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Original research

Profiling of the genetic features of Chinese patients with gastric cancer with HRD germline mutations in a large-scale retrospective study

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ABSTRACT

Background Approximately 10% of gastric cancers (GCs) are associated with strong familial clustering and can be attributed to genetic predisposition. Homologous recombination deficiency (HRD) leads to genomic instability and accumulation of genetic variations, playing an important role in the development and progression of cancer. We aimed to delineate the germline mutation characteristics of patients with HRD-mut GC in Chinese. **Methods** We retrospectively reviewed the genomic sequencing data of 1135 patients with Chinese GC. Patients harbouring at least one loss of function (LoF) germline mutations in *BRCA1*, *BRCA2*, *ATM*, *PALB2*, *BRIP1*, *CHEK1*, *CHEK2*, *FANCA* and *FANCL* were selected for analysis.

Results 89 patients were identified with LoF germline mutations of HRD gene. Germline mutations occurred most commonly in *ATM* (30.33%), followed by *BRIP1* (17.98%), *BRCA2* (14.61%), *BRCA1* (12.36%), *FANCA* (10.11%), *PALB2* (10.11%), *FANCL* (6.74%), *CHEK1* (3.37%) and *CHEK2* (3.37%). 14 out of 89 patients with HRD-mut harboured double mutations in HRD and MMR genes, with the median age of 51.5 years. The decreasing median age would be attributed to five patients with HRD+MMR double-muts harbouring mutations in both HRD and MMR genes. The median age of onset of patients with HRD+MMR double-muts is 47, which is significantly earlier than that of Chinese patients with GC ($p=0.0235$).

Conclusion Our data suggest that carrying both HRD and MMR gene LoF germline mutations may cause early-onset GC. Germline mutations in the HRD gene should be of concern in the study of hereditary GC.

INTRODUCTION

Gastric cancer (GC) remains an important cancer worldwide and is responsible for over one million new cases in 2020 and an estimated 769 000 deaths, ranking fifth for incidence and fourth for mortality globally.¹ Compared with the USA and UK, China had lower GC incidence but higher mortality and disability-adjusted life year rates.² Clustering of GC can be seen in families in approximately 10% of cases. However, a gene defect can be determined in only 1%–3% of cases.^{3,4} The most recognised predisposition syndrome, hereditary diffuse GC, which is caused by heterozygous mutation in the *CDH1* gene, only presents in less than 1%

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Homologous recombination deficiency (HRD) is a well-known predictive biomarker for the response to Poly(ADP-ribose) polymerase (PARP) inhibitors. HRD-related genotypes and high prevalence of HRD mutations have been found in gastric cancer (GC).
- ⇒ Pathogenic germline mutations of HRD genes like *BRCA1*, *BRCA2* and *ATM* are known to confer susceptibility to ovarian and breast cancers.
- ⇒ The role of HRD genes in conferring susceptibility to GC is not clear.

WHAT THIS STUDY ADDS

- ⇒ Our work reported an unexpected phenomenon that carrying both HRD and MMR gene loss of function germline mutations may cause early-onset GC.
- ⇒ There is no significant difference in tumour mutational burden between patients with HRD-mut and patients with HRD-wt among Chinese patients with GC.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ This study suggested the concern of HRD germline mutations in the study of hereditary GC.
- ⇒ These information is to provide an update on the current knowledge about GC and to give a new hint of understanding double germline mutations.

among individuals with GC.⁵ High risk of GC also reported in Lynch syndrome (LS), juvenile polyposis syndrome, Peutz-Jeghers syndrome and familial adenomatous polyposis, which respectively have a 2%–13% lifetime risk, a 1% lifetime risk, a 29% lifetime risk and a 1%–2% lifetime risk of developing GC.⁶ Research about genetic susceptibility for the vast majority of patients with GC diagnosed is limited.

Homologous recombination deficiency (HRD) leads to genetic instability and accumulation of genetic variations, thus playing an important role in the development and progression of many cancer types, especially breast, ovarian and pancreatic cancers.⁷ Research in recent years



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has shown that HRD signature, like COSMIC signature 3, is also enriched in GCs with inactivating germline or somatic mutations of genes regulating homologous DNA recombination such as *BRCA1*, *BRCA2* and *PALB2*.^{8,9} Another analysis of TCGA mutation data revealed that high HRD scores were mostly contributed by germline mutation of *BRCA1/2*, *ATM*, *ATR*, *PALB2*, *CHEK2* and *BRIP1* in GC.¹⁰ In a cohort comprising 484 Chinese patients with GC, 15 of the 484 (3.10%) patients carried Homologous recombination germline mutations occurring in six genes: *ATM* (6/15, 40.00%), *RAD51C* (2/15, 13.33%), *BRCA1* (2/15, 13.33%), *BRCA2* (2/15, 13.33%), *CHEK2* (2/15, 13.33%) and *PALB2* (1/15, 6.67%).¹¹ The association of gastric carcinoma is reported to be stronger with *BRCA2* than *BRCA1*, with an increased relative risk of GC in patients with *BRCA2* mutation (2.59, 95% CI 1.46 to 4.61).¹² The frequency of gastric carcinoma is five times higher than the general population particularly in Ashkenazi Jews with *BRCA2* mutations (5.7%).¹³ Poly(ADP-ribose) polymerase (PARP) inhibitors cause the synthetic lethal effects in HR-deficient cancer cells¹⁴ and are currently approved for the treatment of ovarian cancer, breast cancer and metastatic castration-resistant prostate cancer with *BRCA1/2* mutations,^{15–18} but PARPi drugs investigated in GC are either in the preclinical or clinical phase.¹⁹

LS carriers have a nearly 10-fold increased risk of GC compared with non-carriers.²⁰ Especially in countries with a high prevalence of sporadic gastric carcinoma, LS carriers with a lifetime risk of 30% in Korea²¹ and 44.4% in China²² were diagnosed with GC. In European countries, however, cumulative incidences at 75 years for GC with LS were much less than colorectal cancer (CRC) and endometrial cancer. In the other words, >90% of LS carriers will never develop GC.²³ Germline DNA mismatch repair (MMR) gene aberrations in CRC are associated with high tumour mutational burden (TMB-H) as well as microsatellite instability.^{24,25} The relationship between germline mutation in MMR genes and tumour mutational burden (TMB) in GC remains to be elucidated.

Germline mutations in MMR genes have been reported to be implicated in gastric heritability in Chinese patients, whereas the prevalence and spectrum of HRD germline mutations in Chinese patients with GC remain largely unknown. Here we aimed to retrospectively delineate the germline mutation characteristics of patients with HRD-mut GC.

MATERIALS AND METHODS

Study design and patient characteristics

We retrospectively reviewed the sequencing data from 1135 patients with unrelated GC who underwent mutation profiling from January 2017 to May 2021. This patients were recruited from three centres: Guangdong Provincial People's Hospital, Zhejiang Provincial People's Hospital and Peking University Cancer Hospital, which is located in the southernmost coastal area of China, east coastal area and north area, respectively. We recruited 359 patients from Guangdong Provincial People's Hospital, 413 patients from Zhejiang Provincial People's Hospital and 363 patients from Peking University Cancer Hospital. Recruited patients provided matched white blood cell and tumour tissue samples for genetic test; both somatic and germline mutations were analysed here. In this analysis, we focused on patients harbouring germline mutation in nine HRD genes (*BRCA1*, *BRCA2*, *ATM*, *PALB2*, *BRIP1*, *CHEK1*, *CHEK2*, *FANCA* and *FANCL*).^{26,27}

The overall study design is illustrated in figure 1A. Of the 1135 patients, 881 patients were analysed with next-generation sequencing (Onco PanScan multigene panels covering more than 1 Mb; Genetron Health (Beijing) Company, Beijing, China) for subsequent genetic analysis. Four hundred sixteen patients who had no germline mutations detected in any genes were excluded. Of the remaining 465 patients, 89 patients harbouring germline mutations of the nine core HRD genes were included for further analysis (figure 1A). Among the 89 patients, 29 patients were identified from Guangdong Provincial People's Hospital; 31 patients were identified from Zhejiang Provincial People's Hospital; and 29 patients were identified from Peking University Cancer Hospital.

Somatic mutation analysis was performed on 746 of 881 patients, including TMB (figure 1B).

Germline analysis

Reads were aligned to the reference human genome sequence GRCh37 using Novoalign. Sequencing reads were mapped to a human reference genome (hg19) using the Burrows-Wheeler aligner.^{28–30} Duplicate removal, local realignment and base quality recalibration were performed using PICARD (<http://broadinstitute.github.io/picard/>) and the Genome Analysis Toolkit (GATK).³¹ Sequence mutations were then analysed for indels and single-nucleotide mutations (SNVs) using the GATK. Germline mutations were first filtered as

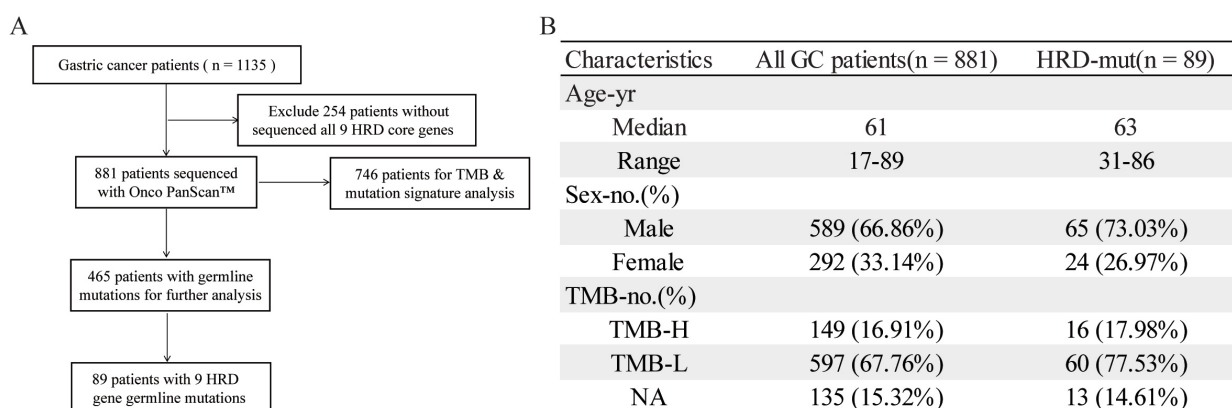


Figure 1 Flowchart of the study design and clinical characteristics. (A) Flowchart of the patients' screening. (B) Clinical characteristics of patients with GC in the genetic analysis cohort and the HRD-mut cohort. GC, gastric cancer; HRD, homologous recombination deficiency; NA, not available; TMB, tumour mutational burden; TMB-H, high tumour mutational burden; TMB-L, high tumour mutational burden.

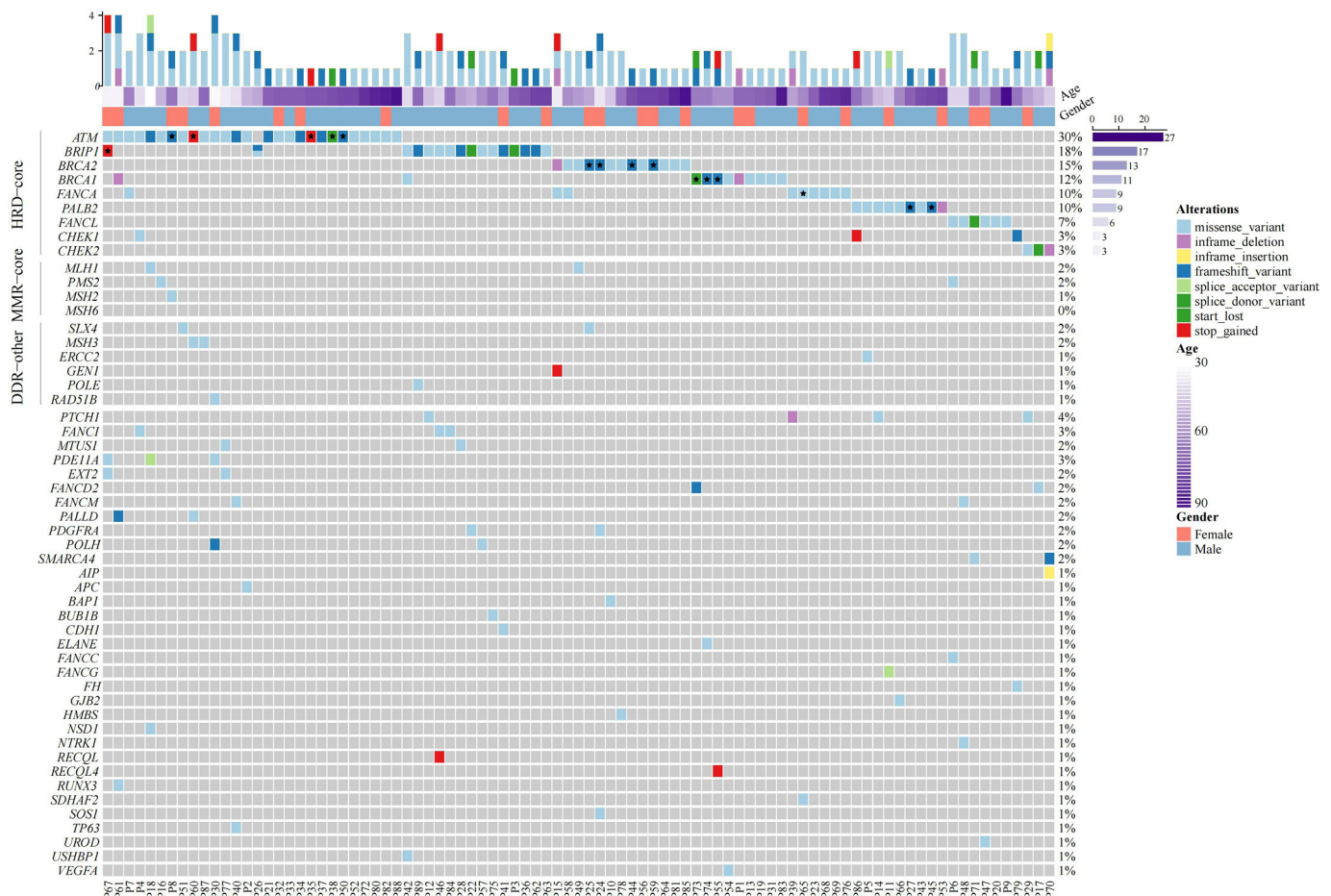


Figure 2 Profiling of germline mutations in 89 HRD-mut patients with gastric cancer. All germline mutations were classified into ‘HRD-core’, ‘MMR-core’, ‘DR-other’ and other signalling pathways. Asterisks indicate pathogenic/likely pathogenic germline mutations in ClinVar. DDR, DNA damage repair; HRD, homologous recombination deficiency; MMR, mismatch repair.

follows: (1) major allele frequency of <0.01% in the database Exome Aggregation Consortium (ExAC_EAS) and (2) recurrence count of ≤ 3 in the cohort. We then performed an analysis of germline mutations which lead to loss of function (LoF) mutations: (1) gain of stop codon, (2) loss of initiation codon, (3) frameshift, (4) deletion of single exon or multiexon (exclude if stop codon occurs in the last exon or the last 50 base pairs of the penultimate exon), (5) splice donor/acceptor effect (canonical ± 1 or 2 splice sites) predicted by FSPLICE (online supplemental table 1) and (6) missense change and inframe indel predicted by silico tools as deleterious (polyphen-2 and PROVEN) (online supplemental table 1).^{32–34} We selected nine core HRD genes (*BRCA1*, *BRCA2*, *ATM*, *PALB2*, *BRIP1*, *CHEK1*, *CHEK2*, *FANCA* and *FANCL*). The HRD-mut cohort included patients with at least one germline LoF mutation in the 10 core HRD genes. Patients with MMR-mut harboured at least one germline LoF mutation in four MMR genes (*MHL1*, *MSH2*, *PMS2* and *MSH6*).

Analysis of the TMB

TMB was extrapolated using the total number of non-synonymous mutations divided by the total genomic target region of PanScan multigene panels of over 1 M bp. A cut-off of the top 20% of the TMB (9.8 mutations/Mb) in this study was selected for defining a tumour as TMB-H. Patients with TMB of <9.8 mutations/Mb were defined as low TMB.

Analysis of somatic copy-number alteration and loss of heterozygosity (LOH)

We performed the copy-number alteration and LOH analysis using the FACETS based on panel data. Facets CNV callers use the somatic BAM files of that patient for nine core HRD genes.³⁵ For each sample, copy-number alterations were defined as $tcn.em \geq 4$ for gains and $tcn.em \geq 1$ with $lcn.em = 0$ for LOH.

Statistical analysis

The difference in age at diagnosis between different groups as well as the TMB scores between different groups was evaluated using Student’s t-test when the variances were equal or Welch’s test when the variances were not equal. Fisher’s exact test was used to assess the differences in other demographic characteristics. Statistical significance was determined as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS

Characteristics of patients with HRD-mut

We retrospectively reviewed the genomic sequencing data of 1135 patients with GC, profiled from January 2017 to May 2021. The cohort had a median age of 61 years, with 67.2% male and 32.8% female. Among them, 881 patients with a median age of 61 years, including 67.88% male and 33.14% female, underwent genetic analysis including germline and

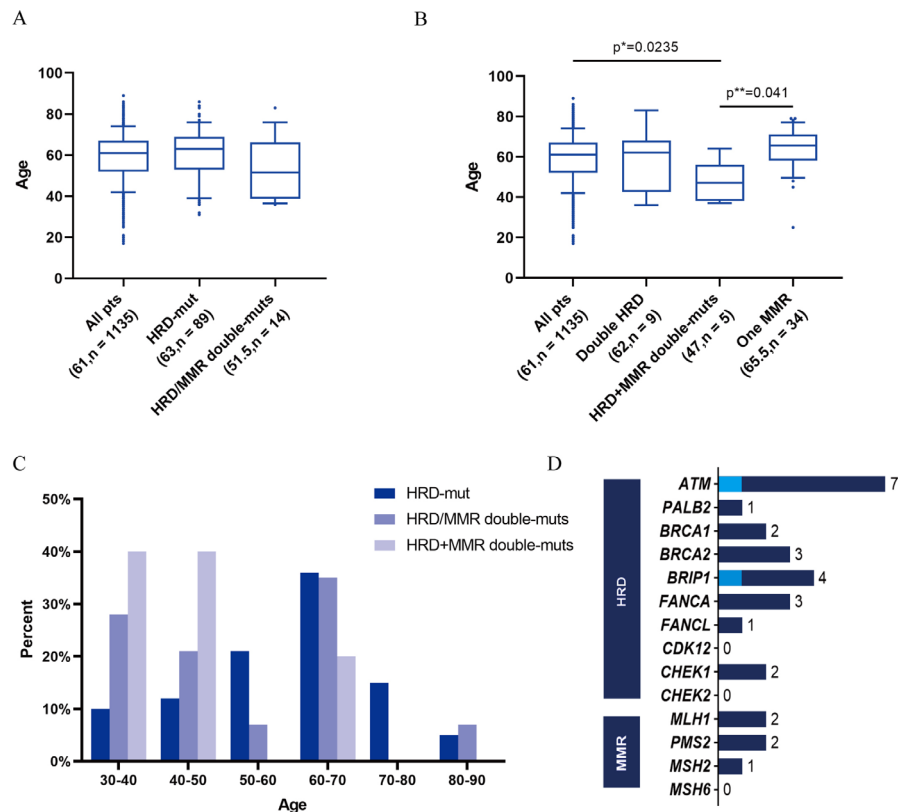


Figure 3 Patients with GC harbouring double germline mutations in both core HRD and MMR genes may have an earlier age of onset. (A) Bar plots display the median age of all patients with GC, patients with HRD-mut and 14 patients with HRD/MMR double-muts. (B) The median age of patients with HRD+MMR double-muts is significantly earlier than that of patients with GC and one patient with MMR gene. (C) Age distributions of patients with HRD-mut, all 14 patients with HRD/MMR double-muts and 5 patients with HRD+MMR double-muts. (D) Proportions of all core HRD genes and MMR gene mutations in 14 patients with HRD/MMR double-muts. The light blue shade in *ATM* and *BRIP1* genes indicated two pathogenic/likely pathogenic mutations classified in ClinVar. GC, gastric cancer; HRD, homologous recombination deficiency; MMR, mismatch repair.

somatic mutation profiling. The median age and gender ratio of this cohort has no significantly difference from the original Chinese GC cohort. Of the 881 patients, 89 patients were identified with germline mutations in core HRD gene (figure 1A). The median age of this HRD-mut cohort was 63 years, with 73% male and 27% female. Collectively, a total of 161 germline mutations were detected in the 89 patients, of which 98 germline mutations occurred in the 9 core

HRD gene. *ATM* (30.33%) was the most frequently mutated HRD gene, followed by *BRIP1* (17.98%), *BRCA2* (14.61%), *BRCA1* (12.36%), *FANCA* (10.11%), *PALB2* (10.11%), *FANCL* (6.74%), *CHEK1* (3.37%) and *CHEK2* (3.37%). ClinVar classified 16 of all 98 HRD gene germline mutations as pathogenic/likely pathogenic (P/LP) in other cancer types like hereditary cancer-predisposing syndrome and breast-ovarian cancer (online supplemental table 2). With

Table 1 Description of 14 patients with homologous recombination deficiency/mismatch repair double-muts

Patient ID	Sex	Mutation A	Mutation B	Other mutation
P6	Male	<i>FANCL</i> c.335C>T, (p.Ser112Leu)	<i>PMS2</i> c.58C>T, (p.Arg20Trp)	<i>FANCC</i> c.239T>C, (p.Ile80Thr)
P8	Male	* <i>ATM</i> c.1402_1403delAA, (p.K468Efs)	<i>MSH2</i> c.2649T>G, (p.Ile883Met)	
P16	Male	<i>ATM</i> c.2944C>T, (p.Arg982Cys)	<i>PMS2</i> c.46A>G, (p.Lys16Glu)	
P18	Female	<i>ATM</i> c.1896del, (p.E632Dfs)	<i>MLH1</i> c.1154G>A, (p.R385H)	<i>PDE11A</i> c.1303-2A>T; <i>NSD1</i> c.487G>T, (p.Asp163Tyr)
P49	Male	<i>BRCA2</i> c.7540A>G, (p.Lys2514Glu)	<i>MLH1</i> c.1937A>G, (p.Tyr646Cys)	
P4	Female	<i>CHEK1</i> c.184C>G, (p.Leu62Val)	<i>ATM</i> c.1351C>T, (p.Arg451Cys)	<i>FANCI</i> c.2183A>G, (p.Asp728Gly)
P7	Female	<i>ATM</i> c.6671T>C, (p.Met2224Thr)	<i>FANCA</i> c.1840C>T, (p.Pro614Ser)	
P15	Male	<i>FANCA</i> c.209A>G, (p.Lys70Arg)	<i>BRCA2</i> c.5218_5223del, (p.Leu1740_Ser1741del)	<i>GEN1</i> c.1201C>T, (p.Arg401Ter)
P26	Male	<i>BRIP1</i> c.3240dup, (p.Ala1081CysfsTer5)	<i>BRIP1</i> c.2301G>C, (p.Glu767Asp)	
P42	Male	<i>BRCA1</i> c.3159A>C, (p.Glu1053Asp)	<i>BRIP1</i> c.1954G>A, (p.Gly652Arg)	<i>USHBP1</i> c.22C>A, (p.Pro8Thr)
P58	Male	<i>BRCA2</i> c.3372G>C, (p.Gln1124His)	<i>FANCA</i> c.209A>G, (p.Lys70Arg)	
P61	Female	<i>ATM</i> c.7382G>A, (p.Arg2461His)	<i>BRCA1</i> c.3327_3329del, (p.Lys1110del)	<i>PALLD</i> c.1017del, (p.Gly340ValfsTer35); <i>RUNX3</i> c.58G>A, (p.Asp20Asn)
P67	Male	* <i>BRIP1</i> c.1315C>T, (p.Arg439Ter)	<i>ATM</i> c.169T>C, (p.Trp57Arg)	<i>EXT2</i> c.995G>A, (p.Ser332Asn); <i>PDE11A</i> c.764C>T, (p.Ser255Phe)
P86	Male	<i>CHEK1</i> c.1135C>T, (p.Arg379Ter)	<i>PALB2</i> c.3296C>G, (p.Thr1099Arg)	

Cell shadow code: light grey: patients with HRD+MMR double-muts; blank background: patients with double HRD-muts.
*These gene mutations are classified as pathogenic/likely pathogenic in ClinVar.

in-depth analysis of the sequencing data of these 89 patients with HRD-mut, we found that 51 patients harboured germline mutations in multiple inherited cancer genes. In addition to the core HRD genes, DNA MMR genes like *MLH1*, *PMS2*, *MSH2* and other DNA damage repair genes like *SLX4*, *MSH3*, *GEN1*, *ERCC2*, *POLE* and *RAD51B* were also detected in these patients with HRD-mut (figure 2).

Patients with GC harbouring double LoF germline mutations in the HRD and MMR pathway may have an earlier age of onset

We screened the germline mutation of the ten core HRD gene and DNA MMR genes (*MLH1*, *MSH2*, *PMS2* and *MSH6*). Out of the 89 patients with HRD-mut, 14 harboured double gene mutations in the 14 genes (HRD/MMR double-muts), with a median age of 51.5 years. Though the median age of the 14 patients with HRD/MMR double-muts was almost 10 years younger than that of the whole GC cohort, the p value of the two cohorts has no obvious difference (Student's t-test, p=0.1139) (figure 3A). Nine out of 14 patients with HRD/MMR double-muts harboured double HRD mutations, with a median age of 62 years, which is even 1 year older than the whole GC cohort. Five patients with double mutation harboured mutations in both one HRD and one MMR genes (HRD+MMR double-muts), with a median age of 47, and the age of onset was significantly earlier than that of patient

cohort with GC (Student's t-test, p=0.0235). In order to rule out the possibility that early onset of GC is caused by MMR germline mutations, we then identified 34 patients who harboured only one of MMR gene mutation, with a median age of 65.5, which is significantly different from that of patients with HRD+MMR double-muts (Student's t-test, p=0.041) (figure 3B,C). This result indicated that patients with double mutation with both HRD and MMR genes in GC may have an earlier age of onset. We found 35 LoF germline mutations in these 14 HRD/MMR double-muts (table 1). *ATM* (NM_000051.3):c.1402_1403delAA, (p.K468Efs) and *BRIP1* NM_032043.2:c.1315C>T, (p.R439X) were classified as P/LP in hereditary cancer-predisposing syndrome and familial cancer of the breast, respectively, by ClinVar database. *ATM* was the most frequently mutated gene in the patients with HRD/MMR double-muts, which is consistent with the highest proportion of mutations in the patients with HRD-mut. Moreover, no alterations in *CHEK2* and *MSH6* genes were detected in the 14 patients with HRD/MMR double-muts patients (figure 3D).

Somatic genomic profile

We also investigated the somatic genomic profiles of the 89 patients with HRD-mut (figure 4). Of 89 patients with HRD germline mutations, 0.561% (n=5) of patients identified no somatic gene alteration. *TP53* (58%), *LRP1B* (18%),

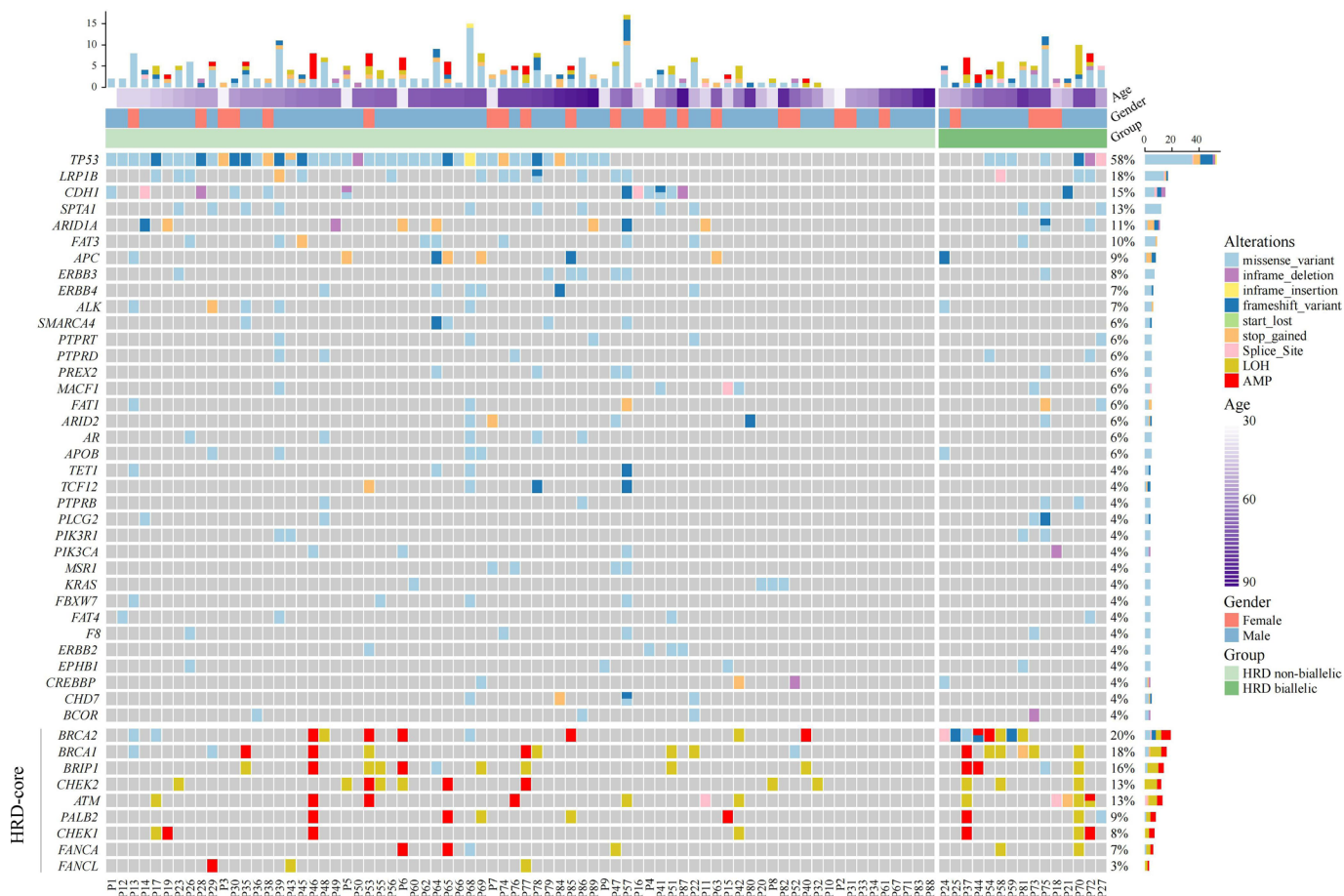


Figure 4 Profiling of somatic mutations in 89 patients with HRD-mut with gastric cancer. Somatic mutations were classified into 'HRD-core' and other signalling pathways. The columns and rows represent patients and genes, respectively, and are sorted in decreasing order by the frequency of gene mutations. The right panel indicates the frequency of gene mutations. All patients were divided into two groups: HRD biallelic and HRD non-biallelic. AMP, amplification; HRD, homologous recombination deficiency; LOH, loss of heterozygosity.

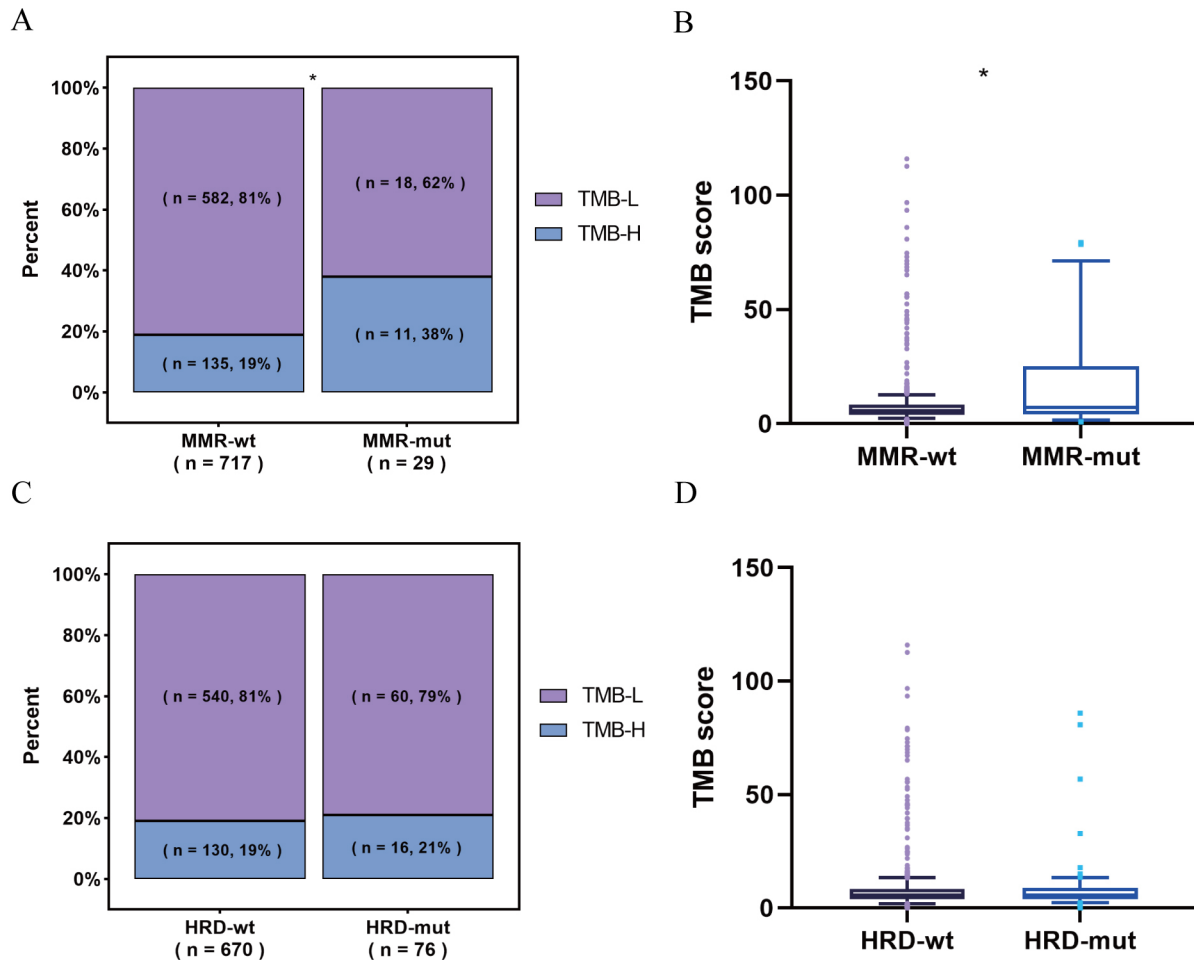


Figure 5 Associations among mutations in HRD genes, MMR genes and TMB status. (A) The percentages of patients with TMB-H in the MMR-mut group is significantly higher than that of the MMR-wt group. (B) Bar plots display the TMB score between the MMR-mut group and MMR-wt group. (C) The percentage of patients with TMB-H in the HRD-mut compared with the MMR-mut group. (D) Bar plots display the TMB score between the HRD-mut group and MMR-mut group. Fisher's exact test was used to examine the percentages of patients with TMB-H among each group. P values of TMB scores between MMR-mut and MMR-wt groups were calculated with the Welch's test as the variances were not equal. P values of TMB scores between HRD-mut and HRD-wt groups were calculated with Student's t-test as the variances were equal. * $P < 0.05$. HRD, homologous recombination deficiency; MMR, mismatch repair; TMB, tumour mutational burden; TMB-H, high tumour mutational burden; TMB-L, high tumour mutational burden.

CDH1 (15%), *SPTA1* (13%) and *ARID1A* (11%) were the most frequently mutated genes in the 89 patients with HRD-mut. It is worth noting that 27.0% ($n=24$) of patients acquired somatic core HRD gene mutation; 9% ($n=8$) of patients acquired somatic SNV in the same HRD gene; and 7.9% ($n=7$) of patients acquired the same HRD LOH as HRD biallelic mutations patients (online supplemental table 3). We identified one patient (P17) with simultaneous HRD missense mutation and LOH event, one patient (P29) with simultaneous HRD missense mutation and amplification event, and one patient (P37) with simultaneous HRD missense mutation, LOH and amplification events. We did not find any mutations that were mutually exclusive with HRD mutations. However, only 46.7% of patients with HRD biallelic mutations have *TP53* mutations that are much less than 60.81% of patients with HRD non-biallelic mutations, though the difference was not significant. Among the 746 patients who underwent TMB analysis, patients with TMB-H were seen in 38% of the MMR-mut group, which was significantly higher than that of 19% in the MMR-wt group (Fisher's exact test, $p=0.0166$). In addition, the level of TMB score was significantly higher in the MMR-mut

group than in the MMR-wt group (Welch's test, $p=0.0356$) (figure 5A,B). Unlike MMR mutations that led to elevated TMB, there was no significant difference in TMB between the HRD-wt and HRD-mut groups. Patients with TMB-H were seen in 21% of the HRD-mut group, not significantly higher than 19% of the HRD-wt group (Fisher's exact test, $p=0.7603$). Moreover, there was no difference in the level of TMB score between the HRD-mut group and the HRD-wt group (Student's t-test, $p=0.9165$) (figure 5C,D).

DISCUSSION

To the best of our knowledge, this is the first study to systematically explore HRD LoF germline mutations in a large Chinese cohort of GCs. Pathogenic germline mutations of HRD genes like *BRCA1*, *BRCA2* and *ATM* are known to confer susceptibility to ovarian and breast cancers.^{23,36} Because the role of HRD genes in conferring susceptibility to GC is not clear,³⁶ we focus on LoF germline mutations instead of pathogenic or likely pathogenic germline mutations.

Among all 881 patients who underwent comprehensive genetic analysis, 10.01% ($n=89$) of the patients harboured

HRD LoF germline mutations. In the 89 patients with HRD-mut, 16 germline mutations can be ClinVar classified as P/LP in other cancer types like hereditary cancer-predisposing syndrome and breast-ovarian cancer (online supplemental table 2). However, according to the American College of Medical Genetics and Genomics (ACMG) guidelines, there is no sufficient evidence to support these 16 pathogenic germline mutations were P/LP in GC, while the rest of LoF germline mutations can all be classified as mutations of uncertain significance (VUS). Of the 89 patients with HRD-muts, 51 harboured LoF germline mutations in multiple genes. The additional mutations occurred most frequently in MMR and HRD-related genes. Out of the 89 patients with HRD-mut, 14 harboured another LoF mutation in core HRD genes or MMR genes.

Carriers of double mutations in the *BRCA* genes expressed their cancer at an earlier age (44.6 ± 13.5) than did patients with single mutation (48.1 ± 13.0).³⁷ Previous also reported one case of co-occurrence germline mutations in *BRCA1* and *MSH6* in a patient with early-onset endometrial cancer.³⁸ In our study, we identified nine patients harbouring double HRD-core gene LoF germline mutations (HRD+HRD). Moreover, five patients harboured both one core HRD and one MMR gene LoF germline mutations (HRD+MMR). Unexpectedly, only patients with HRD+MMR double-muts exhibited early onset of age, but not for patients with HRD+HRD double-muts or HRD-mut patients. In order to rule out the possibility that early onset of GC is caused by MMR germline mutations, we pay attention to the cancer risk of pathogenic_MMR carriers for GC. Cumulative incidences at 75 years (risks) for GC with pathogenic_MMR germline mutations were less than 10%, which is much lower than CRC and often diagnosed predominantly in older age. Pathogenic_MMR carriers do not exhibit the characteristics of early onset for GC.²³ Consistent with previous reports, in our cohort the MMR-mut patients, which harboured with only one of MMR gene germline mutation, did not exhibit early onset of age (median age, 65.5). Our result first reports early onset of GC in HRD+MMR double-muts patients. It implies HRD and MMR mutations synergistically increase tumour risk in GC, which still need more methods like functional studies, family cosegregation analysis and so on to provide more evidence to classify the germline mutations.

Some of the 14 double mutation carriers harboured other germline mutations in *FANCC*, *PDE11A*, *FANCI*, *GEN1*, *USHBP1*, *PALLD*, *EXT2*, *NSD1* and *RUNX3* gene. *EXT2* gene mutations were associated with hereditary multiple exostoses (HMEs). HME is an autosomal dominant disorder characterised by multiple exostoses most commonly arising from the juxtaepiphyseal region of the long bones.³⁹ In a germline genetic study of total 40 Chinese patients with GC also identified one patient harbouring *EXT2* likely pathogenic mutation inherited from his mother, who was cancer-free.⁴⁰ However, whether *EXT2* confers susceptibility to GC needs to be deciphered in future work. *RUNX3* belongs to the runt domain family of transcription factors, and a recent research highlighted the significance and mechanism of *RUNX3*-mediated circDyrk1A in suppressing glutamine metabolism in GC via the miR-889-3 p/FBXO4 axis.⁴¹ However, another report indicated that no germline *CDH1*, *TP53* or *RUNX3* mutations were detected in any of the patients with early-onset GC.⁴² To the best of our knowledge, no literature has clearly reported that *FANCC*, *PDE11A*, *FANCI*,

GEN1, *USHBP1*, *PALLD*, *NSD1* were associated with germline GC susceptibility.

Due to the extremely low incidence of HRD and MMR double mutations, follow-up difficulties and hereditary genetic testing in GC are not widely accepted in China, there is a lack of published large cohort data of germline mutations. Nevertheless, we still collected one family history of double mutations. We obtained detailed family history of one patient with HRD+MMR double-muts (*ATM*+*MLH1*). The patient's younger sister (II-2) was detected with the same double mutation of *ATM* NM_000051.3:c.1896del, (p.E632Dfs) and *MLH1* NM_000249.3:c.1154G>A, (p.R385H) (online supplemental figure 1). We conducted a prospective follow-up of this double mutation family member, but no evidence of GC was found at the time this article was written.

Although double germline mutations are rare, it had been reported before in breast cancer, ovarian cancer, CRC and other hereditary cancers.^{37 38 43 44} In a cohort of 1023 unrelated patients with suspicion of hereditary cancer, 13 (1.37%) patients harbouring two pathogenic germline mutations in dominant cancer-predisposing genes were identified.⁴⁵

According to the ACMG guideline, in the context of dominant disorders, the detection of a mutation in trans with a pathogenic mutation can be considered supporting evidence for a benign impact. The VUS is easily ignored in patients with potential double mutations. In addition, clinical interpretation of germline mutations is a pressing challenge.³⁶ We therefore recommend considering the potential value of VUS in genetic screening.

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