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Title: *BRCA1* and *RAD51C* promotor methylation in human resectable pancreatic adenocarcinoma

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Highlights

BRCA germline mutation is associated with sensitivity to PARP-inhibitors.

After DNA bisulfite conversion, digital droplet PCR and PCR followed by capillary electrophoresis are methods to explore methylation.

Methylation of *BRCA1* and *RAD51C* promoters in pancreatic adenocarcinoma in Europe is probably an extremely rare event.

Abstract:

Background :

Homozygous Recombination Deficiency (HRD) is associated with sensitivity to PARP-inhibitors (PARPi) in different cancer types. In pancreatic adenocarcinoma (PA) the main cause of HRD is *BRCA1/2* germline mutation and patients with mutations in *BRCA1/2* may benefit from PARPi. Recently other mechanisms leading to HRD were described in different cancer types, including gene mutations and epigenetic changes such as promoter hypermethylation. In PA, *BRCA1* promoter hypermethylation, a known mechanism of gene silencing was recently described. However, results are discordant between North American studies (0.7% of PA) and Asian ones (up to 60% of PA) and the association with HRD is not clear.

Methods :

Here, we developed 2 quantifications methods to explore *BRCA1* and *RAD51C* promoter methylation in a series of 121 Formalin Fixed-Paraffin-Embedded (FFPE) specimens from resected PA without neoadjuvant treatment. The methylation-specific PCR was done with 2 different methods after DNA bisulfite conversion: a digital droplet PCR, and a PCR followed by capillary electrophoresis, to score the methylated / non methylated ratios in tumor samples. Methods were validated for specificity and sensibility using 100, 20, 10, 5 and 0% methylated commercial DNA for fragment analysis with a detection cutoff of 5-10%. Limit of blank was defined as 5 droplets/20 μ L for *RAD51C* and 1 droplet/20 μ L for *BRCA1* for ddPCR. Samples were reviewed by a pathologist, macrodissected before DNA extraction to obtain 50-60% of tumoral cells. DNAs were treated for bisulfite conversion and analyzed using both methods in parallel to known positive and negative controls in each run.

Results and conclusion :

No methylation at *BRCA1* or *RAD51C* was found in this series of PA suggesting that HRD gene promoter methylation is a rare event in European patients.

Keywords: PARP inhibitors, DNA methylation, Homologous recombination, *BRCA1* promotor, *RAD51C* promotor, pancreatic adenocarcinoma

Abbreviations

Alb : albumin

Bp : base pairs

CE : Capillary electrophoresis

ddPCR : Digital droplet PCR

FFPE : Formalin fixed-paraffin- embedded

FW : forward HR : Homologous recombination

PA : Pancreatic adenocarcinoma

PARP : Poly-ADP ribose polymerase

PARPi : Poly-ADP ribose polymerase inhibitors

RV : reverse

TCGA : The Cancer Genome Atlas

TMA : Tissue micro arrays

Introduction

Pancreatic adenocarcinoma (PA) develops from ductal pancreatic cells and represents 90% of malignant pancreatic tumors. It is the 7th cause of worldwide cancer mortality¹, with a 5 year overall survival around 9% all stages confounded². Its incidence increases without any explanatory factor and it is expected to become the 2nd cause of cancer mortality in Europe in 2030³.

Surgery is the only curative treatment for localized tumors (10-20% of PA), followed by adjuvant chemotherapy⁴. Palliative treatments consist of chemotherapy^{5,6} and radiotherapy⁷. Up to now, with rare exceptions, PA treatment is not guided by tumor biology. However, new treatments with confirmed clinical value in other cancers types such as ovarian cancer are under investigation in PA patients.

Poly-ADP ribose polymerase (PARP) inhibitors have recently become a standard of care for patients showing an homologous recombination deficiency mostly owing to *BRCA1* and *BRCA2* alterations^{8,9}. In metastatic PA, 4-7% patients harbor germline *BRCA1/2* mutations. For *BRCA1/2* patients that have not progressed under platinum-based chemotherapy, PARP inhibitors (PARPi) maintenance significantly increased progression free survival versus placebo¹⁰.

The mechanisms of inactivation of double strand breaks DNA repair by homologous recombination (HR) are multiple and include alterations (mutations, deletions) in HR pathway genes as well as methylation of *BRCA1* and *RAD51C* promoters which are generally exclusive of gene mutations. Methylation is one of the most studied epigenetic modification leading to gene silencing involved in carcinogenesis¹¹. *BRCA1* promoter is methylated in different tumor type including breast (up to 20%) and ovarian (up to 19%) sporadic cancers¹². *RAD51C* gene which encodes a DNA double-strand break repair protein, has been reported to confer high-penetrance susceptibility to both breast and ovarian cancer¹³ and also a sensitivity to PARPi¹⁴. If methylation of the *BRCA1* promoter was already described in PA, no study exists for *RAD51C*. Here we analyzed large series of European resected PA patients to determine the prevalence of *BRCA1* and *RAD51C* promoter methylation to evaluate their clinical value as markers of PARPi response. Digital droplet PCR (ddPCR) and capillary electrophoresis were used for methylation detection because of their high reported sensitivity and specificity^{15,16}.

Materials and Methods:

Tumor samples

FFPE (Formalin fixed-paraffin- embedded) tissue blocks were centralized in the Centre de Ressources Biologiques (University of Paris). These blocks consisted of macrodissected consecutive samples of

patients resected in 3 French hospitals (La Pitié-Salpêtrière, Saint-Antoine and Ambroise Paré ; Paris) and 1 Belgian hospital (Erasmus ; Brussels) from 1996 to 2018 (mostly from Puleo et al's¹⁷ prospective study). Informed consent was obtained from all subjects involved in the study. Exclusion criteria consisted of post operative death (within the 30 days after surgery), macroscopically invaded resection margins (R2 resection), pre operative treatment or other than ductal adenocarcinoma histology.

All the blocks were subject to tissue micro arrays (TMA): tumor-enriched zones on Hemateine Eosine-stained slides were selected by specialized pathologists in pancreatic diseases (Drs Jérôme CROS and Jérémy AUGUSTIN) to extract cores from paraffin blocks with 50-60% tumoral cells.

DNA extraction

After deparaffinization, samples were processed for DNA extraction.

For all samples, DNA was extracted using the AllPrep (Dr Jérôme CROS) or the QIAamp DNA (Dr Jérémy AUGUSTIN) FFPE tissue kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions.

DNA bisulfite conversion

In order to study genes methylation status, extracted DNA (10 ng per well) then underwent bisulfite conversion with EZ DNA Methylation Gold (Zymo Research, Irvine, USA) following the manufacturer's instructions. The principle is to convert unmethylated cytosines to uraciles as methylated cytosine remain cytosine after bisulfite treatment. Positive control consisted of 100% methylated DNA (Zymo Research). Negative control consisted of unmethylated genomic DNA for *BRCA1* and *RAD51C* promoters (Promega, Madison, USA). The final eluted volume corresponded to 10 µL per well.

PCR amplification of bisulfited DNA

Digital droplet PCR (ddPCR)

We then performed ddPCR amplification of DNA sequences of methylated *BRCA1* and *RAD51C* gene promoters from bisulfite converted DNA samples (Table 1).

Positive control consisted of ubiquitous albumin gene (Table 1).

After amplification mix preparation, samples underwent droplet generation (QX200 Droplet Generator), PCR (S1000 Thermal Cycler), droplets reading (QX200 Droplet Reader); and droplets clusters were determined manually on QuantaSoft Analysis Pro software according to the manufacturer's recommendations (Bio Rad, Hercules, USA).

Methylation level was determined as the ratio of methylated droplets concentration (after limit of blank – LOB - subtraction) out to albumin droplets concentration. LOB corresponded to the concentration of methylated droplets in negative control samples. LOB was determined as 5

droplets/20 μ L for *RAD51C* and 1 droplet/20 μ L for *BRCA1*. LOB subtraction aimed at eliminating false positives¹⁸.

The protocol is detailed on Table 2.

PCR followed by capillary electrophoresis

Capillary electrophoresis (CE) constituted a 2nd technique to study DNA methylation.

We used forward primers for methylated and unmethylated sequences and a 3rd reverse primer of an unmethylated universal region. Samples were thus tested using 2 sets of primers testing 2 CpG loci for each gene (*BRCA1* and *RAD51C*). Methylated (marked in blue) and unmethylated (marked in green) amplicons length differed of some base pairs to identify them on CE (Table 3).

We used 5 μ L of bisulfite converted DNA per well. Amplification mix preparation and hybridation temperatures are shown in table 4. Diluted PCR products were analyzed in formamide on a 3730xl DNA Analyzer sequencer.

The protocol is detailed on Table 4.

Results were analyzed with Gene Mapper software (ThermoFischer, Waltham, USA). Methylated fragments were differentiated from unmethylated ones according to their length and to the color of the fluorophore linked to the methylation specific primer.

DNA fragments with height < 300 were considered as background noise.

Results:

We first screened 121 PA samples by methyl specific PCR amplification and capillary electrophoresis (fragment analysis). No methylation was detected for the 2 genes *BRCA1* and *RAD51C*. Each positive control (100% methylated DNA) was found methylated for *BRCA1* and *RAD51C*. Each negative control (unmethylated DNA) was found unmethylated for these 2 genes.

To rule out a lack of sensitivity of the method, we then used ddPCR with a different set of primers. In each run, methylated and un-methylated controls were analyzed in parallel. We tested the sensitivity of this method and showed that we are able to detect methylation in all positive controls (100% methylated DNA before bisulfite conversion) with methylated/unmethylated ratios from 35% to 60%. No methylation of the promoter region of these two genes was detected in negative controls, nor in tumor samples by ddPCR.

Discussion:

No *BRCA1* nor *RAD51C* gene promoter hypermethylation was detected in our resected pancreatic adenocarcinoma collection by 2 different techniques. The capillary electrophoresis based technique has the advantage to consider as the reference the unmethylated sequence of the gene studied demonstrating that we were able to amplify DNA fragments of the genes of interest (*BRCA1* and *RAD51C*). Moreover, the concordance between ddPCR and capillary electrophoresis-based technique for *BRCA1* and *RAD51C* methylation study has been previously verified in our laboratory in ovarian tumors¹⁹. Results from previously published series are discrepant as 2 Asian studies^{20,21} showed a prevalence of 28% to 60% (respectively from 58 and 18 PA); and one on TCGA (The Cancer Genome Atlas) data²² showed a prevalence of 0.7% (from 150 PA). Our series and the Asian ones do not differ by the material used (FFPE tissue blocks) nor in the choice of the CpG loci tested. Our amplicons generated after ddPCR (10 CpG isles, 119 base pairs, position 43125350-43125469, chromosome 17) and capillary electrophoresis (10 CpG isles, 120 base pairs, position 43125319-43125440, chromosome 17) are located in the same region as the one described in the 2 Asian studies (9 CpG isles, 75 base pairs, position 43125355 – 43125429, chromosome 17) (Figure 1). Nevertheless, some differences between our study and the Asian ones could explain this discordance for *BRCA1*. The Asian studies used nested PCR before electrophoresis to increase sensitivity, which can be responsible of false positives owing to contamination²³. In the Japanese study *BRCA1* methylation assessment was performed in 147 areas after macrodissection of FFPE from 58 patients to increase sensitivity²¹.

Nevertheless, there are also some limitations in our study, such as the low amount of DNA undergoing bisulfite conversion (10 ng per well, versus > 100 ng per well in the previous Asian and American studies). The relative low methylated/unmethylated ratios (from 35 to 60%) of 100% methylated DNA samples shown with ddPCR also represent a limit of our study, possibly due to the low amount of DNA in the samples before bisulfite conversion.

Conclusions:

BRCA1 and *RAD51C* genes involved in DNA repair by homologous recombination were not methylated in any of our 121 European resected PA sample, in line with TCGA data²² but contrarily to what have been published in two Asian studies^{20,21}.

We cannot exclude the hypothesis of a late involvement of *BRCA1* or *RAD51C* promoter methylation in PA carcinogenesis as the two Asian studies and TCGA data only involved resected PA. It could be interesting to explore this hypothesis or to study other actionable biomarkers (as *RAD51* other paralogs) in order to increase therapeutic possibilities in PA. Indeed, in PA, treatment with

molecularly matched therapy after molecular screening improved median overall survival (2.6 years [2.4 – not reached] vs 1.5 year [1.3 – 1.9], $p < 0.0001$) compared to patients with targetable molecular alteration but treated with unmatched therapies in a real life study including 189 patients with targetable molecular alteration²⁴.

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Tables

Target	Amplicon length (bp)	Number of CpG	Target sequences (before bisulfite conversion), primers and probes used for ddPCR
methyated <i>RAD51C</i>	85	12	<p>Target sequence : TGTAAGGTTTCGGAGTTTCGTGGTTAGGTCGTAGAGTCGGTT TTTTTCGTTTTACGTTTGACGTTTGACGTTACGTCGTACGTT TTAGCGA</p> <p>FW primer : 5'-TGTAAGGTTTCGGAGTTTCGTGC-3' RV primer : 5'-TCGCTAAAACGTACGACGTAACG-3' RV probe: 6-FAM-AAAACCGACTCTACGACCTAACCG-MGB</p>
methyated <i>BRCA1</i>	119	10	<p>Target sequence : CAAAAAATCTCAACGAACTCACGCCGCGCAATCGCAATTTT AATTTATCTATAATTCCCGCGCTTTTCCGTTACCACGAAAAC CAAAAACTACCGCTAAACAACAACCTCAAAAATA</p> <p>FW primer: 5'-CAAAAAATCTCAACGAACTCACG-3' RV primer : 5'-TATTTTGAGAGGTTGTTGTTTAGC-3' VIC (RV) probe : 6-VIC-GGTAACGGAAAAGC-MGB FAM (RV) probe : 6-FAM-GGTAACGGAAAAGC-MGB</p>
Alb	76	0	<p>Target sequence : GGGATGGAAAGAATTTTATGTTTGGTGAAGGTTAAGGGTTTT TATAATTTATAGAGAATTTGGGGTTAGTTTGTTT</p> <p>FW primer: GGGATGGAAAGAATTTTATGTT RV primer : AACAACTAACCCCAAATTCT FW probe : 6-VIC-AGGGTTTTTATAATTTA-MGB</p>

(in bold : CpG isles), bp : base pairs, Alb : albumin, FW : forward, RV : reverse

Table 1. DNA sequences of *BRCA1* and *RAD51C* gene promoters, probes and primers amplified for ddPCR

Assay Mix 20Xpreparation	For 40 reactions (with 100 μ mol/L reagents) : 4 μ L of methylated <i>RAD51</i> CFW primer 4 μ L of methylated <i>RAD51</i> C RV primer 2 μ L of methylated <i>RAD51</i> C FAM probe 6 μ L of albumin FW primer 6 μ L of albumin RV primer 2 μ L of albumin VIC probe 4 μ L of methylated <i>BRCA1</i> FW primer 4 μ L of methylated <i>BRCA1</i> RV primer 2 μ L of methylated <i>BRCA1</i> FAM probe 1 μ L of methylated <i>BRCA1</i> VIC probe 15 μ L of water
Amplification mix preparation	For 1 well : 11 μ L of Biorad Supermaster mix (Probe) 1,1 μ L of Assay Mix 20X 10 ng of bisulfite converted DNA Complete with water to obtain 22 μ L in total
Compartmentation	QX200 Droplet Generator : 20 μ L of amplification mix within 50 μ L of oil
PCR	S1000 Thermal Cycler (Biorad) : 10 min at 95°C 15 sec at 94°C, 1 min at 56°C (45 fold) 10 min at 98°C Then conservation at 12°C
Detection	QX200 Droplet Reader (Biorad)
Interpretationof results	QuantaSoft Analysis Pro software : Normalization on albumin droplets concentration. Limit of blank subtraction. Methylation percentage calculation.

Table 2. ddPCR protocol.

Gene	Sens	Methylation status	Primer/Probe	Number of CpG isles	Amplification length (bp)	T°C of hybridization of primers for PCR
<i>BRCA1</i>	FW	Methylated	5'FAM- TTTTGGTTTTTCGTGGTA AC-3'	8	120	52
	FW	Unmethylated	5'VIC- TTTTTTGGTTTTTGTGG TAAT-3'		122	
	RV	Universal	TATCTAAAAAACC CAACCTATC			
<i>RAD51C</i>	RV	Universal	CCTCTAAAAATTCCTC ACAATCTAAA	7		58
	FW	Methylated	5'FAM- CGTTTCGTTTTGAAAT GTAGCG-3'		110	
	FW	Unmethylated	5'VIC- GATGTTTTGTTTTGAA ATGTAGTG-3'		108	

Bp : base pairs, *FW* : forward, *RV* : Reverse

«Methylated» and «unmethylated» primers sequences were identical to the respective probes sequences (FAM for «methylated», VIC for «unmethylated»)

Table 3. Primers used for *BRCA1* and *RAD51C* fragment genes amplification before capillary electrophoresis

Reagents	Amplification mix (per well, in μL)
H ₂ O	7,9
10X Buffer	2,0
MgCl ₂	0,8
dNTP	3,2
Universal primer (20ng/ μl)	0,3
Methylated primer (20ng/ μl)	0,3
Unmethylated primer (20ng/ μl)	0,3
Hot Start Qiagen	0,2
DNA (1 ng/ μl)	5
Total	20

A.

Polymerase activation	95°C	10 min	X1
Denaturation	94°C	30 sec	X40
Primer hybridization	52°C (<i>BRCA1</i>) 58°C (<i>RAD51C</i>)	40 sec	
Elongation	72°C	30 sec	
Incubation	72°C	10 min	X1

B.

Reagents	For 1 well (in μl)
Formamide	18,4
Rox 400HD	0,1
Diluted PCR products (dilution factor of 1:100)	1,5

C.

Table 4 (A, B and C). Protocol of DNA fragments analysis after PCR and CE.

A. Protocol for amplification mix preparation.

B. Thermocycler program for PCR

C. Post PCR treatments

Figure

CE
ddPCR 5'-TATCTAAAAAACCCACACCTATCCCCCGTCCAAAAAATCTCAA**CGAACTCA**
5'-CAAAAAATCTCAA**CGAACTCA**

CE
ddPCR **CGCCGCGCAATCGCAATTTTAATTTATCTATAATTCCCGCGCTTTTC**CGTTACCA****
CGCCGCGCAATCGCAATTTTAATTTATCTATAATTCCCGCGCTTTTCCGTTACCA****

CE
ddPCR **CGAAAACCAAAA**-3'
CGAAAACCAAAAAACTAC**CG**CTAAACAACAACCCTCAAATA-3'

***In bold and italics:** sequence of BRCA1 methylated promoter amplified in the 2 Asian studies^{20,21} (75 pb)*

Underlined : primers and probes used in our study

Overlined in blue : common amplified sequence between CE and ddPCR

Figure 1. Comparison of the sequences and CpG isles of BRCA1 methylated promoter amplified in our study and the 2 Asian ones^{20,21}.

CE 5'-TATCTAAAAAACCCACACCTATCCCC**CGTC**CAAAAAATCTCAA**CGAACTCA**
ddPCR 5'-CAAAAAATCTCAA**CGAACTCA**

CE **CGCCGCGCAATCGCAATTTTAATTTATCTATAATTCCCGCGCTTTTCGTTACCA**
ddPCR **CGCCGCGCAATCGCAATTTTAATTTATCTATAATTCCCGCGCTTTTCGTTACCA**

CE **CGAAAACCAAAA**-3'
ddPCR **CGAAAACCAAAAAACTACCGCTAAACAACAACCCCTCAAATA**-3'

***In bold and italics:** sequence of BRCA1 methylated promoter amplified in the 2 Asian studies^{16,17}(75 pb)*

***Underlined :** primers and probes used in our study*

***Overlined in blue :** common amplified sequence between CE and ddPCR*

Figure 1. Comparison of the sequences and CpG isles of BRCA1 methylated promoter amplified in our study and the 2 Asian ones^{16,17}.