Methylation Analyses Reveal Promoter Hypermethylation as a Rare Cause of “Second Hit” in Germline BRCA1-Associated Pancreatic Ductal Adenocarcinoma

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Abstract

Background and Objective Pancreatic ductal adenocarcinoma (PDAC) is characterized by the occurrence of pathogenic variants in BRCA1/2 in 5–6% of patients. Biallelic loss of BRCA1/2 enriches for response to platinum agents and poly (ADP-ribose) polymerase 1 inhibitors. There is a dearth of evidence on the mechanism of inactivation of the wild-type BRCA1 allele in PDAC tumors with a germline gBRCA1 (gBRCA1) pathogenic or likely pathogenic variant (P/LPV). Herein, we examine promotor hypermethylation as a “second hit” mechanism in patients with gBRCA1-PDAC.

Methods We evaluated patients with PDAC who underwent Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) somatic and germline testing from an institutional database. DNA isolated from tumor tissue and matched normal peripheral blood were sequenced by MSK-IMPACT. In patients with gBRCA1-PDAC, we examined the somatic BRCA1 mutation status and promotor methylation status of the tumor BRCA1 allele via a methylation array analysis. In patients with sufficient remaining DNA, a second methylation analysis by pyrosequencing was performed.

Results Of 1012 patients with PDAC, 19 (1.9%) were identified to harbor a gBRCA1 P/LPV. Fifteen patients underwent a methylation array and the mean percentage of BRCA1 promotor methylation was 3.62%. In seven patients in whom sufficient DNA was available, subsequent pyrosequencing confirmed an unmethylated BRCA1 promotor. Loss of heterozygosity was detected in 12 of 19 (63%, 95% confidence interval 38–84) patients, demonstrating loss of heterozygosity is the major molecular mechanism of BRCA1 inactivation in PDAC. Two (10.5%) cases had a somatic BRCA1 mutation.

Conclusions In patients with gBRCA1-P/LPV-PDAC, loss of heterozygosity is the main inactivating mechanism of the wild-type BRCA1 allele in the tumor, and methylation of the BRCA1 promotor is a distinctly uncommon occurrence.

1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) has a rising incidence and is projected to surpass colorectal cancer in 2026 as the second cause of cancer death in the USA [1]. The extraordinarily challenging prognosis of PDAC is partially driven by inherent resistance to cytotoxic therapy and immunotherapy and a lack of early detection with validated screening intervention along with intrinsic tumor resistance mechanisms innate to the tumor microenvironment and immune system and other considerations [2]. Up to 9.7% of patients with PDAC have a pathogenic or likely pathogenic variant (P/LPV) that affects DNA damage repair genes [2]. Among these, altered BRReast Cancer gene 1 (BRCA1) and BReast Cancer gene 2 (BRCA2) expression impairs DNA double-strand break repair resulting in homologous recombination deficiency (HRD). Pancreatic ductal adenocarcinoma tumor cells with HRD experience synthetic lethality when exposed to DNA-damaging agents, which enables treatment opportunities with poly (ADP-ribose) polymerase (PARP) 1 inhibitors and platinum chemotherapy [3, 4]. In individuals with a germline BRCA1/2 (gBRCA1/2) P/LPV, an acquired mutation in the tumor wild-type
Furthermore, concomitant somatic has been reported in up to 57% and 20% of patients, respectively for ovarian cancer, hypermethylated BRCA1 promotor is frequent and gene expression. In triple-negative breast cancer and epithelial mline variants, epigenetic modifications may silence BRCA1/2 allele as one mechanism of inactivation of the wild-type BRCA1 allele. In patients with PDAC and a brca1 P/LPV, we examined the LOH status in the tumor tissue using a methylation array analysis. In patients with sufficient leftover DNA input was used for bisulfite conversion (EZ DNA Methylation Kit; Zymo Research; catalog number D5002), followed by an FFPE restoration step using the Infinium HD FFPE DNA Restore Kit (Illumina; catalog number WG-321-1002).

### 2.1 Somatic BRCA1 Mutation Status and LOH Analyses

Genomic DNA from FFPE tumor tissues and matched normal peripheral blood from each patient were extracted and sequenced on Illumina HiSeq2500. Genomic DNA was extracted from FFPE tissue using the Chemagic DNA Tissue kit (PerkinElmer Chemagen Technologie, GmbH, Baesweiler, Germany) after manual macrodissection to ensure at least a 10% tumor content. Please refer to our previous publication for detailed wet-lab used procedures, quality control, and variant calling pipelines [15].

We followed the established workflow for calling somatic mutations in oncology genes [15]. As BRCA1 is a cancer predisposition gene, we follow the American College of Medical Genetics and Genomics guidelines as well as Association for Molecular Pathology/American Society of Clinical Oncology/Cancer College of American Pathologists guidelines for variant interpretations of germline [16] and somatic variants [17]. Germline variants are classified as pathogenic, likely pathogenic, variants of uncertain significance, likely benign, or benign based on the scoring scheme delineated in the American College of Medical Genetics and Genomics guidelines [14]. In individuals who harbored a gBRCA1 P/LPV, we examined the LOH status in the tumor tissue using a FACETS analysis [18].

### 2.2 Methylation Analysis

In individuals found to have a gBRCA1 P/LPV, we examined the BRCA1 promoter methylation status in the tumor using a methylation array analysis. In patients with sufficient leftover DNA samples, a second methylation analysis by pyrosequencing was performed.

#### 2.2.1 Methylation Array

For each sample of genomic DNA, 250 nanograms (ng) of input was used for bisulfite conversion (EZ DNA Methylation Kit; Zymo Research; catalog number D5002), followed by an FFPE restoration step using the Infinium HD FFPE DNA Restore Kit (Illumina; catalog number WG-321-1002).
All samples were processed on the Infinium 850k array and scanned using the Illumina iScan, according to the manufacturer’s recommended protocol. Each CpG site interrogated by the Infinium array is identified by a unique cg identifier in the format of cg#, where # is a number (e.g., cg17301289 is the cg identifier of a BRCA1 promoter CpG site) [19]. CpG loci associated with the BRCA1 promoter covered by the assay included cg17301289, cg04658354, cg04110421, cg21253966, cg16630982, cg16963062, cg15419295, cg20187250, and cg24806953 (Table 1). Methylation level was measured using beta values and a beta value less than 0.20 indicated a qualitatively unmethylated CpG loci.

2.2.2 Pyrosequencing

The assay was designed to detect the level of methylation in a region of transcription start site in exon 1 of the BRCA1 gene (Ensembl Transcript ID ENST00000357654.9). Tumor DNA was extracted and bisulfite treated using the EZ-DNA Methylation Kit (Cat#D5020; Zymo Research, Irvine, CA, USA). Five hundred nanograms of DNA was used for each analysis [15]. A positive control specimen (CpGenome universal methylated DNA, Cat# S7821; Millipore Corporate, Billerica, MA, USA) and a negative control specimen (peripheral blood DNA) were included in the entire procedure along with patient samples. A single polymerase chain reaction (PCR) fragment spanning a transcription start site of BRCA1 exon 1 was amplified, and the degree of methylation of 11 CpG sites was analyzed in a single pyrosequencing reaction (Qiagen, Germantown, MD, USA) (Fig. 1). The PCR products (10 μL) were sequenced by pyrosequencing on a PyroMark Q24 Workstation (Qiagen) following the manufacturer’s instructions. The BRCA1 methylation levels were graded as unmethylated or methylated if the average level of methylation across all 11 CpG sites is lower or higher than 10%, respectively.

2.3 Statistics

Baseline sociodemographic characteristics and genomic features were summarized using the frequency and percentages for categorical covariates, and the median and interquartile range for continuous variables. No formal statistical analyses were conducted, and summary descriptive statistics were used to describe what we have observed from this cohort of patients.

3 Results

From our institutional database, we identified 1182 patients with pancreatic tumors, including 1012 patients with PDAC (Fig. 2). All patients underwent somatic and germline testing. Germline P/LPVs were detected in 212 (20.9%) patients with PDAC. The most frequently altered genes were BRCA2 (N = 45 out of 1012, 4.4%), APC (N = 27, 2.7%), and ATM (N = 21, 2.1%) (Fig. 3). Nineteen (1.9%) patients with PDAC harbored a gBRCA1 P/LPV. Of these, the median age at diagnosis was 53 years (interquartile range 47–66), 15 (79%) were male, and four patients (21%) had Ashkenazi Jewish heritage (Table 2).

Of the 19 patients with a gBRCA1 P/LPV, we analyzed the tumor for the secondary hits that inactivate the normal allele. Notably, 12 (63%, 95% confidence interval [CI] 38–84%) tumors had LOH, indicating LOH is the major molecular mechanism that inactivates the BRCA1 normal allele in the tumor. Two (10.5%) cases had a sBRCA1 P/LPV. Interestingly, in addition to a gBRCA1 P/LPV, a concomitant sBRCA1 variant and LOH were detected in the tumor of case #16. Altogether, a second hit by LOH and/or somatic mutations was detected in 13 of

| Table 1 Description of CpG sites associated with BRCA1 gene variants |
|---------------------------------------------------------------|
| CpG site | Illumina cg identifier | GRCh37 coordinate on chromosome 17 | Position relative to transcription start site |
|---------|------------------------|---------------------------------|-------------------------------------------|
| 1       | cg17301289             | 41277462                        | −81                                       |
| 2       | cg04658354             | 41277444                        | −63                                       |
| 3       | NA                     | 41277436                        | −55                                       |
| 4       | cg04110421             | 41277428                        | −47                                       |
| 5       | cg21253966             | 41277426                        | −45                                       |
| 6       | NA                     | 41277400                        | −19                                       |
| 7       | cg16630982             | 41277394                        | −13                                       |
| 8       | cg16963062             | 41277392                        | −11                                       |
| 9       | cg15419295             | 41277389                        | −8                                        |
| 10      | cg20187250             | 41277381                        | −1                                        |
| 11      | cg24806953             | 41277364                        | 18                                        |

NA Not available

△ Adis
19 (68.4%) gBRCA1-PDAC tumors. Table 3 summarizes the molecular events that inactivate both BRCA1 alleles in the tumor in 19 patients included in this study.

As five out of 19 (26%) cases had no second hit detected in the tumor DNA, we decided to pursue a methylation analysis to explore the role of DNA methylation in BRCA1 inactivation in pancreatic cancer. Using the leftover DNA from clinical testing, we first conducted a methylation array that was successfully completed in 15 (79%) cases, while four cases had an insufficient sample for analysis. The mean percentage of methylated DNA of the BRCA1 CpG islands was 3.62% (range 2.6–5.1%), which suggested that neither BRCA1 allele was methylated. Seven patients had sufficient leftover DNA for a second analysis by pyrosequencing. All seven cases had methylation levels less than 10% across all 11 CpG sites, suggesting unmethylated BRCA1 promoters.

4 Discussion

The tumor suppressor genes BRCA1/2 and their encoded proteins are crucial components of DNA double-strand break repair by homologous recombination. In gBRCA1/2-associated tumors, an acquired alteration of the wild-type allele leads to complete loss of BRCA function and a higher sensitivity to DNA-damaging agents [5–7]. There is a complete lack of data on epigenetic silencing of the wild-type BRCA1 in gBRCA1-PDAC, albeit it has been described in breast and ovarian tumors. Maxwell et al. reported BRCA1 locus-specific LOH in 37 of 41 (90%) gBRCA1 carriers diagnosed with breast cancer, and in 48 of 52 (93%) of patients with gBRCA1-ovarian tumors [5]. In their sub-cohort of patients who underwent methylation-specific PCR, somatic promoter methylation was observed in eight of 23 (35%, range not reported) gBRCA1-breast tumors and three of 15 (20%, range not reported) gBRCA1-ovarian tumors. In our study, we examined the methylation status of the BRCA1 gene using two distinct assays and observed that promoter hypermethylation is an uncommon phenomenon, and in fact did not occur as a mechanism of wild-type BRCA1 allele inactivation. Our results are consistent with a recent study by Zhou et al. who measured the promoter methylation of germline ATM, BRCA1, and BRCA2 genes using real-time PCR in 655 patients with PDAC. They observed minimal levels of promoter methylation affecting these three genes. Of note, 113 (17.2%) of the cohort met criteria for familial pancreatic cancer, defined as having a family history of at least two first-degree relatives with PDAC, albeit the frequency of gBRCA1/2 variants in this cohort was not reported [10].

Other molecular inactivating mechanisms of wild-type BRCA1 have been described in patients with gBRCA1-PDAC. In our analysis, LOH was detected in 12 of 19 (63%, 95% CI 38–84) of
patients, demonstrating LOH is the major molecular mechanism of \textit{“second hit”} for \textit{BRCA1} inactivation in PDAC. Additionally, we observed a low prevalence of a second somatic mutation of \textit{BRCA1}, which occurred in two (10.5\%) patients in our cohort. In a prior study, Sokol et al. analyzed 12,248 patients with pancreatic neoplasms, 5.2\% of whom had a \textit{BRCA1/2} variant. Among patients with computationally predicted \textit{gBRCA1} and \textit{gBRCA2} variants, respectively, 79.2\% and 79.7\% had a biallelic alteration in the tumor. The group did not report the frequency of different types of biallelic alteration, which included LOH of the wild-type allele, homozygous deletion, and two or more \textit{BRCA1/2} alterations in the same tumor sample [11]. In a cohort of seven patients with \textit{gBRCA1}-associated PDAC, Al-Sukhni et al. observed that LOH of the \textit{BRCA1} locus occurred in five cases. Of these, three had tumor DNA sequenced, which all had loss of the wild-type \textit{BRCA1} allele [13]. Nguyen et al. examined the HRD landscape of a large pan-cancer cohort. Among 370 patients with PDAC, three were carriers of a \textit{gBRCA1} P/LPV. They observed LOH in all three patients, albeit the exact mechanism was not reported [20]. Similarly, Lowery et al. interrogated the somatic and germline profiles of 615 patients with exocrine pancreatic tumors, which included primarily PDAC, adenosquamous carcinoma, acinar cell carcinoma, and undifferentiated tumors. Loss of heterozygosity analysis was performed in ten patients with a \textit{gBRCA1} variant. Of these, three had LOH and three had copy neutral LOH [12].

**Table 2** Baseline demographic characteristics of patients with pancreatic ductal adenocarcinoma and a germline \textit{BRCA1} pathogenic or likely pathogenic variant

| Parameter                                             | \(N = 19\) |
|-------------------------------------------------------|------------|
| Sex, \(N\) (%)                                       |            |
| Female                                                | 4 (21)     |
| Male                                                  | 15 (79)    |
| Ethnicity, \(N\) (%)                                  |            |
| African American                                      | 1 (5.3)    |
| Ashkenazi Jewish/Caucasian                            | 4 (21)     |
| Caucasian                                             | 13 (68)    |
| Declined to answer                                    | 1 (5.3)    |
| Age at diagnosis, years, median (IQR)                 | 53 (47–66) |
| Initial stage at diagnosis, \(N\) (%)                 |            |
| Stage 2                                               | 2 (11)     |
| Stage 3                                               | 3 (16)     |
| Stage 4                                               | 14 (74)    |
| Past history of other malignancy, \(N\) (%)           |            |
| Ampullary cancer                                      | 1 (5.3)    |
| Breast cancer                                         | 1 (5.3)    |
| No                                                    | 17 (89)    |
| First-degree relative with pancreas cancer, \(N\) (%) | 2 (11)     |

\(\text{IQR} \) interquartile range
In unselected populations, somatic alterations of BRCA1/2 are rare and have been reported in 1.5–3.9% of PDAC tumors [21, 22]. In patients with gBRCA1-PDAC, prior studies have found a high prevalence of somatic BRCA1 mutations, albeit their findings were limited in the sample size. Yurgelun et al. described concomitant sBRCA1 mutations in one (33%) out of three patients [23], whereas Borazanci et al. observed the same finding in one of two PDAC tumors [24]. Our results indicate that second sBRCA1 mutations are infrequently associated with gBRCA1 P/LPVs and occurred in only two (10.5%) of 19 patients in our cohort.

BRCA1/2-associated PDAC delineates a subpopulation of patients with key clinical features. Healthy individuals with a gBRCA1/2 P/LPV carry a two-fold to six-fold increase in a lifetime risk of PDAC and are typically diagnosed with this disease at a median age of 60 years, younger than sporadic cases [25–27]. Moreover, tumors with deficient DNA repair experience synthetic lethality when exposed to DNA-damaging agents including PARP inhibitors and platinum chemotherapy. gBRCA1/2 status is a validated surrogate biomarker for HRD in patients with PDAC, albeit its predictive value differs based on zygosity status [5, 28]. In a study by Momtaz et al. with 136 patients with metastatic PDAC, 116 had a gBRCA1/2 variant and 20 had a sBRCA1/2 mutation. Zygosity analysis identified biallelic BRCA1/2 alterations in 65 (56%) patients within the germline cohort and in 12 (60%) patients in the somatic cohort. Survival analyses showed numerically higher median overall survival in the biallelic group versus the group of patients with a heterozygous gBRCA1/2 or sBRCA1/2 when treated with frontline platinum therapy (respective median overall survival were 26 months, 95% CI 20–52 months vs 8.7 months, 95% CI 6.2 months to not reached) and when treated with a PARP inhibitor (26 months, 95% CI 24–53 months versus 8.7 months, 95% CI 7.2 months to not reached, respectively) [28]. Despite not reaching statistical significance because of the small sample size, these results suggest that monoallelic loss of BRCA1 is likely a bystander passenger alteration instead of a driver of PDAC tumor phenotype in some patients. Similarly, Park et al. reported higher median overall survival in patients with advanced PDAC with HRD, defined as having a somatic or germline pathogenic variant of BRCA1, BRCA2, PALB2, ATM, BAP1, BARD1, BLM, BRIP1, CHEK2, FAM175A, FANCA, FANCC, NBN, RAD50, RAD51, RAD51C, or RTEL1 [29]. Of note, the survival benefit seen in the HRD group remained statistically significant after adjusting for first-line platinum treatment, which suggests an independent prognostic impact of the underlying tumor biology.

The inherent resistance of PDAC to the one-size-fits-all chemotherapy points to the necessity of new therapy development centered on precision medicine strategies. Presently, gBRCA1/2 variant status is used to select patients with PDAC who may benefit from platinum-based chemotherapy and PARP inhibitors [3, 4]. However, only a small portion of individuals benefit from such therapies because of the rarity of gBRCA1/2 variants. Therefore, it is an imperative to examine and potentially expand surrogate

| Case number | Germline BRCA1 change | Germline BRCA1 amino acid change | Somatic BRCA1 variants | Loss of heterozygosity |
|-------------|-----------------------|--------------------------------|------------------------|-----------------------|
| 1           | c.427G>T              | p.Glu143*                      | No                     | No                    |
| 2           | c.4986+5G>A           | n/a                            | No                     | Yes                   |
| 3           | c.1953dupG            | p.Lys652Glufs*21               | No                     | Yes                   |
| 4           | c.5266dupC            | p.Gln1756Profs*74              | No                     | Yes                   |
| 5           | c.68_69delAG          | p.Glu23Valfs*17                | No                     | No                    |
| 6           | c.68_69delAG          | p.Glu23Valfs*17                | No                     | Yes                   |
| 7           | c.68_69delAG          | p.Glu23Valfs*17                | BRCA1 in-frame deletion V1688del (c.5062_5064del) | No |
| 8           | c.68_69delAG          | p.Glu23Valfs*17                | No                     | Yes                   |
| 9           | c.5266dupC            | p.Gln1756Profs*74              | No                     | Yes                   |
| 10          | c.65T>C               | p.Leu22Ser                     | No                     | No                    |
| 11          | c.5266dupC            | p.Gln1756Profs*74              | No                     | Yes                   |
| 12          | c.68_69delAG          | p.Glu23Valfs*17                | No                     | Yes                   |
| 13          | c.68_69delAG          | p.Glu23Valfs*17                | No                     | No                    |
| 14          | Deletion exons 13-19  | n/a                            | No                     | Indeterminate         |
| 15          | c.68_69delAG          | p.Glu23Valfs*17                | No                     | Yes                   |
| 16          | c.181T>G              | p.Cys61Gly                     | BRCA1 (NM_007294) exon10 p/E1258* (c.3772G>T) | Yes |
| 17          | c.2389G>T             | p.Glu797*                      | No                     | Yes                   |
| 18          | c.68_69delAG          | p.Glu23Valfs*17                | No                     | No                    |
| 19          | c.68_69delAG          | p.Glu23Valfs*17                | No                     | Yes                   |
5 Conclusions

In summary, we have demonstrated that LOH is the major molecular mechanism of “second hit” in patients with PDAC and a gBRCA1/P/LPV. Concomitant sBRCA1 mutations are rare, whereas methylation of promoter CpG islands of BRCA1 is a distinctly uncommon mechanism of inactivation of the wild-type BRCA1 in these patients. Further investigation will clarify whether epigenetic modifications (e.g., histone acetylation and microRNAs) occur as potential mechanisms for biallelic loss of DNA damage repair genes in PDAC.

Declarations

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