BRCA2 Germline Mutations Identify Gastric Cancers Responsive to PARP Inhibitors

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ABSTRACT

Despite negative results of clinical trials conducted on the overall population of patients with gastric cancer, PARP inhibitor (PARPi) therapeutic strategy still might represent a window of opportunity for a subpopulation of patients with gastric cancer. An estimated 7% to 12% of gastric cancers exhibit a mutational signature associated with homologous recombination (HR) failure, suggesting that these patients could potentially benefit from PARPi. To analyze responsiveness of gastric cancer to PARPi, we exploited a gastroesophageal adenocarcinoma (GEA) platform of patient-derived xenografts (PDX) and PDX-derived primary cells and selected 10 PDXs with loss-of-function mutations in HR pathway genes. Cell viability assays and preclinical trials showed that olaparib treatment was effective in PDXs harboring *BRCA2* germline mutations and somatic inactivation of the second allele. Olaparib responsive tumors were sensitive to oxaliplatin as well. Evaluation of HR deficiency (HRD) and mutational signatures efficiently stratified responder and nonresponder PDXs. A retrospective analysis on 57 patients with GEA showed that *BRCA2* inactivating variants were associated with longer progression-free survival upon platinum-based regimens. Five of 7 patients with *BRCA2* germline mutations carried the p.K3326* variant, classified as "benign". However, familial history of cancer, the absence of RAD51 foci in tumor cells and a high HRD score suggest a deleterious effect of this mutation in gastric cancer. In conclusion, PARPi could represent an effective therapeutic option for *BRCA2*-mutated and/or high HRD score patients with GEA, including patients with familial intestinal gastric cancer.

Significance: PARP inhibition is a potential strategy for treating patients with gastric cancer with mutated *BRCA2* or homologous repair deficiency, including patients with familial intestinal gastric cancer, for whom *BRCA2* germline testing should be recommended.

Introduction

Despite the efforts spent in translational and clinical research to identify novel molecular targets and develop new therapeutic strategies, gastroesophageal adenocarcinoma (GEA) has a major impact on global health (1). Due to the absence of early symptoms, most cases are diagnosed at late stages and patients' outcome is still unsatisfactory, with 70% of patients dying of the disease within 5 years.

From a histologic point of view, GEAs are classified based on the Lauren criteria into diffuse and intestinal adenocarcinomas. In 2014, The Cancer Genome Atlas (TCGA) released a comprehensive genomic characterization of these tumors (2) that allowed a molecular classification of GEAs into four major subtypes: (i) CIN: tumors presenting Chromosomal Instability, that account for >50% of all GEAs and are characterized by gross genomic alterations; (ii) MSI: tumors with Microsatellite Instability, endowed with a high mutation rate due to defects in the Mismatch Repair machinery; (iii) GS: Genomically

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Stable tumors without CIN and MSI traits; and (iv) EBV-positive: associated to Epstein–Barr Virus infection.

The approved therapeutic options for GEAs are limited, with surgery and systemic chemotherapy based on the combination or sequence of various chemotherapy agents (platinum agents, fluoropyrimidines, taxanes, irinotecan, and trifluridine/tipiracil) as mainstay of care. Regarding targeted therapies, trastuzumab is approved in the first-line in association to chemotherapy for unresectable or metastatic HER2-positive gastric cancer. Ramucirumab (targeting VEGFR2) can be used alone or in combination with paclitaxel in the second-line setting. However, the addition of trastuzumab increases objective response rate of only 12% compared with chemotherapy alone (47% vs. 35% with chemo alone; ref. 3); a similar clinical improvement is observed when considering ramucirumab (28% vs. 16%; ref. 4). Regarding immune checkpoint inhibitors, pembrolizumab in combination with trastuzumab and chemotherapy achieved promising preliminary activity (5), and has been granted accelerated approval by FDA. FDA has also approved nivolumab plus chemotherapy as first-line treatment for advanced metastatic gastric cancer, with a subset of patients who achieved long-term benefit (6). Despite such advances and the current development of promising therapeutic strategies, there are a number of molecular subgroups with low prevalence, but potential actionability that are at risk of being neglected.

A still open and debated question is whether PARP inhibitors (PARPi), which are approved for other tumor types such as breast, ovarian, prostate, and pancreatic cancer (7), may still represent a potentially valuable option for patients with GEA (8). Indeed, the percentage of patients showing alterations in DNA double-strand break (DSB) repair and homologous recombination deficiency (HRD), who might potentially benefit from PARP inhibition, is relevant: around 18% in esophageal adenocarcinoma (9) and 7% to 12% in gastric cancer (10). Unfortunately, clinical trials conducted in patients with molecularly unselected gastric cancer to address this hypothesis provided negative results (11), further suggesting that patients' selection based on HRD is mandatory to potentially achieve treatment benefit.

On these premises, taking advantage of a proprietary annotated platform of GEA patient-derived xenografts (PDX), we studied the response to PARPis by performing preclinical trials on gastric cancer PDXs, with the aim of identifying sensitive tumors and discovering genetic alterations useful for their selection. We identified patients characterized by germline mutations in the *BRCA2* gene and loss of the wild-type (WT) allele as optimal candidates for a therapeutic strategy with PARPi in gastric cancer.

Materials and Methods

Primary cell preparation, dose–response cell viability assays and GR50 evaluation

Primary cells used in *in vitro* experiments were obtained from GEA PDX specimens following the procedure described in (12) and maintained in culture in Iscove's medium supplemented with 10% FBS and antibiotics. Genetic identity between primary cells and the original tumor was verified by short tandem repeat profiling (Cell ID, Promega); the presence of the indicated gene mutations was confirmed by Sanger sequencing. *Mycoplasma* testing was routinely performed using the PCR Mycoplasma Detection Kit (Applied Biological Materials Inc., Richmond, BC, Canada). Verified cells are generally thawed few weeks before the experiments and kept in culture for 3 to 6 months. In all the experiments, cell viability was assessed by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) which measures the ATP content of the cells. Primary cells were seeded in 96-well plates (3,000–5,000 cells/well) and cultured in the presence of increasing concentrations of PARPis (1.25–40 μmol/L concentration range for olaparib and rucaparib; 0.312–10 μmol/L concentration range for niraparib) for 6 days. In **Fig. 1**, we compared PARPi response in the different models using the Growth Rate Inhibition 50 (GR50) method that, besides normalizing to the plating efficiency, also considers the doubling time of the cells. This computation is recommended when comparing cells endowed with very different proliferation rates that could confound the pharmacologic effect (13). The GR50 was calculated starting from dose–response data using the GRcalculator tool (http://www.grcalculator.org) as described in (14). Olaparib, rucaparib, and niraparib were purchased from Selleckchem.

Preclinical trials in PDXs

Experiments were performed on 8 weeks old female immunocompromised NOD/SCID mice (Charles River). GTR0210, GTR0126, GTR0222, GTR0264, GTR0324, GTR0459, GTR0503, and GTR0213

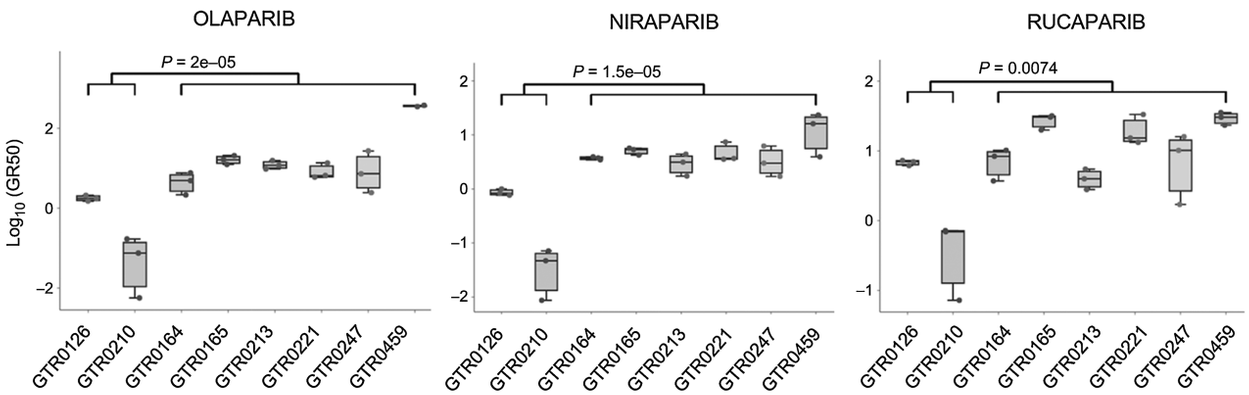

Figure 1.
GEA primary cells bearing BRCA2 germline mutations and loss of the WT allele are sensitive to PARPis. Boxplots showing the GR50 of primary cells derived from gastric cancer PDXs exposed to 3 different PARPis: olaparib, niraparib, and rucaparib. Boxes indicate the median ± standard deviation of GR50 values of 3 independent dose–response experiments (dots). GR50 and statistical significance (Wilcoxon rank-sum test) were calculated using the GRcalculator tool [(14); see Methods for details].

PDXs were expanded for 2 to 3 generations to obtain 5 to 7 mice per treatment arm. The presence of the indicated gene mutations was verified by gDNA sequencing for each model before PDX expansion. When tumors reached an average volume of 220 to 250 mm^3 mice were randomized and treated for the indicated days with either vehicle (saline) or 100 mg/kg olaparib [5 days/ week, per overall survival (OS)] or 5 mg/kg oxaliplatin (once/week for 3 weeks, IP). Tumor size was evaluated once weekly by caliper measurements and approximate volume of the mass was calculated using the formula $4/3\pi(D/2)(d/2)^2$, where D and d are the major and minor tumor axes, respectively. As often done in PDX models, the response in mice has been evaluated using RECIST 1.1-like criteria, i.e., progressive disease (PD): ≥35% increase from baseline; partial response (PR): ≥ 50% reduction from baseline; stable disease (SD): intermediate variations from baseline (15). Statistical analysis was performed with GraphPad PRISM 8.0, using the two-way ANOVA Bonferroni corrected method. Statistical significance: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Olaparib for *in vivo* experiments was purchased from Biosynth Carbosynth Ltd. Oxaliplatin was kindly provided by the Hospital Pharmacy. No a priori criteria were used for including and excluding animals, experimental units or data points; no confounders were controlled.

Evaluation of HRD score and mutational signatures in PDXs

Genomic DNA extracted from PDX models was captured with Agilent SureSelect XT Human All Exon V6 (Agilent Technologies, Santa Clara, CA) and Illumina Exome Panel – enrichment oligos (Illumina Inc., San Diego, CA) covering 45 Mb of exonic content; libraries were subjected to paired-end sequencing on Illumina NextSeq500 and NovaSeq (Illumina, San Diego, CA) producing 150-bp reads. Raw data were deposited in the EGA Archive (EGAS00001006790). Reads were aligned to a concatenated human-mouse genome reference (hg38-mm10) with BWA (16) and subsequently processed with GATK (17) public best practice workflows for duplicate removal and base quality recalibration. After the removal of reads mapping to murine chromosomes, somatic single-nucleotide variants and Insertion/Deletions were identified using Mutect2 and annotated with Annovar (18). Sequenza (19) was used to detect somatic copy-number alterations (SCNA). Genomic HRD signatures were estimated using scarHRD (20) from SCNAs and sigLASSO (21) to assign COSMIC mutational signatures version 2 (22) and Somatic Signatures from Secrier and colleagues (9) using passing filter mutations from Mutect2 as input.

RAD51 foci assay

Immunofluorescence stainings were performed as described in (23) at Vall d'Hebron Institute of Oncology with the antibodies described in the Supplementary Methods. Biomarkers were quantified on formalin-fixed, paraffin-embedded (FFPE) patient tumor samples by scoring the percentage of geminin-positive cells with 5 or more nuclear foci. Geminin is a master regulator of cell-cycle progression that enables to mark for S–G_2-cell cycle phase (24). Scoring was performed onto live images using a 60x-immersion oil lens. One hundred geminin-positive cells from at least three representative areas of each sample were analyzed. Samples with low yH2AX (<25% of geminin-positive cells with yH2AX foci) or with <40 geminin-positive cells were not included in the analyses, due to insufficient endogenous DNA damage or tumor cells in the S–G_2-phase of the cell cycle, respectively. Scoring was performed twice using the microscope Nikon TiE at the University of Parma. RAD51 score was defined as the number of geminin-positive cells that express more than 5 RAD51 nuclear foci. The predefined cutoff of 10% for the RAD51 score was used to qualify tumors as HRD (≤10%) or homologous recombination proficient (HRP, >10%; ref. 25).

Patients

Patients included in the clinical dataset had metastatic gastric or gastroesophageal junction cancers and were treated with platinum- and fluoropyrimidine-based chemotherapy at Fondazione IRCCS Istituto Nazionale dei Tumori of Milano. In patients with HER2-positive disease, trastuzumab was added to doublet chemotherapy as per standard practice. Pretreatment FFPE tumor samples obtained for diagnostic purpose were molecularly profiled by means of Foundation One CDx test. All patients provided written informed consent.

MLH1 gene editing

Cells (2×10^5 to 3×10^5) were transduced overnight with a Cas9 encoding lentiviral vector (pKLV2-EF1a-Cas9Bsd-W; Addgene, 68343), in the presence of 8 μg/mL polybrene (Millipore). Lentivirus-containing medium was refreshed with complete medium the following day. Positively infected cells were selected with 20 μg/mL blasticidin (Thermo Fisher Scientific, A1113903), starting 48 hours after cell transduction. A subsequent infection with the lentiviral vector pkLV.hygro.ccdb_3173 (Kindly provided by Drs. G. Picco and M. Garnett) containing a single-guide RNA (sgRNA) targeting *MLH1* exonic region (GCTACCCAATGCCTCAACCG) was done. Hygromicin (500 μg/mL; Invitrogen, 10687010) was used to select infected cells. To identify *MLH1*-knockout (KO) clones, infected populations were single-cell cloned in 96-well plates; at least 30 clones were expanded and analyzed. Gene inactivation was ascertained by Western blot analysis.

Sanger sequencing of homologous recombination genes

Genomic DNA was extracted from PDXs or primary gastric carcinoma cells with Reliaprep gDNA Miniprep system (Promega) or QIAamp DNA Mini kit (Qiagen) respectively, according to the manufacturer's instructions. RNA was extracted with RSC miRNA tissue kit (Promega) and retrotranscribed to cDNA with the High capacity cDNA retrotranscription kit (Applied Biosystems). The region of interest was amplified by PCR with the primers reported in the Supplementary Methods. p.R2336C and ATM were analyzed on cDNA; all the other mutations on gDNA. The DNA region of interest was sequenced with Sanger standard method.

IHC

MMR proteins were probed by IHC with antibodies raised against MLH1 (G168–15, BD Biosciences), MSH2 (FE11, Calbiochem, Merck), MSH6 (44, BD Biosciences), and PMS2 (A16–4, BD Biosciences). Pathologist reviewed the IHC slides, providing the presence or not of positive tumor cells showing MMR expression.

Statistics

GR50 was calculated starting from dose–response data using the GRcalculator tool (http://www.grcalculator.org) as described in (14). For PDX trials, statistical significance was calculated using the two-way ANOVA with Bonferroni correction.

Study approval

The generation of the GEA PDX platform used in this study and the molecular and genomic characterization thereof have been extensively

described in (26). All animal procedures adhered to the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) standards and were approved by the Ethical Commission of the Candiolo Cancer Institute (Candiolo, Torino, Italy), and by the Italian Ministry of Health (authorization n. 58/2021PR). All patients provided written informed consent; samples were collected, and the study was conducted under the approval of the review boards of all the institutions. The study was done in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonization, and Good Clinical Practice guidelines and GDPR (General Data Protection Regulation).

PDX models data and metadata will be openly available in PDX Finder (https://doi.org/10.1093/nar/gky984; pdxfinder.org) and in the EurOPDX data portal (http://dataportal.europdx.eu) that will be updated with the newly generated models.

Data availability statement

The exome sequencing data generated in this study are publicly available in EGA Archive (EGAS00001006790). Other raw data generated in this study are available upon request from the corresponding author.

Results

GEA primary cells carrying *BRCA2* germline mutations and loss of the WT allele are sensitive to PARP inhibition *in vitro*

We exploited a proprietary annotated platform of human GEA PDXs (26), to analyze their responsiveness to PARPis and unveil molecular predictors of treatment benefit. We started with a candidate gene approach and searched for GEA models carrying genetic alterations in genes of the homologous recombination (HR) pathway, some of which had been previously correlated with pharmacologic response in patients with ovarian cancer *(BRCA1*, *BRCA2*, *ATM*, *ATR*, *BRIP1*, *CDK12*, and *PALB2*; refs. 13, 27). We focused our attention only on loss-of-function (LOF) mutations, such as nonsense and frameshift variants that introduced a premature STOP codon in the protein. Of 165 genomically annotated PDXs, we selected 6 mutated models (GTR0126, GTR0164, GTR0210, GTR0213, GTR0247, GTR0459) for which PDX-derived primary cells were available for *in vitro* experiments (see **Table 1**). The most frequently mutated HR genes were *BRCA2* and *ATM* with 4 and 2 LOF models, respectively. One *BRCA2*-mutated PDX (GTR0164) also presented LOF mutations in *PALB2* and *CDK12*. *In vitro* viability assays were performed on these 6 primary cell models and, as negative controls, on primary cells derived from PDXs not exhibiting HR gene mutations (GTR0165 and GTR0221). Cells were exposed to 3 different clinically approved PARPis—olaparib, niraparib and rucaparib—and cell viability was evaluated at increasing drug concentrations in dose–response assays. To compare responsiveness, we calculated the GR50 (28), as the proliferation rate and the cell doubling time were strikingly different among the different models (see Materials and Methods for details). Two models carrying a LOF mutation in *BRCA2*, namely GTR0126 and GTR0210, displayed high sensitivity to PARPis, especially olaparib and niraparib (**Fig. 1**). *BRCA2* LOF variants were also present in GTR0164 and GTR0459 that showed sensitivity comparable to non-mutated cells (GTR0165 and GTR0221). When we analyzed more in depth the mutational status of *BRCA2*, we found that in GTR0126 and GTR0210 models the nonsense mutations were of germline origin, because they were present also in the patient's matched normal gastric mucosa (Supplementary Fig. S1). In addition, in both cases the WT allele had undergone loss-of-heterozygosity (LOH) in the tumor. On the contrary, the GTR0459

Q7

Table 1. HR gene variants and clinical features of GEA models used in the study.

Tumor ID	HR gene	Variant	Type of variant	Second hit	Lauren class	MSS/MSI status	Age at diagnosis	Gender	Familial history of cancer	*In vitro* models	*In vivo* models
GTR0247	ATM	p.S214Pfs	Frameshift	NO	Intestinal	MSI	60	F	NA	V	
GTR0213	ATM	p.I158Nfs	Frameshift	NO	Intestinal	MSI	77	M	NA	V	V
	ATM	p.K2303Rfs	Frameshift	NO							
GTR0164	BRCA2	p.K1691Nfs	Frameshift	NO	Intestinal	MSI	72	F	Gastric k (father, brother)	V	
	PALB2	p.N280Tfs	Frameshift	NO							
	CDK12	p.K404Efs	Frameshift	NO							
GTR0210	**BRCA2**	**p.K3326***	Nonsense	LOH	Intestinal	MSS	43	M	Pancreas K (father)	V	V
GTR0264	**BRCA2**	**p.K3326***	Nonsense	LOH	Intestinal	MSI	66	M	Colon K (proband and father)		V
GTR0324	**BRCA2**	**p.K3326***	Nonsense	p.K3360Nfs	Intestinal	MSI	73	M	Gastric K (4 uncles, grandmother), gastric benign disease (brother), breast K (sister)		V
GTR0459	**BRCA2**	**p.K3326***	Nonsense	NO	Intestinal	MSS	74	F	Gastric K (father, brother), breast K (sister), lymphoma (daughter)	V	V
GTR0503	**BRCA2**	**p.K3326***	Nonsense	NO	intestinal	MSS	77	M	Larynx K (father), otolaryngo K (brother)		V
GTR0126	**BRCA2**	**p.S599***	Nonsense	LOH	Intestinal	MSS	77	M	Lung K (father), breast K (mother, aunt, daughter, nice)	V	V
GTR0222	**BRCA2**	**p.E462***	Nonsense	p.R2336C#	Intestinal	MSS	NA	M	NO		V

In bold: germline variants.
#Conflicting Interpretation of pathogenicity, according to the ClinVar database
NA, not available.

PDX, although bearing the same germline *BRCA2* nonsense variant as GTR0210, retained the WT allele (Supplementary Fig. S1). In the GTR0164 PDX, the identified *BRCA2* mutation was not germline but only somatic and the second allele was again WT. Considering these results, we hypothesized that GEAs bearing germline inactivating mutations in the *BRCA2* gene and loss of the WT allele might be the right candidates for PARP inhibition. Interestingly, genetic alterations of *ATM* did not seem to confer significant responsiveness to PARPi, neither in the presence of a single *ATM* frameshift mutation (GTR0247) nor if both alleles were affected (GTR0213; **Fig. 1** and Supplementary Fig. S1).

GEA PDXs bearing *BRCA2* germline mutations and loss of the WT allele are responsive to olaparib

To verify responsiveness in patients' tumors we then moved to *in vivo* experiments and performed preclinical trials in gastric cancer PDXs using olaparib. On the basis of the results obtained in cell viability assays, we focused our attention on *BRCA2* germline mutated tumors. Besides GTR0126 and GTR0210, already used in *in vitro* experiments, in our GEA platform we found 5 additional PDX models carrying *BRCA2* deleterious germline mutations, available for xenotrials but from which we did not succeed in deriving primary cells for *in vitro* assays. In total, 7 *BRCA2* germline mutated PDXs (7 cases of

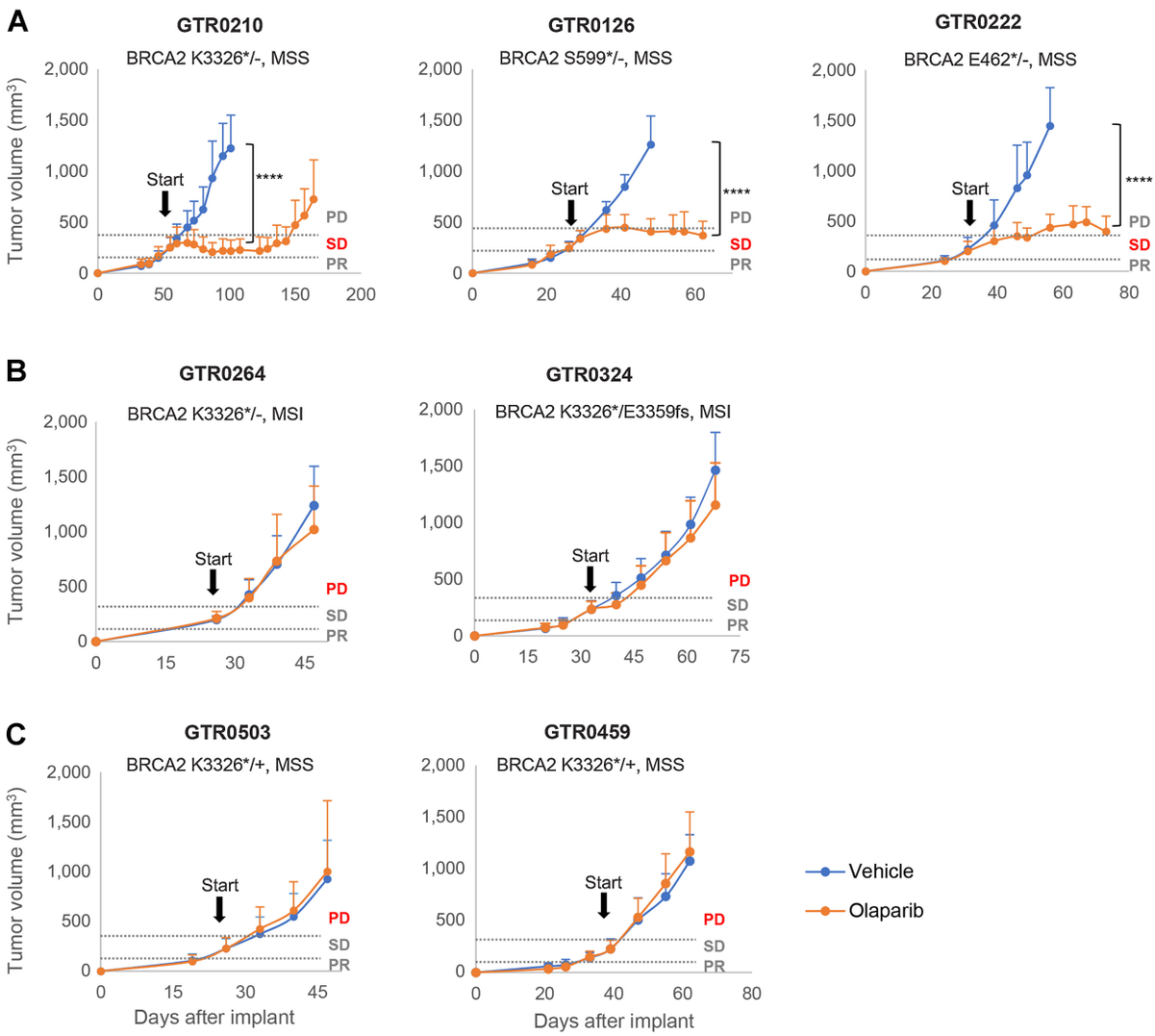

Figure 2.

MSS gastric cancers carrying BRCA2 germline mutations and loss of the WT allele are responsive to olaparib in preclinical trials. Tumor growth curves of the PDX cohorts derived from the BRCA2 germline mutated human specimens of the indicated models. After reaching an average tumor volume of 220 to 250 mm^3, PDXs were treated either with placebo (vehicle, blue lines) or olaparib (2 mg/mouse, 5 days/week per OS; orange lines). Lines represent average tumor volume + standard deviation. $N = 5–7$ animals. The response has been evaluated using RECIST 1.1-like criteria: PD: ≥ 35% increase from baseline; PR: ≥ 50% reduction from baseline; SD: intermediate variations from baseline (dashed lines). The clinical response of each PDX is indicated in red). On the top of the graphs the BRCA2 genotype and the MSS/MSI status of the treated model are indicated; group A comprises BRCA2 germline mutations and loss of the WT allele in a MSS context; group B shows BRCA2 germline mutations and loss of the WT allele in a MSI context; group C carries BRCA2 germline mutations without loss of the WT allele in a MSS context. Arrows = treatment start. Statistical significance was calculated using the Two-way ANOVA with Bonferroni correction. For GTR0126 and GTR0222 the olaparib arm at the end of the trial was compared with the Vehicle arm at the time of mice sacrifice (****, $P < 0.0001$).

165 sequenced PDXs, 4%) were challenged with olaparib. The *BRCA2* genotype and clinical characteristics are summarized in **Table 1**. It is worth noting that 5 *BRCA2*-mutated patients of 7 carried the same germline p.K3326* truncating variant (GTR0210, GTR0264, GTR0324, GTR0459, and GTR0503; Supplementary Figs. S1 and S2) and 2 of them (GTR0324 and GTR0459) reported a familial history of gastric cancer (**Table 1** and Supplementary Fig. S3). On the basis of the Lauren histologic classification, all tumors had Intestinal histotype. Most of them were microsatellite stable (MSS), whereas GTR0264 and GTR0324 showed microsatellite instability (MSI; **Table 1**). All the mutations were confirmed in the PDXs by DNA sequencing prior to starting the xenotrials (Supplementary Figs. S1 and S2).

According to the modified RECIST (26), three models achieved SD upon olaparib administration (**Fig. 2**). Among them, the GTR0126 and GTR0210 models had shown responsiveness also in *in vitro* experiments. A third model, GTR0222, for which primary cells for *in vitro* assays were not available, achieved SD in the preclinical trial. Interestingly this model, besides the germline premature STOP codon in *BRCA2* at p.E462*, as putative second hit bore the somatic missense variant p.R2336C (**Table 1** and Supplementary Fig. S2), that has conflicting interpretation of pathogenicity in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/variation/96845/). GTR0264 and GTR0324 were refractory to PARP inhibition, although in both cases the second *BRCA2* allele was inactivated by LOH and a frameshift mutation, respectively. As reported for other tumor types, tumor growth of GTR0459 and GTR0503 that retained the normal allele (Supplementary Figs. S1 and S2) was not affected by olaparib (**Fig. 2**). A similar result, confirming the *in vitro* assays, was observed with GTR0213 carrying two truncating frameshifts in the *ATM* gene (Supplementary Fig. S4), suggesting that *ATM* inactivation might not be sufficient to confer responsiveness to PARPi.

Trying to explain primary resistance in GTR0264 and GTR0324, we hypothesized that it could be associated with the MSI status, which could activate molecular mechanisms counteracting PARP inhibition. Indeed, GTR0264 and GTR0324 tumors had MSI-high status by microsatellite PCR assay (Supplementary Fig. S5A). We also evaluated by IHC the expression of different mismatch repair (MMR) genes (*MLH1, MSH2, MSH6, PMS2*) and found that the GTR0264 model did not express *MSH2*, likely due to a germline truncating mutation (p.Q690*) and a pathogenic splice site variant already reported in Lynch Syndrome (c.1511–2A>G, ClinVar VCV000090688.13), while GTR0324 lacked *MLH1* and *PMS2* expression (Supplementary Fig. S5B). To verify the possible association between a MSI status and insensitivity to PARP inhibition, we inactivated in GTR0210 responsive cells the *MLH1* gene, the MMR gene most frequently lost in MSI gastric tumors, by means of genome editing with the CRISPR–Cas9 system. sgRNAs targeting *MLH1* exonic region were used and multiple clones were isolated. Loss of MLH1 protein expression was confirmed by Western blot analysis in 4 clones (**Fig. 3A**). Parental and *MLH1* KO cells were grown for 6 days in the presence of increasing doses of olaparib. As shown in **Fig. 3B**, *MLH1* inactivation led to loss of drug sensitivity. Interestingly, as assessed by PCR assays, at the time of experiment execution the *MLH1* KO cells had not yet developed MSI (Supplementary Fig. S6). To evaluate whether this was a gastric cancer specific effect, we knocked out *MLH1* in CAPAN1, a *BRCA2* mutant MMR proficient pancreatic carcinoma cell line sensitive to olaparib (Supplementary Fig. S7A), and performed similar assays. Consistently, we confirmed the resistance to PARPi upon MLH1 editing in these cells (Supplementary Fig. S7B), suggesting that the interplay between HR and MMR is not restricted to gastric cancer cells.

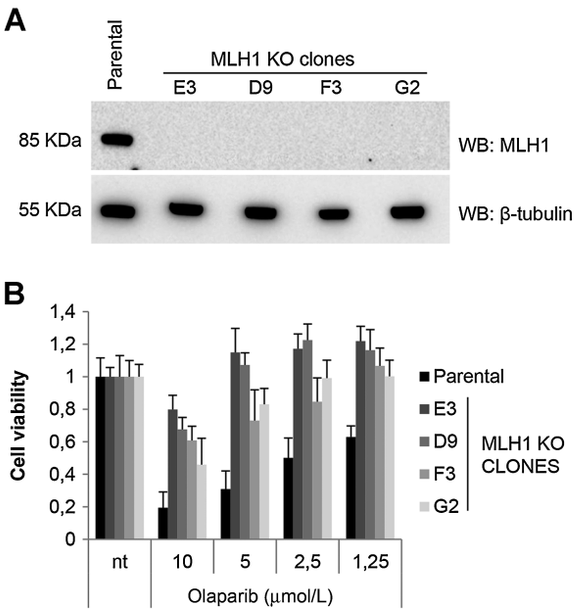

Figure 3.
MLH1 gene KO abrogates responsiveness to olaparib. **A,** Western blot analysis of 4 different MLH1 KO clones (E3, D9, F3, G2) obtained from GTR0210 primary cells (parental) by CRISPR-Cas9 genome editing. **B,** Cell viability of GTR0210 parental cells and MLH1 KO clones derived thereof, exposed at the indicated increasing concentrations of olaparib for 6 days.

Genomic HRD signatures predict response to PARPis in GEA

It is generally agreed that HRD could represent a predictor of response to PARPis (29) and that the use of HRD testing should enter clinical practice for patients' selection (30). Molecular signatures able to highlight HRD beyond *BRCA1/2* inactivating mutations have been recently described to identify patients with pancreatic and ovarian cancer experiencing objective response or longer OS upon platinum regimens (31, 32). To evaluate whether a HRD phenotype could be informative also for gastric cancer patient selection, we applied different tools estimating HRD signatures to the whole-exome sequencing of the 7 PDXs used in the preclinical trials. We exploited scarHRD (20) and SigLASSO (21) based on the COSMIC mutational Signature 3 previously found in breast, ovarian, and pancreatic cancers with failure of the DNA DSB repair (22) and on the signature S3 reported in HR defective esophageal adenocarcinomas (9). Overall, we observed good concordance across HRD signature levels estimated by the different tools (**Fig. 4A**; Supplementary Table S1). Models that showed sensitivity to olaparib were associated with higher values of HRD score and HR impaired mutational signatures (**Fig. 4B**). Of note, the two signatures estimated by SigLASSO [Signature 3 COSMIC and S3 from (22)] provided perfect classification of responsiveness to olaparib (AUC = 1).

Low RAD51 foci score is associated with responsiveness to PARPis in GEA

Another technique has been recently proposed to envisage tumor patients with sensitivity to PARPis, based on the assessment of RAD51 foci formation in tumor specimens (23). Because RAD51 is recruited to DSBs by BRCA1/2 upon DNA damage, the amount of RAD51 foci in a sample can be used as a marker of a proficient or deficient HR machinery. We challenged this method on 6 of the 7 GEA PDX models used in preclinical trials, as one of them (GTR0222) was not

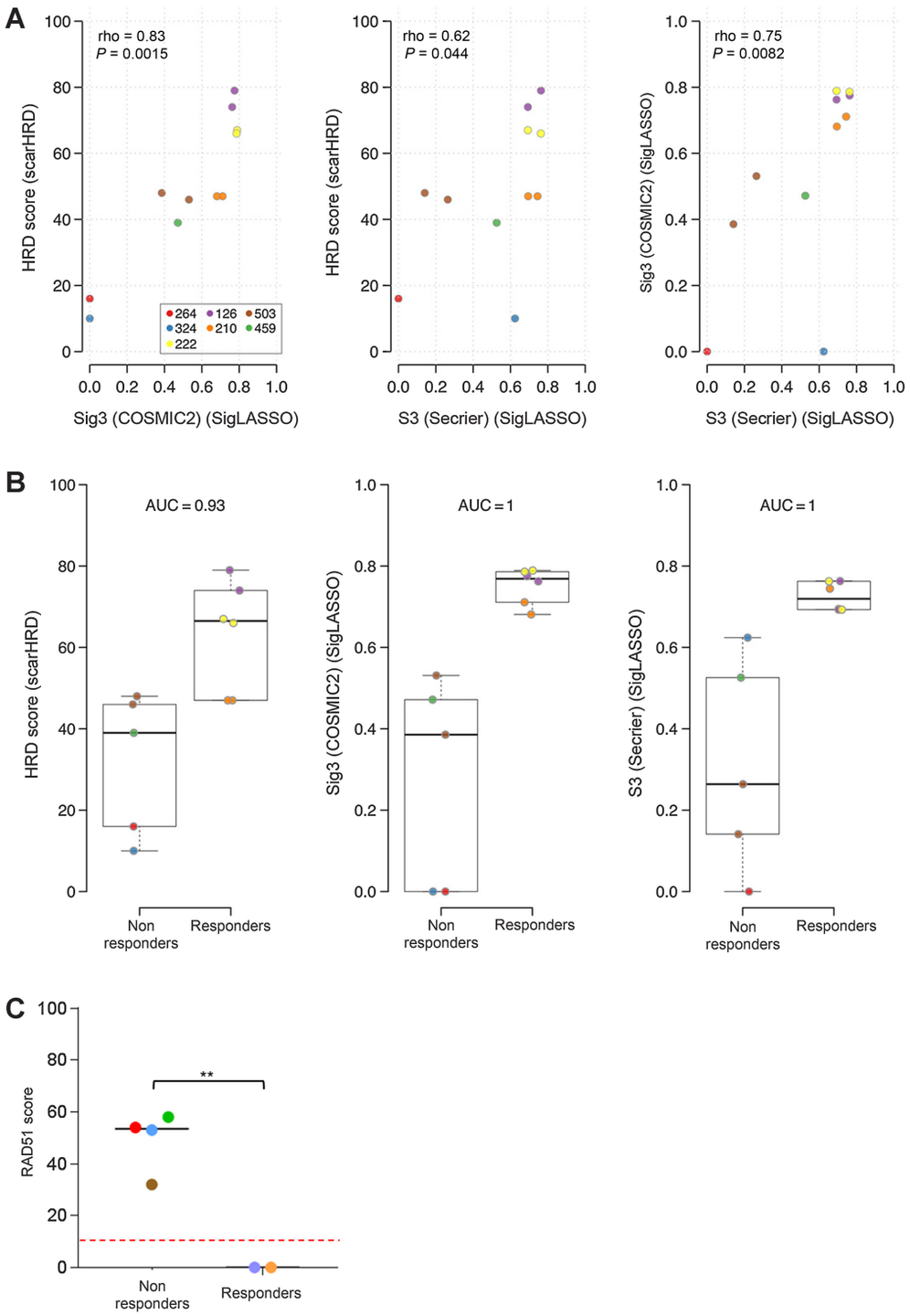

Figure 4.

HRD score and mutational signatures predict responsiveness to olaparib. **A,** Scatter plots showing values of HRD and mutational signature score (colored dots) obtained with the indicated tools in the PDXs used in preclinical trials. For the GTR0126, GTR0210, GTR0222, and GTR0503 models the analysis was performed on two different mice. GTR0126, GTR0210, and GTR0222 = responder PDXs; GTR0264, GTR0324, GTR0459, and GTR0503 = nonresponder PDXs. **B,** Boxplot showing distribution of HRD score, COSMIC Signature 3 and S3 signature from (9) in responder and nonresponder PDXs. **C,** Evaluation of the RAD51 score in the PARPi responsive and resistant models used in the preclinical trials shown in **Fig. 2**. GTR0222 tumor tissue was not evaluable due to technical issues. RAD51 score was defined as the number of geminin-positive cells that express at least 5 RAD51 nuclear foci. The predefined cutoff of 10% (red dashed line) for the RAD51 score was used to qualify tumors as HRD (≤10%) or HRP (>10%). **, $P = 0.005$.

suitable for evaluation. The two evaluable olaparib sensitive models (GTR0126 and GTR0210) were endowed with a low RAD51 foci score, while all the other tumors showed RAD51 foci levels above the 10% threshold (**Fig. 4C**), suggesting that also scoring for RAD51 foci formation on routinely prepared tumor specimens could be an informative method for GEA patient selection. Importantly, the GTR0210 tumor sample bearing the p.K3326* variant and loss of the WT allele, showed lack of RAD51 foci (Supplementary Fig. S8A) reminiscent of an impaired HR pathway, strongly suggesting a LOF effect of the mutation.

Interestingly, while the parental GTR0210 primary cells responsive to olaparib were HR-deficient with a RAD51 score below 10%, the derived *MLH1* KO clones resulted HR-proficient (Supplementary Fig. S8B), suggesting that *MLH1* inactivation may have restored HR and rendered the cells insensitive to PARP inhibition.

GEAs sensitive to olaparib are cross-sensitive to oxaliplatin

A well consolidated observation in clinical practice is that tumors sensitive to PARPis are also responsive to platinum-based chemotherapy (33, 34). To evaluate whether this is the case also in gastric cancers, we performed xenotrials with oxaliplatin in the same PDX models described above. Basically, tumors that had displayed disease stabilization upon olaparib treatment showed a similar response upon oxaliplatin administration (**Fig. 5**). On the basis of RECIST-like criteria, nonresponders to olaparib confirmed absence of objective response also to oxaliplatin.

BRCA2-mutated patients with GEA achieve a prolonged progression-free survival upon platinum-based chemotherapy

Finally, to fuel a potential clinical translation of our preclinical observations indicating germline *BRCA2*-mutated tumors lacking the WT allele as optimal candidates for PARP inhibition, we assembled a cohort of patients with advanced GEA treated at the Fondazione IRCCS Istituto Nazionale dei Tumori of Milano with available comprehensive genomic profiling data. Because PARPi are not approved in gastric cancer, we considered up-front therapy with platinum agents as a surrogate, based on evidence in other tumors (33, 34) and on our observation of cross-sensitivity between olaparib and oxaliplatin. We included in the analysis 57 patients with advanced GEA treated with up-front platinum-fluoropyrimidine regimens (with or without docetaxel) and with the addition of trastuzumab in HER2-positive disease (Supplementary Table S2). The cohort included 4 patients with *BRCA2* LOF mutations, 2 of which were germline (see Supplementary Table S3). A third patient, with a very early tumor onset, carried the p.K3326* mutation; unfortunately, we did not have suitable material available to confirm the germline nature of this variant. It is worth noting that 3 of these 4 patients reported a familial history of gastric cancer. As shown in the swimmer plot in **Fig. 6**, patients harboring *BRCA2* inactivating variants (red bars) were among the best responders, with a progression-free survival (PFS) above the median of 6.4 months (13.1, 12.5, and 8.0 months). In the same cohort, we also evaluated the presence of mutations in genes other than *BRCA1/2* involved in the HR machinery including *ATM*, *ATR*, *RAD51*, and *FANCA*. Notably, patients bearing deleterious variants in these genes (yellow bars) were mostly associated with longer PFS (**Fig. 6**; Supplementary Table S3).

Discussion

GEAs are aggressive and heterogeneous tumors with a 5-year survival of less than 20% (1). Because the therapeutic options are limited, in the last years a compelling challenge to find novel pharmacologic approaches has guided the efforts of translational and clinical gastric cancer research. Great hope was generated by the comprehensive genomic characterization in the context of TCGA, which allowed the molecular classification of GEA into four molecular subtypes (2). However, this knowledge and the multitude of potential new targets have been only marginally translated into novel therapeutic opportunities. Currently, the possibility to tailor therapy on patients' needs by exploiting specific tumor vulnerabilities, as envisaged by precision medicine, is an unmet medical need for several patients' subgroups.

A heated debate, instead, accompanies the still open question whether an "old" therapeutic strategy, such as inhibition of PARP activity in HR-deficient tumors, might be repurposed in GEAs. This approach exploits synthetic lethality in tumor cells that have lost the mechanisms of HR repair and is already approved for breast, ovarian, prostate, and pancreatic cancer. Indeed, 50% of all gastric cancers display chromosomal instability (CIN subtype), which is frequently related to defects in the HR repair. Around 7% to 18% of GEAs carry alterations in the HR pathway (9, 10) and they are catalogued among platinum-sensitive ones. Drawing from these assumptions, PARP inhibition is potentially a promising therapeutic tool. However, initial clinical trials designed to verify this hypothesis were inconclusive: after the enthusiasm for the results of a phase II clinical trial comparing olaparib+paclitaxel versus paclitaxel alone (35), which highlighted a longer OS in patients with ATM-low expressing tumors, the confirmatory phase III GOLD trial failed to meet the primary endpoint (11).

With the aim to refine the molecular selection of patients potentially eligible for PARPi, we exploited our proprietary platform of GEA PDXs and started with a candidate gene approach, thus selecting models carrying gene alterations in the HR pathway. We limited our study to genes whose alterations had already been associated with a good response in patients with cancer (8) and found available models with inactivating mutations (truncating frameshifts and premature STOP codons) in *BRCA2*, *ATM*, *CDK12* and *PALB2*. Detailed analysis of genetic alterations unveiled that tumors with sensitivity to PARP inhibition, both *in vitro* and in PDX trials, bore germline inactivating variants in the *BRCA2* gene and somatic LOH or mutation of the second allele. Interestingly, no germline LOF mutation in *BRCA1* was found in our GEA PDX platform nor in our patients' cohort, suggesting that *BRCA1* inactivation is not likely to be significantly involved in aetiology of gastric cancer.

From our results, gastric cancer appears to behave as a classic BRCA-associated cancer, affected by PARP inhibition when both *BRCA2* alleles are functionally inactivated, in accordance with the synthetic lethality concept (7, 36). It is worth noting that in our GEA PDX platform, 5 of 7 *BRCA2* germline mutated tumors carried the same p.K3326* nonsense mutation. This truncating variant is currently classified as "benign" in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/variation/38266/). However, the 5 PDX originating patients reported a relevant familial history of BRCA-associated and nonassociated tumors and two of them (GTR0324 and GTR0459) showed a clear familial history of gastric cancer. In addition, another patient bearing the p.K3326* variant, with a familial history of gastric cancer and a very early tumor onset (18 years), was independently found in the retrospective patient cohort. Albeit we were not able to perform segregation studies to confirm hereditability of the p.K3326* in affected relatives due to unavailability of tumor specimens, our data suggest that considering this alteration as a neutral polymorphism in the pathophysiology/aetiology of GEAs would be questionable. Indeed, the same variant was previously associated to familial

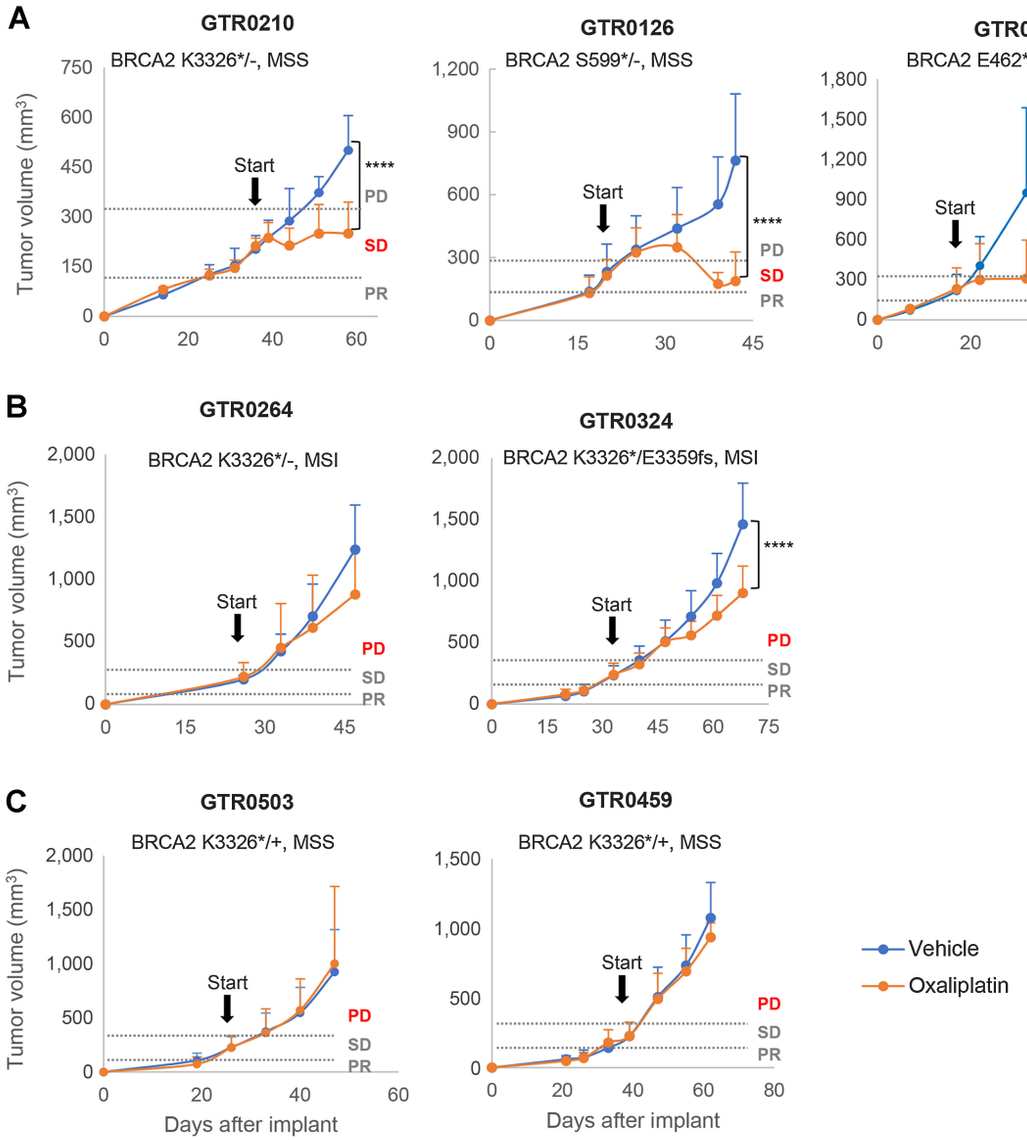

Figure 5.

Gastric cancer PDXs responsive to olaparib exhibit cross-sensitivity to platinum agents. Tumor growth curves in the same BRCA2 germline mutated PDX models shown in **Fig. 2**. When reaching an average tumor volume of 220 to 250 mm^3, mice were treated either with placebo (vehicle, blue lines) or oxaliplatin (0.1 mg/mouse, once a week, IP, for 3 weeks; orange lines). Lines represent average tumor volume + standard deviation. $N = 4–7$ animals. The response has been evaluated using RECIST 1.1-like criteria: PD: ≥ 35% increase from baseline; PR: ≥ 50% reduction from baseline; SD: intermediate variations from baseline (dashed lines). The clinical response of each PDX is indicated in red). On the top of the graphs the BRCA2 genotype and the MSS/MSI status of the model are indicated (groups A, B, and C as in **Fig. 2**) Arrows = treatment start. Statistical significance was calculated using the two-way ANOVA with Bonferroni correction. For GTR0222 the Oxaliplatin arm at the end of the trial was compared with the Vehicle arm at the time of mice sacrifice (****, $P < 0.01$).

cases of esophageal squamous cell carcinoma (37), pancreatic adenocarcinoma (38), small cell lung cancer, and squamous cell cancer of the skin (39). In addition, genome wide association studies demonstrated that the p.K3326* variant is a predisposing factor in lung squamous cell carcinoma (40) and upper aerodigestive tract cancer (41). Unlike the best known *BRCA2* mutations, p.K3226* has a mild effect on hormone related cancers (39) but is associated with tumors characterized by strong environmental genotoxic risk factors, suggesting that affected individuals may be more sensitive to genotoxic stress. The p.K3326* is in the last of the 27 exons of the *BRCA2* gene, resulting in the loss of the 93 C-terminal amino acids of the protein (39). Because the RAD51 interaction domain required for the stabilization of the stalled replication forks (42) is very close to this site, it was hypothesized that this mutation could interfere with the interaction between *BRCA2* and RAD51 (39, 43). Indeed, our observation of the absence of RAD51 foci in the GTR0210 PDX tumor specimen confirms this hypothesis, suggesting that this variant is less suited to prevent degradation of stalled replication forks. This effect could be particularly dangerous in tissues exposed to genotoxic stress, where replication fork progression is continuously challenged. In light of the results

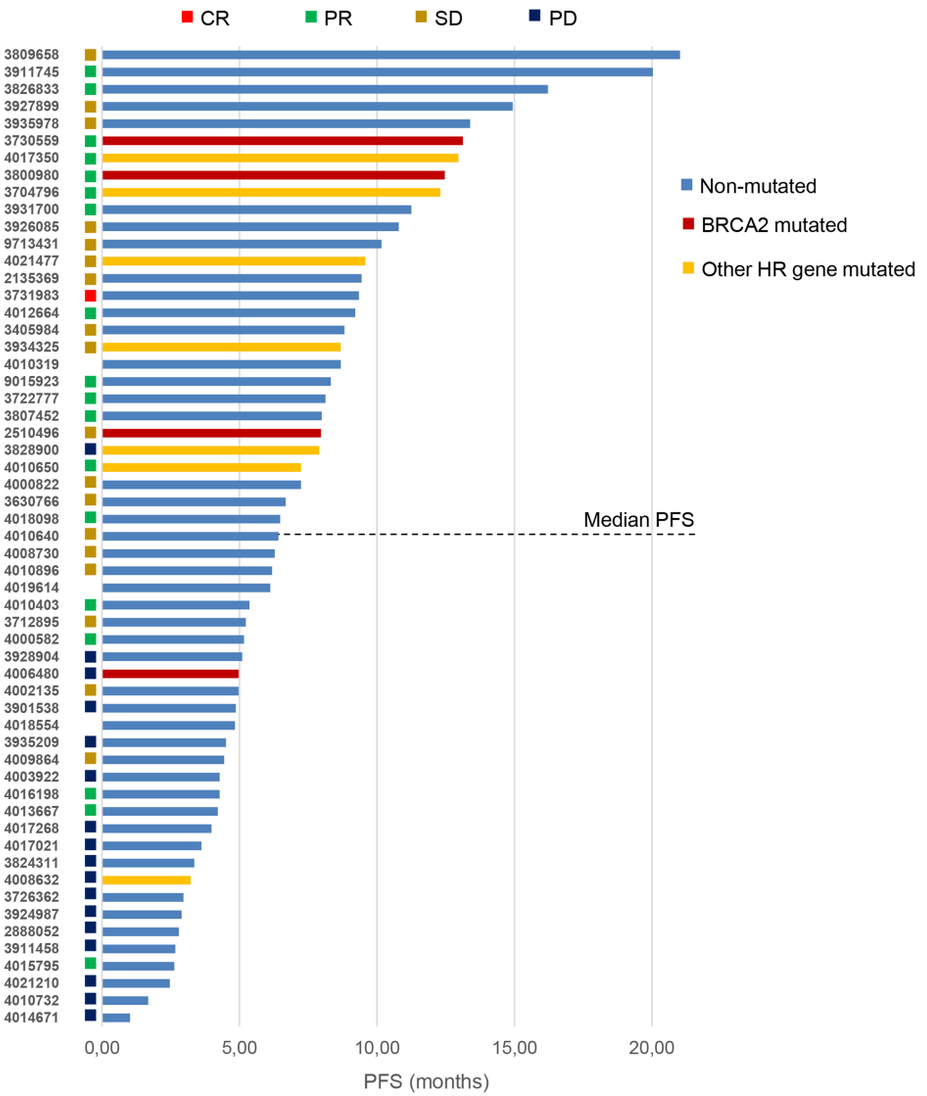

Figure 6.
BRCA2-mutated patients with GEA achieve prolonged PFS upon platinum-based chemotherapy. Waterfall plot of PFS in patients with GEA administered platinum agents. Red bars = patients with BRCA2-mutated tumors; yellow bars = patients with LOF mutations in other HR genes (see Supplementary Table S3). The horizontal dashed line indicates the patient with the median PFS (= 6.4 months). CR, complete response.

obtained with GTR0222 (*BRCA2* genotype p.E426*/p.R2336C), it is conceivable that also the p.R2336C missense mutation, currently annotated with conflicting interpretation of pathogenicity (https://www.ncbi.nlm.nih.gov/clinvar/variation/96845/) may be reconsidered as "likely pathogenic".

Another interesting observation deriving from our studies is that the responsiveness to PARPi in GEA seems to be restricted to MSS cancers. Indeed, in our preclinical trials, PDXs carrying inactivating alterations of both *BRCA2* alleles but characterized by a MSI phenotype (GTR0264 and GTR0324) did not respond to treatment. Interestingly, KO of the *MLH1* gene through CRISPR/Cas9 genome editing in a *BRCA2*-mutated primary cell line responsive to olaparib, was sufficient to abrogate the response to treatment. This effect is unlikely due to the acquisition of a high mutational burden because the *MLH1* KO cells did not exhibit a clear MSI at the time of experiment execution. Therefore, olaparib resistance in MSI tumors is not necessarily due to the accumulation of mutations typical of the MSI status, but it appears causally linked to alterations in the MMR machinery. Indeed we show that HR-deficient cells (responsive to PARPi) upon MLH1 KO not only became nonresponsive to PARPi but also reacquired HR proficiency, since they regained the ability to form RAD51 foci. We have also observed that this behaviour is not restricted to gastric cancer cells but can be more general, as we noticed it also in pancreatic cancers cells. Concerning patients, the co-occurrence of inactivation of both HR and MMR is quite rare in most tumors except for prostate cancer (44). Interestingly, Sokol and colleagues found that two patients with prostate cancer with co-occurring BRCA and MMR mutations were not responsive to PARPi. Even if it is possible that in MSI tumors BRCA2 mutations are not real drivers (but rather passenger alterations associated with hypermutation status), it is also conceivable that the interplay between the two repair systems might play a role in refractoriness to PARPi.

Interestingly, also the PDX carrying 2 inactivating mutations in *ATM* (GTR0213) and not responding to olaparib displayed a MSI phenotype. It would be of interest to establish whether gastric cancers bearing inactivating mutations in both *ATM* alleles coupled with MSS status would benefit from PARP inhibition. Unfortunately, our GEA platform did not include suitable models to address this possibility, which remains to be explored in future works. Currently, there is no clear explanation for our experimental observation even though it is

known that HR and MMR are physiologically linked, with MMR regulating Homeologous Recombination during meiosis (45–47). Albeit the identification of the mechanism through which MLH1 expression impacts on response to olaparib is out of the aim of this work, it represents a very interesting field of investigation.

In line with the general agreement that HRD might be an agnostic biomarker of responsiveness to PARPi, genetic analysis of the PDX models used in preclinical trials revealed that responsive tumors were associated with high values of HRD score and with BRCA-associated mutational signatures. It is worth noting that the COSMIC Signature 3 (10) and the esophageal adenocarcinoma specific signature S3 (9) were able to discriminate responder from nonresponder tumors with surprising accuracy, thus representing a promising tool for GEA patient selection. Notably, the prevalence of patients with gastric cancer with HRD identified by Signature 3 was about 7–12% (10). Thus, the assessment of germline *BRCA* mutations alone may miss a relevant proportion of patients with platinum-sensitive disease and/or PARPi sensitivity potentially driven by HRD, but lacking specific genomic alterations in the HR pathway. In this perspective, performing a post-hoc analysis of these mutational signatures and/or *BRCA2* germline mutations in the cohort of patients enrolled in the GOLD trial would be of outstanding interest to verify their predictive clinical value. The results obtained with the *in silico* analysis are in line with those obtained experimentally with the RAD51 foci evaluation. Indeed, high values of HRD score were associated with low RAD51 scores, suggesting that also the analysis of RAD51 foci formation on routinely prepared tumor specimens could be an informative method for GEA patient selection.

Our findings support the idea that germline deleterious variants in the *BRCA2* gene could act as predisposing factors in the development of GEA, because patients harboring those mutations often reported a familial history of gastric cancer. GEAs associated to *BRCA2* mutations are almost invariably classified as intestinal according to the Lauren classification, indicating that *BRCA2* germline mutations could account for a percentage of familial intestinal gastric cancer (FIGC) cases. It is also tempting to speculate that germline LOF mutations in other classical HR genes such as *ATM* and *PALB2*, albeit rare, might predispose to the development of FIGC HR-deficient tumors vulnerable to PARP inhibition. Indeed, a recent study performed on patients with unselected gastric and esophageal cancer highlighted the presence of pathogenic germline variants in these genes as well (48). Unfortunately, our GEA PDX platform does not include informative models to address this issue. In a scenario where the molecular basis of FIGC still remains unresolved, our results suggest that *BRCA2* germline genetic testing and PARPi based therapies in positive cases could represent a new clinical strategy for patient management.

In conclusion, we indicate *BRCA2* germline mutated GEAs bearing loss of the WT allele and MSS traits as optimal candidates for a PARPi strategy. Clinical trials with PARPis in a proper molecularly selected GEA patient population would be of outstanding interest to confirm our preclinical data.

Authors' Disclosures

A. Petrelli reports grants from Italian Association for Cancer Research (AIRC) during the conduct of the study. S. Rizzolio reports grants from AIRC during the conduct of the study. F. Pietrantonio reports personal fees from Amgen, Lilly, Merck Serono, Bayer, Servier, Pierre Fabre, BMS, MSD; grants from AstraZeneca, Incyte, Agenus, BMS; and personal fees from Astellas outside the submitted work. M. Benelli reports personal fees from Novartis outside the submitted work. C. Orru reports grants from AIRC during the conduct of the study. C. Migliore reports grants from AIRC during the conduct of the study. I.M. Maina reports grants from AIRC during the conduct of the study. E. Puliga reports grants from AIRC during the conduct of the study. V. Serra reports grants from Instituto de Salud Carlos III during the conduct of the study; grants from AstraZeneca outside the submitted work; in addition, V. Serra has a patent for PCT/EP2018/086759 pending. B. Pellegrino reports other support from Lilly, Pfizer, Novartis; and personal fees from MSD outside the submitted work. A. Llop-Guevara reports grants from Asociación Española Contra el Cáncer (AECC) outside the submitted work; in addition, A. Llop-Guevara has a patent for WO2019122411A1 pending. A. Musolino reports grants from Lilly; personal fees from Seagen, Daiichi, Gilead, Novartis; and personal fees from AstraZeneca outside the submitted work. S. Siena reports other support from Agenus, AstraZeneca, BMS, CheckMab, Daiichi-Sankyo, GSK, Seagen; and other support from T-One Therapeutics outside the submitted work. A. Sartore-Bianchi reports personal fees from Amgen, Bayer, Novartis, Servier; and personal fees from Guardant Health outside the submitted work. F. Morano reports personal fees from Servier, Lilly, Pierre-Fabre; and grants from Incyte outside the submitted work. C. Marchiò reports personal fees from Bayer, Roche, AstraZeneca; and personal fees from Daiichi-Sankyo outside the submitted work. S. Corso reports grants from AIRC during the conduct of the study. S. Giordano reports grants from AIRC; and grants from ministry of health during the conduct of the study. No disclosures were reported by the other authors.

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