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Germline Mutations in *PALB2*, *BRCA1*, and *RAD51C*, Which Regulate DNA Recombination Repair, in Patients With Gastric Cancer

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Up to 10% of cases of gastric cancer are familial, but so far, only mutations in *CDH1* have been associated with gastric cancer risk. To identify genetic variants that affect risk for gastric cancer, we collected blood samples from 28 patients with hereditary diffuse gastric cancer (HDGC) not associated with mutations in *CDH1* and performed whole-exome sequence analysis. We then analyzed sequences of candidate genes in 333 independent HDGC and non-HDGC cases. We identified 11 cases with mutations in *PALB2*, *BRCA1*, or *RAD51C* genes, which regulate homologous DNA recombination. We found these mutations in 2 of 31 patients with HDGC (6.5%) and 9 of 331 patients with sporadic gastric cancer (2.8%). Most of these mutations had been previously associated with other types of tumors and partially co-segregated with gastric cancer in our study. Tumors that developed in patients with these mutations had a mutation signature associated with somatic homologous recombination deficiency. Our findings indicate that defects in homologous recombination increase risk for gastric cancer.

Keywords: Stomach; Tumor; WES; Interaction.

Worldwide, gastric cancer (GC) is the fifth most commonly diagnosed malignancy and the third cause of cancer-related deaths.[1] Up to 10% of cases show familial clustering, suggesting a genetic basis.[2] *CDH1* mutations are a known cause of hereditary diffuse gastric cancer

[†]In memoriam.

Abbreviations used in this paper: **GC, gastric cancer; HDGC, hereditary diffuse gastric cancer; HR, homologous recombination; WES, whole-exome sequencing.**

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(HDGC), explaining approximately 40% of cases,[3,4] but the genetics of non-HDGC remain largely unknown. To identify novel GC genes, we analyzed *CDH1* mutation-negative HDGC cases using whole-exome sequencing (WES) followed by candidate gene targeted analyses in independent HDGC and non-HDGC cases.

WES of 28 *CDH1*-negative European HDGC cases identified three with candidate causal variants (Table 1): nonsense (p.Arg414Ter) and splice site (c.3201+1G>T) *PALB2* mutations, and a nonsense *RAD51C* (p.Arg237Ter) mutation. No deleterious mutations were seen in other known cancer genes (Supplementary Methods). *PALB2* and *RAD51C* are both critical in homologous recombination (HR), a major DNA repair pathway.[5] Both of the *PALB2* mutations have been reported previously as pathogenic in breast cancer families[6] and *RAD51C* p.Arg237Ter is reported as pathogenic in ClinVar.[7]

We then performed targeted sequencing of *PALB2* and *RAD51C*, their interaction partners *BRCA1/2* and *CDH1* in 173 additional Latin-American GC cases. Based on enrichment of HR mutations in our discovery cohort and a recent report showing multiple intestinal, diffuse, and mixed histology gastric tumors with a somatic HR deficiency signature,[8] our validation cohort included both HDGC and non-HDGC cases of diffuse and nondiffuse histology (Supplementary Methods). Targeted sequencing identified 4 additional mutation carriers: 2 sharing a known Hispanic *BRCA1* founder mutation (p.Gln1111Asnfs)[9] and 2 with novel *PALB2* mutations (p.Pro918Gln and p.Lys628_Cys630del) with predicted deleterious effects. Residue Pro918 falls in the PALB2 WD40 domain, which mediates interactions with BRCA2, RAD51, and RAD51C, whereas Lys628-Cys630 resides in the binding domain of MRG15, a transcription regulator and whose PALB2 interaction is required for homology-directed DNA double-strand break repair indicating potential pathogenicity of these 2 novel mutations.[10,11]

In a third phase of the study, we genotyped all 6 *PALB2, RAD51C*, and *BRCA1* mutations described plus 4 known Hispanic *BRCA1/2* founder mutations (Supplementary Methods) in 160 independent Latin-American non-HDGC cases and found 3 additional mutation carriers, 1 with a *BRCA1* mutation (p.Gly559Valfs) and 2 with *PALB2* mutations (p.Lys628_Cys630del and p.Arg414Ter) (Table 1). Interestingly, during the preparation of this article, our clinic-based Portuguese collaborator (MRT and GS) identified 1 additional GC case (GM037589) with *PALB2* p.Arg414Ter. None of the 7 *PALB2*, *RAD51C*, and *BRCA1* mutations detected in 11 unrelated Caucasian and Latin-American cases was detected in 1,170 population-matched controls (see mutation details in Supplementary Table 1).

Clinical details of our mutation carriers are presented in Table 1. Most of them had diffuse histology, 2 had HDGC syndrome (CG-05 and GM022584), and 1 reported a history of hereditary breast and ovarian cancer (case CG-36, not shown). These mutation carriers were predominantly nonsmokers and/or negative for *Helicobacter pylori* infection (Table 1), which suggest that GC risk in most of these cases was not driven by these 2 known environmental risk factors.[12]

To obtain additional evidence of the causality of our HR gene mutations, we carried out loss of heterozygosity, mutational signature, and co-segregation analyses in available samples from tumors and relatives. For loss of

Table 1. Details of Clinical Information of the Mutation Carriers

Mutation details	ID	Age of onset	Sex	Histology	Satisfied HDGC criteria?	*Helicobacter pylori* infection	History of smoking
PALB2	CG-12[a,d]	69	M	Intestinal	No	NA	NA
c.1240C>T, p.Arg414Ter	CG-008[c]	48	F	Diffuse	NA	NA	Yes
	GM037589	46	F	NA	No	Negative	No
PALB2	CG-05[a]	50	M	Diffuse	Yes	Negative	No
c.3201+1G>T							
PALB2	CG-039[b]	47	F	Diffuse	NA	Negative	No
c.1882_1890delAAGTCCTGC, p.Lys628_Cys630del	CG-028[c,d]	81	M	Intestinal	NA	Negative	Yes
PALB2	3CG-103[b,d]	79	F	Mixed	No	Negative	Yes
c.2753C>A, p.Pro918Gln							
BRCA1	CG-036[b]	67	F	Diffuse	No	NA	No
c.3331_3334delCAAG, p.Gln1111Asnfs	CG-059[b]	54	M	Diffuse	No	NA	No
BRCA1	CG-001[c]	65	M	NA	No	Positive	Yes
c.1674delA, p.Gly559Valfs							
RAD51C	GM022584[a,d]	73	M	Diffuse	Yes	Negative	No
c.709 C>T, p.Arg237Ter							

NA, Not available.
[a]Identified by WES.
[b]Identified by targeted sequencing.
[c]Identified by genotyping.
[d]LOH and mutational signature analyzed.

heterozygosity and mutational signatures, we performed WES in 4 available tumor samples from 3 *PALB2* (CG-12/p.Arg414Ter, CG-028/p.Lys628_Cys630del and 3CG-103/p.Pro918Gln) and *RAD51C* mutation carriers (Table 1). We found no loss of heterozygosity or compound heterozygosity in these tumor samples (not shown). Interestingly, when we analyzed the somatic WES data for mutational signatures, we found that all 4 tumors were enriched for a signature indicative of HR defects,[13,14] providing evidence for the causality of these mutations (Supplementary Methods, Supplementary Figures 1 and 2).

Figure 1 shows available pedigrees from mutation carriers. Case 3CG-103 and her daughter were both diagnosed with GC and carried the *PALB2* p.Pro918Gln mutation (Figure 1*A*). GM037589, a *PALB2* p.Arg414Ter carrier, developed GC and breast cancer and had a sister diagnosed with ovarian and endometrial cancer who also carried *PALB2* p.Arg414Ter (Figure 1*B*). The *RAD51C* p.Arg237Ter carrier's son died of colon cancer but did not carry the mutation (Figure 1*C*). We found that GC was the predominantly diagnosed malignancy among unavailable relatives of these carriers (Figures 1*A*−*D*). Although we did not have access to samples from relatives of the *PALB2* p.Lys628_Cys630del carriers, our local collaborators found this mutation co-segregating in an unrelated breast cancer family (unpublished data). Albeit limited, our co-segregation data partially support GC causality of *PALB2* mutations. The *RAD51C* co-segregation data are, however, inconclusive, but the presence of a strong HR signature in the gastric tumor of this mutation carrier warrants further studies on *RAD51C* as a candidate GC gene.

In summary, our study identified 11 cases with mutations in *PALB2*, *BRCA1*, and *RAD51C*, 3 closely related HR genes. Some of these mutations are known to be pathogenic in other cancer types. Out of 362 cases analyzed, 6.45% of the HDGC cases (2 of 31) and 2.7% (9 of 331) of non-HDGC cases had *PALB2, BRCA1*, or *RAD51C* mutations, suggesting that HR genes play a role in GC risk. Our data also provide

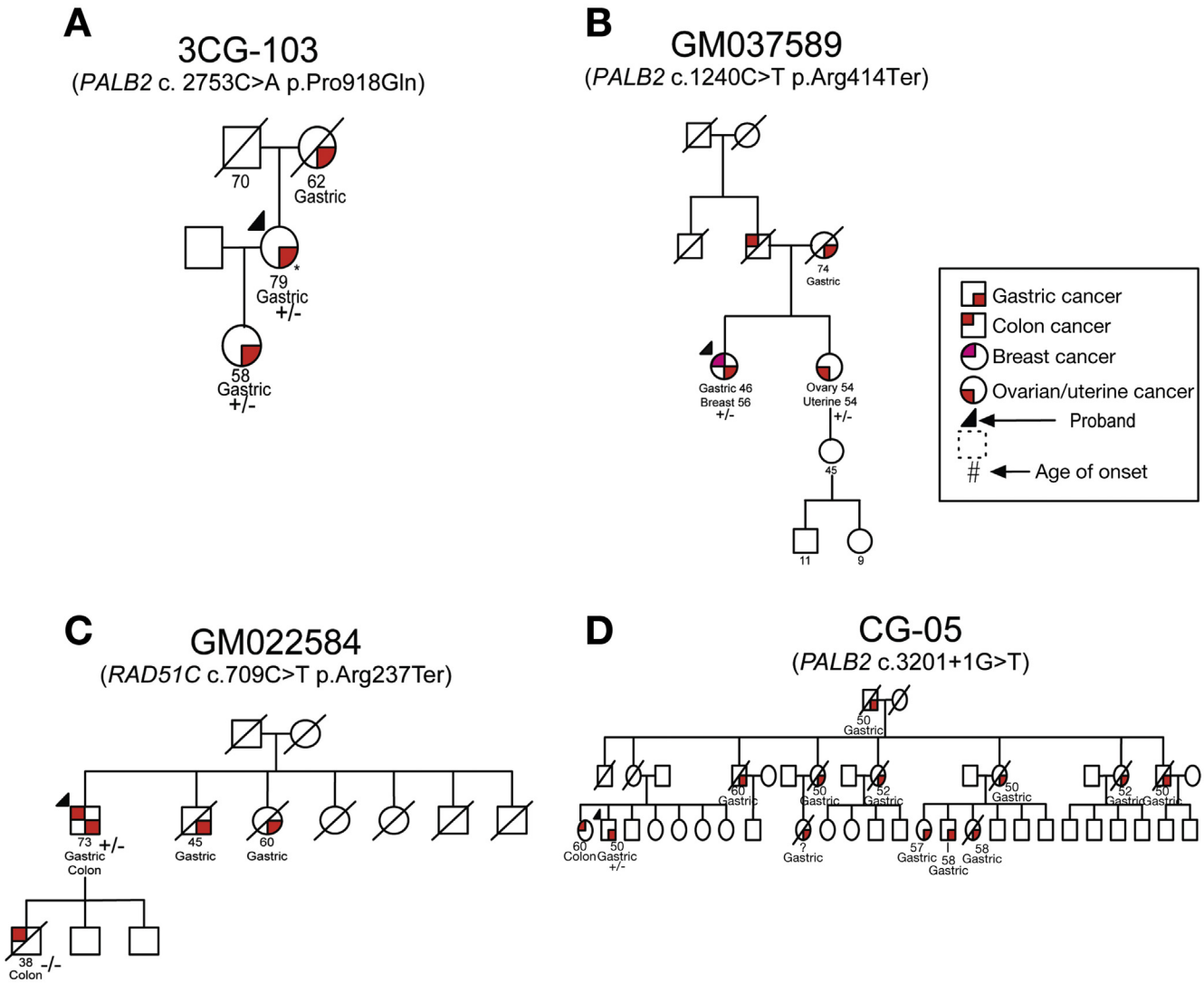

Figure 1. Available pedigrees of mutation carriers. (*A*) Pedigree of patient 3CG-103 carrying *PALB2* missense mutation. (*B*) Pedigree of patient GM037589 carrying *PALB2* nonsense mutation. (*C*) Pedigree of patient GM022584 carrying *RAD51C* nonsense mutation. (*D*) Pedigree of patient CG-05 carrying *PALB2* splice site mutation.

evidence of a germline basis for the recently reported HR mutational signature in gastric tumors and strengthens the evidence for a causal role of these genes, specifically *PALB2,* in GC, as observed previously.[4,15] Future larger studies are needed to definitively assign causality and understand the penetrance and prevalence of HR gene mutations in GC and to further understand if and why some individuals from hereditary breast and ovarian cancer families with HR gene mutations develop GC. Further characterizations of the GC histology in HR gene mutation carriers are also needed, as we found instances where the same mutation was found in cases with different histologies (CG-12 and CG-008 with *PALB2* p.Arg414ter and CG-039 and CG-028 with PALB2 p.Lys628_Cys630del; Table 1). *CDH1* mutation−negative families might benefit from HR gene testing and increased endoscopic surveillance and targeted therapies, such as poly ADP ribose polymerase inhibitors.[8]

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.12.010.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Phase 1: Variant Discovery by Whole-Exome Sequencing

Patient recruitment. For WES analysis, we included 28 GC cases (and 6 relatives from 4 different families) with HDGC (defined according to the published guidelines[1]) recruited in the Portuguese Oncology Institute (University of Porto, Portugal) and in the Genomic Medicine group (Santiago de Compostela, Spain). Sample collection was undertaken with informed consent and ethical review board approval of the corresponding institution, in accordance with the tenets of the Declaration of Helsinki. All of these 28 index HDGC cases tested negative for *CDH1* mutations at clinical laboratories in these 2 Portuguese and Spanish institutions. Mean age of HDGC index patients was 48.2 years (SD, 13.2 years). Fifteen of these patients were male and 13 were female. Interestingly, 1 of these patients (CG-12), who was initially included as an HDGC case, on histologic re-examination by 2 independent surgical pathologists (JC-T and AB) was reclassified as having intestinal histology. This case was therefore reclassified as a non-HDGC in our study.

Whole-exome sequencing library preparation. Samples were prepared for WES using Agilent SureSelect XT2 protocol. Briefly, up to 1 μg DNA was sheared using Covaris E220 sonicator. Fragments were end-repaired, A-tailed and Illumina-compatible adaptors were ligated at the ends. The fragments were then enriched using PCR. Eight multiplexed samples were hybridized to the bait set, washed, and captured fragments were amplified by PCR. Samples were then sequenced on an Illumina HiSeq2000 sequencer with 100PE sequencing.

Whole-exome sequencing data analysis. For data analysis, publically available tools as well as custom shell scripts were used. Raw data was trimmed for adaptors and sequence quality and then aligned to the human reference genome GRCh37 with decoy sequences using BWA-mem, version 0.7.12 (Scythe, version 0.994 Beta, 2011, https://github.com/vsbuffalo/scythe; Sickle, version 1.33, 2011, https://github.com/najoshi/sickle).[2] For WES, duplicates were removed with Picard, version 1.129 (http://picard.sourceforge.net). BAM files were locally realigned using GATK IndelRealigner v3.3 and recalibration of the quality scores was performed using GATK BaseRecalibrator, version 3.3.[3] Multiple callers were used to call variants: GATK HaplotypeCaller non-joint, version 3.3[4]; Freebayes, version 0.9.14–17[5]; SNVER[6]; Varscan, version 2.3.7[7]; and Samtools mpileup, version 1.2.[8] Calls were filtered based on: coverage $\geq$10, number of reads supporting variant $\geq$5, minimum variant frequency $\geq$0.20, minimum frequency of variant reads present on opposite strand $>$0.10, and minimum average read quality $\geq$22. Variants were annotated using Annovar.[9] In addition, single-nucleotide polymorphisms and INDEL calling was performed using GATK HaplotypeCaller joint genotyping. Calling, variant filtering, and variant score recalibration were performed using GATK, version 3.3 Best Practices.[4,10] Variants called by at least 2 different callers were considered for further analysis. To select the most informative single-nucleotide variants (SNVs), filtering of the initial data was performed to exclude all synonymous SNVs, SNVs that map to pseudo-genes, repeated regions, segmental duplications, and "dispensable" genes. The remaining protein sequence-altering variants were subjected to frequency filtering using data from publicly available data sets, such as the Exome Variant Server, the UK10K study, dbSNP, and the 1000 Genomes Project to exclude variants with $>$1% minor allele frequency. Of the remaining 7781 variants, SNVs in known cancer predisposition genes[11] were identified (n = 45). Of those, 2 SNVs were protein-truncating (*PALB2*: p.Arg414Ter and *RAD51C*: p.Arg237Ter) with predicted deleterious amino acid substitutions (based on Polyphen, SIFT, MutationAssessor, and MutationTaster) and 1 variant resulted in disruption of a splice site. For the 3 candidate causal variants, pileups were visually inspected in the Integrative Genomics Viewer.[12] No truncating, deleterious mutations were seen in any other cancer genes.

Phase 2: Candidate Gene Validation by Targeted Sequencing

Patient recruitment. For WES replication by targeted sequencing, we included 14 Chilean GC cases recruited in a local cancer clinic, 4 of which satisfied HDGC criteria. Our study included a total of 31 HDGC index cases in the discovery (n = 27) and validation (n =4) phases. Of the remaining 10 Chilean non-HDGC cases, 5 had intestinal GC and 5 were of unknown histology. For targeted sequencing, we also included additional GC cases from Colombia (n = 90) and Mexico (n = 69), of which 104 cases had diffuse histology, 42 had mixed histology, 1 had intestinal histology, and in 12 cases histology was unknown. Together, 53 cases had early-onset GC (younger than 50 years). Chilean cases were recruited in Dr Sótero del Río Hospital, and Clinical Hospital Pontificia Universidad Católica (both in Santiago, Chile). The Ethics Committees of Dr Sótero del Río Hospital and Clinical Hospital Pontificia Universidad Católica de Chile approved the recruitment protocols. Colombian cases for validation phases 2 and 3 were recruited from a multicenter study in Colombia and in the Instituto Mexicano de Seguro Social following protocols approved by University of Tolima (Ibague, Colombia) and Instituto Mexicano de Seguro Social National Council for Research on Health (Mexico City, Mexico).

Targeted sequencing library preparation and data analysis. Approximately 350-bp PCR amplicons covering the entire coding regions of *BRCA1, BRCA2, CDH1, PALB2*, and *RAD51C* were amplified from 50 ng genomic DNA using Fluidigm Access array system and libraries were sequenced on a MiSeq platform with 250PE reads. Sequence data analysis was performed with a bioinformatics pipeline similar to the one described for WES.

Phase 3: Mutation validation by genotyping. Patient recruitment and genotyping: For genotyping, we included

160 non-HDGC cases from Colombia (n = 93) and Mexico (n = 67) that included 24 cases with diffuse histology, 117 with intestinal histology, 8 with mixed histology, and 11 with unknown of histology. All 6 sequence-identified *PALB2, RAD51C*, and *BRCA1* mutations in phase 1 and 2 (see Phase 1 and Phase 2 description in supplementary methods and main text), as well as 4 additional known Hispanic *BRCA1/2* founder mutations (c.5123C>T /p.Ala1708Val and c.1674delA/p.Gly559Valfs in *BRCA1* and c.2808_2811delTAAA/p.Ala938Profs and c.4889C>G/ p.Ser1630Ter in *BRCA2*) were included in phase 3 of genotyping. Genotyping of these 10 mutations was performed using competitive allele-specific PCR using KASP assays (LGC Genomics, Beverly, MA), following manufacturer's guidelines.

Sanger sequencing. All mutations identified using WES, targeted sequencing, and genotyping in phases 1, 2, and 3 were verified using Sanger sequencing. Details of the sequencing primers are as follows: *PALB*_p.Arg414 Ter - Forward: TGAACTTGGTTGTCCTGTGC, Reverse: TGACACTCTTGATGGCAGGA. *PALB2*_c.3201+1G, Forward: TTTG CCCTCAGGTCCTACAG, Reverse: TGGTTTGTTGGAAGAATGT GA, *PALB2*_p.Lys628_Cys630del, Forward: CCTCCATTTCTG-TATCCATGC, Reverse: AAGAGGATTCCCTTTCTTGGA, *PAL B2*_p.Pro918Gln – Forward : CCAGCTGACAGAGACAAAGATG, Reverse: TCTGAGCCTTCAAATGATGAAA, *BRCA1*_p.Gln1111 Asnf – Forward: GGGTGAAAGGGCTAGGACTC, Reverse: CAGAG GGCCAAAATTGAATG, *BRCA1*_p.Gly559Valfs – Forward: ACCA AACGGAGCAGAATGGT, Reverse: GCAATTCAGTACAATTAG GTGGGC, *RAD51C*_p.Arg237Ter - Forward: GGTCCCTGCTC TCTTGGAGA, Reverse: ACCAACCAAACGTAACTTTACTCAA.

Whole-Exome Sequencing of Tumor DNA for Loss of Heterozygosity and Mutational Signature Analysis

DNA was extracted, using a Qiagen tissue kit, from formalin-fixed paraffin-embedded tumor tissue samples from 4 cases: CG-12 (*PALB2* nonsense mutation carrier), 3CG-103 (*PALB2* missense mutation carrier), CG-028 (*PALB2* in-frame deletion carrier), and GM022584 (*RAD51C* nonsense mutation carrier). WES was performed using KAPA and Agilent SureSelect XT kits following manufacturer's guidelines. Samples were sequenced on a HiSeq4000 using PE150 sequencing. Sequence data analysis was performed using GATK best practices as described, and somatic variants were called with GATK MuTect2.[13]

Mutational signature analysis. Mutational signature analysis in somatic tissue is a recent field that is undergoing active development, improvement, and statistical grounding. The first general signature model for mutation signature analysis was developed by Alexandrov et al[14] and was used to analyze The Cancer Genome Atlas dataset, leading to the first defined mutational signature resulting from defects in homologous recombinational DNA repair (HR), annotated as "Signature 3."[15] A conceptually different theoretical model of mutation signatures was developed by Shiraishi et al[16] with an accompanying computational framework called pmsignature. This model pools all mutations from all the samples and seeks signatures that occur relatively frequently in the mutation pool. The output from the analysis is a matrix of estimated signature parameters defining the signatures, and a membership weight matrix that estimates the relative contribution of each signature to the mutations in each sample. The number of signatures that is found, K, is a parameter that must be specified a priori. The Shiraishi signature model differs from the earlier model in that it assumes independence of the adjacent bases, so the number of parameters with a single surrounding base is far fewer than with the Alexandrov model, leading to more statistically stable parameter estimates. We combined the mutations of our 4 tumor samples with 40 TCGA GC whole exomes to increase the power to detect common GC signatures and to provide positive and negative HR signature controls. Of the 40 samples, 20 were selected from the 27 samples with non-zero value for "Signature 3" and 20 were selected from the remaining samples with a zero value.[17] We configured the Shiraishi framework to use 5 bases of total context (the mutated base plus 2 bases upstream and 2 bases downstream) and to include the transcription strand as a mutation feature. The mutation signature analysis was done using the R language (http://www.R-project.org/). In order to detect an HR signature, we first determined which of the 27 Shiraishi signatures was most similar to the Alexandrov "signature 3" by using both Frobenius and cosine similarity measures. Heatmaps depicting the Frobenius and cosine similarity of each of the 27 Shiraishi cancer signatures to each of the 30 Alexandrov (COSMIC) cancer signatures are shown in Supplementary Figure 1*A* and *B*, respectively. For Frobenius similarity, Shiraishi signatures 16, 23, 24, and 25 all have similarity $\geq$0.7 to COSMIC signature 3. For cosine similarity, Shiraishi signatures 16, 23, and 25 all have similarity >0.7 to COSMIC signature 3. We have designated Shiraishi signatures 16 and 23−25 as HR signatures on heatmaps that show Shiraishi signatures. Knowing which Shiraishi signatures correspond to an HR signature, we proceeded to determine which signature, if any, of K signatures produced by our analysis, are similar to one of those Shiraishi HR signatures. We used Frobenius similarity in that case, because both signatures being compared are Shiraishi signatures, and the comparison is more reliable than the Alexandrov-Shiraishi comparison. Frobenius similarity showed that, at K = 3, signature #1 [noted as 1(HR)] was most similar to the Shiraishi HR signatures 16, 23, and 25 (full analysis, Supplementary Figure 1*C*). Tumor DNA from our study samples was derived from formalin-fixed paraffin-embedded tissue, and was expected to have a higher percentage of C: G>T: A mutations. Therefore, we analyzed mutational signatures after removing C:G>T:A from our study samples as well as from control samples (restricted analysis). Similar to the full analysis, we first identified signatures with high Frobenius similarity to Shiraishi HR signatures, using K = 3 (Supplementary Figure 1*D*). After optimizing the method, we proceeded to determine whether an HR signature was demonstrated by the 4 study samples where somatic WES data was available. As shown in Supplementary Figure 2, our study samples as

well as the TCGA positive controls, at K = 3, in full and restricted analysis have a significantly higher relative contribution or membership weight for the HR signature compared to the negative controls. Interestingly, another hallmark of somatic HR deficiency is a high frequency of large indels.[14,17] Consistently, similar to The Cancer Genome Atlas HR-positive controls, the mean deletion length found in the tumors from our 4 *PALB2/RAD51C* mutation carriers was higher than in TCGA non-HR GC cases (31.6 bp vs 15.4 bp; $P = 3 \times 10^{-7}$).

References

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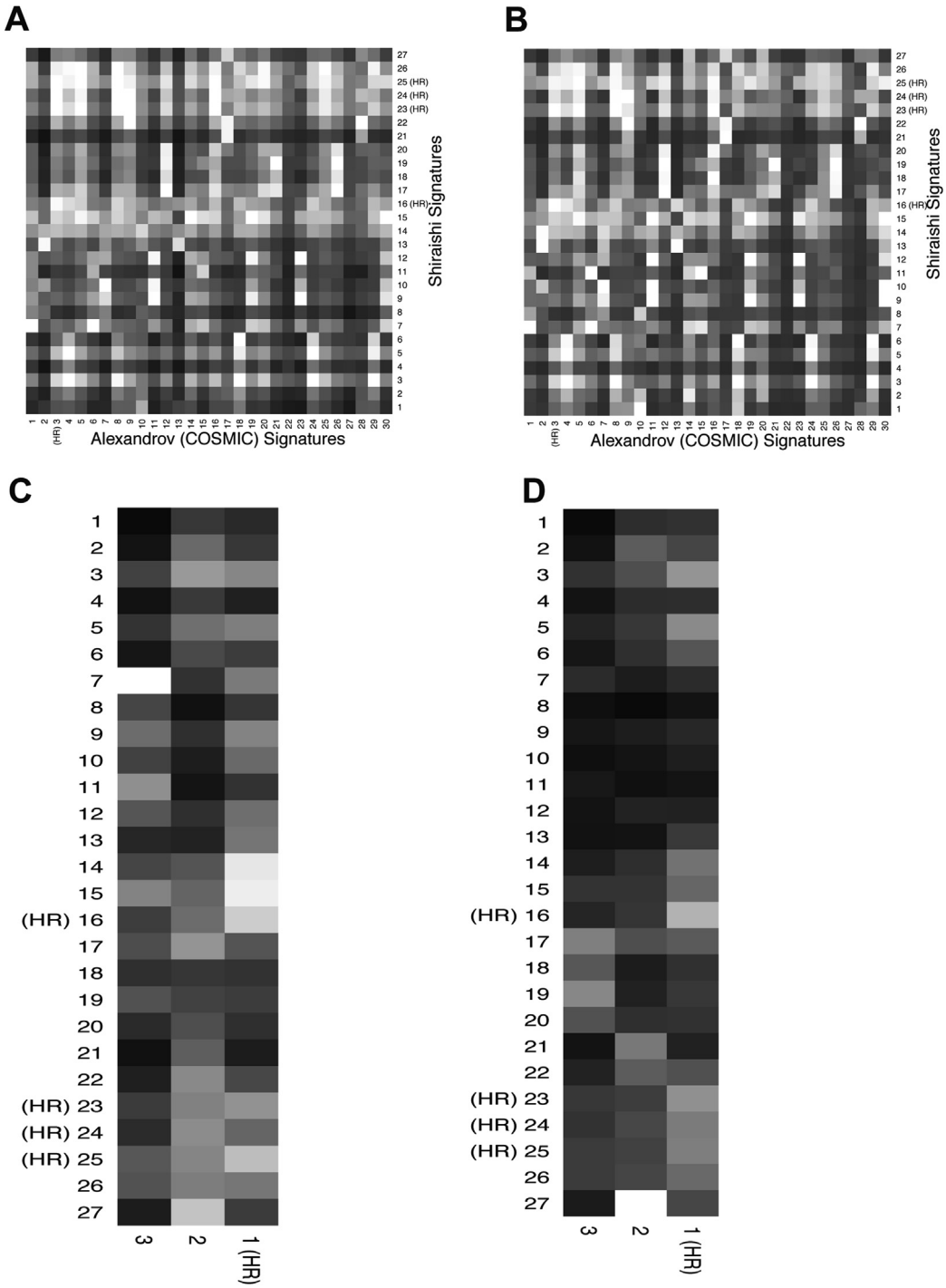
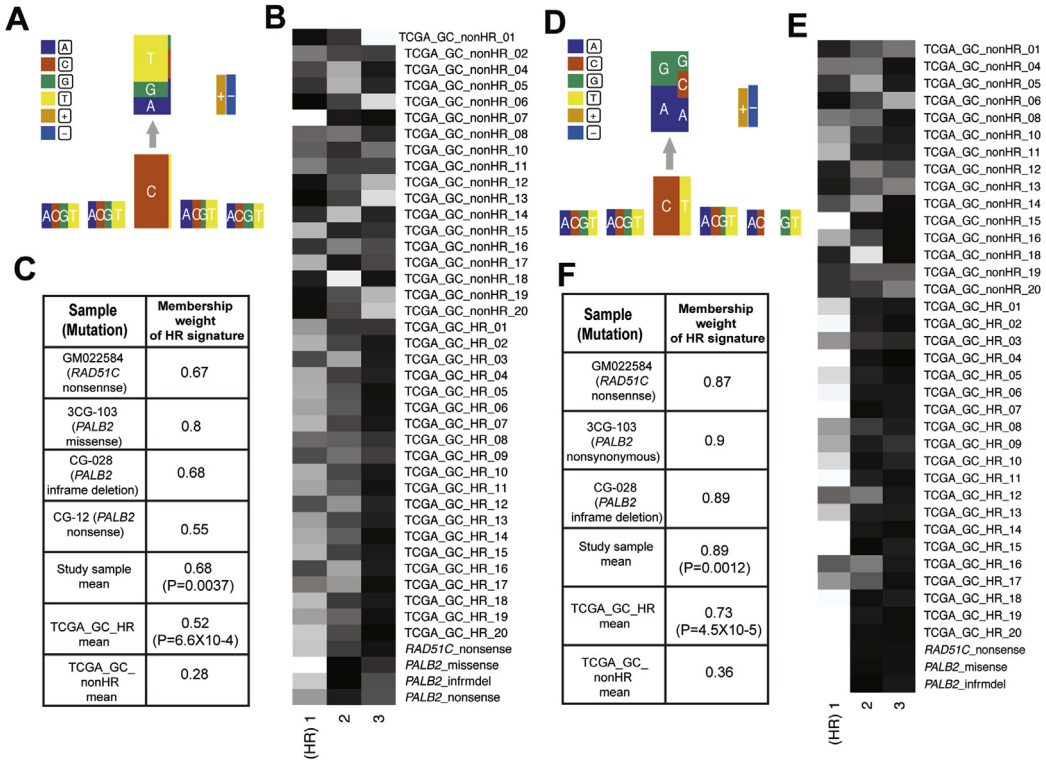


Supplementary Figure 1. Mutational signature analysis methods. Similarity between mutation signatures identified by Alexandrov et al[15] (COSMIC) and Shiraishi et al[16] using A = Frobenius similarity measures and B = Cosine similarity measures. For Frobenius similarity, Shiraishi signatures 16, 23, 24, and 25 all have similarity $\geq$0.7 to COSMIC signature #3, and for cosine similarity, Shiraishi signatures 16, 23, and 25 all have similarity $>$0.7 to COSMIC signature #3. Frobenius similarity was used to determine, at K = 3, which signature showed most similarity to the Shiraishi HR signatures. Considering that our study samples were derived from formalin-fixed paraffin-embedded tumor DNA, this analysis was performed on the full set of SNV mutations (full analysis) as well as after removal of C:G$>$T:A changes (restricted analysis), a known artifact of formalin-fixed paraffin-embedded tissue processing. Signatures with high Frobenius similarity to the Shiraishi HR signatures were identified for K = 3 for C = full analysis and D = restricted analysis. As shown in *C* and *D*, signature #1 (noted on axis as HR) is most similar to Shiraishi HR signature.



Sample (Mutation)	Membership weight of HR signature
GM022584 (*RAD51C* nonsennse)	0.67
3CG-103 (*PALB2* missense)	0.8
CG-028 (*PALB2* inframe deletion)	0.68
CG-12 (*PALB2* nonsense)	0.55
Study sample mean	0.68 (P=0.0037)
TCGA_GC_HR mean	0.52 (P=6.6X10-4)
TCGA_GC_ nonHR mean	0.28

Sample (Mutation)	Membership weight of HR signature
GM022584 (*RAD51C* nonsennse)	0.87
3CG-103 (*PALB2* nonsynonymous)	0.9
CG-028 (*PALB2* inframe deletion)	0.89
Study sample mean	0.89 (P=0.0012)
TCGA_GC_HR mean	0.73 (P=4.5X10-5)
TCGA_GC_ nonHR mean	0.36

Supplementary Figure 2. Analysis of mutational signatures in tumor samples. We used WES data from 4 *PALB2* and *RAD51C* mutation carriers (GM022584, 3CG-103, CG-028, and CG-12) and from 40 HR-defective (TCGA_GC_HR, n = 20) and HR-proficient (TCGA_GC_non-HR, n = 20) cases from The Cancer Genome Atlas (TCGA) study. These analyses included all mutations (full analyses, *A*–*C*, *left panel*) and removal of C:G>T:A changes (restricted analyses, *D*–*E*, *left panel*) as our WES data was generated from archival tumors, which are known to accumulate artifactual C:G>T:A mutations. (*A*) and (*D*) Logos of somatic HR signatures. The central base represents the frequency of the mutation, which is surrounded by the frequency of bases at positions −2 and −1 (*left*) and +1 and +2 (*right*). The *top right bars* indicate the frequency of such mutations in the + and − transcription strand polarities (see Robinson et al[12] for more details). (*B*) and (*E*) Heatmaps of relative contribution or membership weights of each signature within each sample. *Dark shading* indicates low contribution of the mutation signature and *light shading* represents high contribution of the mutation signature. Our 4 samples had highest membership weight to signature #1 (the HR signature) and clustered in the full (which included all mutations, *panel B, right*) and restricted (which excluded C:G>T:A changes, *panel E, left*) analyses with the TCGA HR-positive cases. The pattern involving signatures #2 (unknown cases but very similar to a previously reported signature by Shiraishi et al[16] in gastric and colorectal tumors) and # 3 (cytosine deamination) showed stronger membership weights with the non-HR samples. The *PALB2* nonsense mutation carrier and 5 TCGA_GC_non-HR samples were removed from the restricted analysis as they had few mutations after removal of C:G>T:A changes. (*C*) and (*F*) Tables indicating membership weights for each sample. Table indicates the estimated fraction of mutations associated with the HR signature pattern. Study sample mean indicates mean membership weight of HR signature. *P* value from Mann–Whitney 2-sample U test compares membership weight of the study sample mean or TCGA_GC_HR sample mean to TCGA_GC_non-HR sample mean (row 6 and 8 and row 7 and 8), respectively.

Supplementary Table 1. Details of Mutations Identified in the Study

Chr position (genome assembly = GRCh37/hg19)	Ref	Alt	Gene name	Transcript ID	Complementary DNA change	Protein change and effect	Pathogenicity prediction	Type, effect on protein	ExAC frequency
16: 23646627	G	A	*PALB2*	NM_024675.3	c.1240C>T	p.Arg414Ter	Reported Pathogenic in ClinVar	Nonsense, truncates protein	NA
16: 23625324	C	A	*PALB2*	NM_024675.3	c.3201+1G>T		Reported Pathogenic in ClinVar	Splice-donor variant	NA
16: 23641585-23641593	GCAGGACTT	—	*PALB2*	NM_024675.3	c.1882_1890delAAGTCCTGC	p.Lys628_Cys630del	Reported as VUS in ClinVar,	In-frame deletion, possible effect on recruitment to DNA damage site (see text)	3.31×10^{-5}
16: 23635411	G	T	*PALB2*	NM_024675.3	c. 2753C>A	p.Pro918Gln	Reported as VUS in ClinVar, predicted deleterious in SIFT, PolyPhen, LRT and MutationTaster	Missense, possible effect on protein−protein interaction	1.742×10^{-5}
17: 41244214-41244217	CAAG	—	*BRCA1*	NM_007294.3	c. 3331_3334del CAAG	p.Gln1111Asnfs	Pathogenic	Frameshift deletion, truncates protein	NA
17: 41245874	A	—	*BRCA1*	NM_007294.3	c.1674delA	p.Gly559Valfs	Reported Pathogenic in ClinVar	Frameshift deletion, truncates protein	NA
17: 56787223	C	T	*RAD51C*	NM_058216.2	c.709C>T	p.Arg237Ter	Reported Pathogenic in ClinVar	Nonsense, truncates protein	8.23×10^{-6}

ExAC, exome aggregation consortium; NA, not available; VUS, variant of uncertain significance.