. 23, 581–587. https://doi.org/10.1038/ejhg.2014.157 (2015).
8. Hong, S. et al. Cancer statistics in korea: incidence, mortality, survival, and prevalence in 2017. Cancer Res. Treat. 52, 335–350. https://doi.org/10.4143/crt.2020.206 (2020).
9. Cho, I. et al. Risk factors for double primary malignancies and their clinical implications in patients with sporadic gastric cancer. Eur. J. Surg. Oncol. 40, 338–344. https://doi.org/10.1016/j.ejso.2013.02.027 (2014).
10. Yun, H. R. et al. Double primary malignancy in colorectal cancer patients—MSI is the useful marker for predicting double primary tumors. Int. J. Colorectal. Dis. 24, 369–375. https://doi.org/10.1007/s00384-009-0541-x (2009).
11. Cancer Genome Atlas Research. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 513, 202–209. https://doi.org/10.1038/nature13480 (2014).
12. Cancer Genome Atlas. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337. https://doi.org/10.1038/nature11252 (2012).
13. Choi, Y. Y. et al. Microsatellite instability and programmed cell death-ligand 1 expression in stage II/III gastric cancer: post hoc analysis of the CLASSIC randomized controlled study. Ann. Surg. 270, 309–316. https://doi.org/10.1097/SLA.0000000000002803 (2019).
14. Kim, C. G. et al. Effects of microsatellite instability on recurrence patterns and outcomes in colorectal cancers. Br. J. Cancer 115, 25–33. https://doi.org/10.1038/bjc.2016.161 (2016).
15. Gustadisegni, C., Colafranceschi, M., Ottini, L. & Dogliotti, E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. Eur. J. Cancer 46, 2788–2798. https://doi.org/10.1016/j.ejca.2010.05.009 (2010).
16. Cancer Genome Atlas Research. Integrated genomic characterization of endometrial carcinoma. Nature 497, 67–73. https://doi.org/10.1038/nature12113 (2013).
23. Desai, D. C. et al. Recurrent germline mutation in MSH2 arises frequently de novo. J. Med. Genet. 37, 646–652. https://doi.org/10.1136/jmg.37.9.646 (2000).

24. Woods, M. O. et al. High frequency of hereditary colorectal cancer in Newfoundland likely involves novel susceptibility genes. Clin. Cancer Res. 11, 6853–6861. https://doi.org/10.1158/1078-0432.CCR-05-0726 (2005).

25. Sarode, V. R. & Robinson, L. Screening for Lynch syndrome by immunohistochemistry of mismatch repair proteins: significance of indeterminate result and correlation with mutational studies. Arch. Pathol. Lab Med. 143, 1225–1233. https://doi.org/10.5888/arpa.2018-0201-OA (2019).

26. Buckley, A. R., Ideker, T., Carter, H., Harismendy, O. & Schork, N. J. Exome-wide analysis of bi-allelic alterations identifies a Lynch phenotype in The Cancer Genome Atlas. J. Med. Genet. 60, 373–379. https://doi.org/10.1001/jamaoncol.2016.5719 (2016).

27. Karczewski, K. J. et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 581, 434–443. https://doi.org/10.1038/s41586-020-2308-7 (2020).

28. Knudson, A. G. Jr. Mutation and cancer: statistical study of retinoblastoma. Hum. Mutat. 208–218. https://doi.org/10.1038/341073a0 (1971).

29. Pirinen, M., Donnelly, P. & Spencer, C. C. Including known covariates can reduce power to detect genetic effects in case-control studies. Nat. Commun. 7, 1363–1372. https://doi.org/10.1038/ncomms13551 (2016).

30. Galvan, A., Ioannidis, J. P. & Dragani, T. A. Beyond genome-wide association studies: genetic heterogeneity and individual prediction. J. Natl. Cancer Inst. 101, 1633–1637. https://doi.org/10.1093/jnci/dj635 (2011).

31. Pearlman, R. et al. Clinical characteristics of patients with colorectal cancer with double somatic mismatch repair mutations compared with Lynch syndrome. J. Med. Genet. 56, 462–470. https://doi.org/10.1136/jmedgenet-2018-105699 (2019).

32. Win, A. K. et al. Risks of primary extracolonic cancers following colorectal cancer in Lynch syndrome. J. Natl. Cancer Inst. 104, 1456–1473. https://doi.org/10.1093/jnci/dju335 (2012).

33. Gupta, S. et al. NCCN Guidelines insights: genetic/familial high-risk assessment: colorectal, Version 3.2017. J. Natl Compr Canc Netw 15, 1465–1475. https://doi.org/10.6004/jnccn.2017.0176 (2017).

34. Hlampel, H. et al. Screening for Lynch syndrome (hereditary non-polyposis colorectal cancer) among endometrial cancer patients. Cancer Res. 70, 7810–7817. https://doi.org/10.1158/0008-5472.CAN-10-1114 (2010).

35. Le, D. T. et al. PD-1 blockade in tumors with mismatch-repair deficiency. N. Engl. J. Med. 372, 2509–2520. https://doi.org/10.1056/NEJMoa1500596 (2015).

36. Latham, A. et al. Microsatellite instability is associated with the presence of Lynch syndrome pan-cancer. J. Clin. Oncol. 37, 286–295. https://doi.org/10.1200/JCO.2018.80.0283 (2019).

37. Galvan, A., Ioannidis, J. P. & Dragani, T. A. Beyond genome-wide association studies: genetic heterogeneity and individual predisposition to cancer. Trends Genet. 26, 132–141. https://doi.org/10.1016/j.tig.2009.12.008 (2010).

38. Pirinen, M., Donnelly, P. & Spencer, C. C. Including known covariates can reduce power to detect genetic effects in case-control studies. Nat. Genet. 44, 848–851. https://doi.org/10.1038/ng.2346 (2012).

39. Hindlof, A. L. et al. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. Proc. Natl. Acad. Sci. USA 106, 9362–9367. https://doi.org/10.1073/pnas.0903103106 (2009).

40. Chen, S. et al. Prediction of germline mutations and cancer risk in the Lynch syndrome. JAMA 296, 1479–1487. https://doi.org/10.1001/jama.296.12.1479 (2006).

41. Sun, T. et al. A six-nucleotide insertion–deletion polymorphism in the CASP8 promoter is associated with susceptibility to multiple cancers. Nat. Genet. 39, 605–613. https://doi.org/10.1038/ng0300 (2007).

42. Zhang, Y. et al. A systematic analysis of the association studies between CASP8 D302H polymorphisms and breast cancer risk. J. Gene. Med. 9, 283–289. https://doi.org/10.1002/jgm.1172 (2017).

43. Ying, Y., Xu, J., Qi, Y., Zhang, M. & Yang, Y. CASP8 rs3834129 (-652 G/N insertion/deletion) polymorphism and colorectal cancer susceptibility: an updated meta-analysis. J. Cancer 9, 4166–4171. https://doi.org/10.7150/jca.27110 (2018).

44. Chen, H., Zhang, S. & Wu, Z. Fanconi anemia pathway defects in inherited and sporadic cancers. Transl. Pediatr. 3, 300–304. https://doi.org/10.3978/j.issn.2224-4336.2014.07.05 (2015).

45. Esteban-Jurado, C. et al. The Fanconi anemia DNA damage repair pathway in the spotlight for germline predisposition to colorectal cancer. Eur. J. Hum. Genet. 24, 1501–1505. https://doi.org/10.1038/ejhg.2016.144 (2016).

46. Huang, J. P. et al. FANCA D1359Y mutation in a patient with gastric polyposis and cancer susceptibility: A case report and review of literature. World J. Gastroenterol. 24, 4412–4418. https://doi.org/10.3748/wjg.v24.i38.4412 (2018).

47. Soung, Y. H. et al. CASPASE-8 gene is inactivated by somatic mutations in gastric carcinomas. Cancer Res. 65, 815–821 (2005).

48. Soung, Y. H. et al. Caspase-8 gene is frequently inactivated by the frameshift somatic mutation 1225_1226delCTG in hepatocellular carcinomas. Oncogene 24, 141–147. https://doi.org/10.1080/096074004100016484 (2005).

49. Ramanagoudr-Bhojappa, R. et al. Multiplexed CRISPR/Cas9-mediated knockout of 19 Fanconi anemia pathway genes in zebrafish revealed their roles in growth, sexual development and fertility. PLoS Genet. 14, e1007821. https://doi.org/10.1371/journal.pgen.1007821 (2018).

50. Pulliam-Leath, A. C. et al. Genetic disruption of both Fanc C and Fanc D in mice recapitulates the hematopoietic manifestations of Fanconi anemia. Blood 116, 2915–2920. https://doi.org/10.1182/blood-2009-08-240747 (2010).

51. Hashibe, M. et al. Contribution of tobacco and alcohol to the high rates of squamous cell carcinoma of the supraglottis and glottis in Central Europe. Am. J. Epidemiol. 165, 814–820. https://doi.org/10.1093/aje/kwk066 (2007).

52. Gandini, S. et al. Tobacco smoking and cancer: a meta-analysis. Int. J. Cancer 122, 155–164. https://doi.org/10.1002/ijc.23033 (2008).

53. Services, U. S. D. o. H. a. H. 14th report on carcinogens. https://ntp.niesh.nih.gov/whatwesay/studies/cancer/roc/index. html (2016).

54. Li, X. et al. MLH1 promoter methylation frequency in colorectal cancer patients and related clinicopathological and molecular features. PLoS ONE 8, e59064. https://doi.org/10.1371/journal.pone.0059064 (2013).

55. Umak, A. et al. Revised Bethesda Guidelines for hereditary non-polyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J. Natl. Cancer Inst. 96, 261–268. https://doi.org/10.1093/jnci/djh034 (2004).

56. Niu, B. et al. MSIsensor: microsatellite instability detection using paired tumor-normal sequence data. Bioinformatics 30, 1015–1016. https://doi.org/10.1093/bioinformatics/btt755 (2014).
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Author contributions

Y.Y.C., J.H.C., and O.H. designed and supervised the research. X.X., D.N., Y.Y.C., J.E.L. generated sequencing data and Y.Y.C. performed the statistical analysis, prepared the figures and tables, and drafted the manuscript. Y.Y.C., J.H.C., and O.H. designed the experiments. S.S., J.E.L. conducted experiment. S.I., J.P.S., J.J., H.K., S.H.N. screened patients and collected specimen. Y.Y.C., L.M. and O.H. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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