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REVIEW

Circulating tumor DNA tracking in patients with pancreatic cancer using next-generation sequencing

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KRAS;
Mutation;
Pancreatic cancer;
Next generation sequencing;
Target therapy

Abstract
Background: Pancreatic cancer remains one of the most devastating malignancies due to the absence of techniques for early diagnosis and the lack of target therapeutic options for advanced disease. Next Generation Sequencing (NGS) generates high throughput and valuable genetic information when evaluating circulating tumor DNA (ctDNA); however clinical utility of liquid biopsy in pancreatic cancer has not been demonstrated yet.

The aim of this study was to evaluate whether results from a Next Generation Sequencing panel on plasma samples from pancreatic cancer patients could have a clinical significance.
Methods: From December 2016 to January 2020, plasma samples from 27 patients with pancreatic ductal adenocarcinoma at two different tertiary Spanish Hospitals underwent ctDNA testing using a commercial NGS panel of 65 genes. Clinical data were available for these patients. VarsSome Clinical software was used to analyse NGS data and establish pathogenicity.
Results: Evaluable NGS results were obtained in 18 out of the 27 plasma samples. Somatic pathogenic mutations were found mainly in KRAS, BRCA2, FLT3 and HNF1A, genes. Pathogenic mutations were detected in 50% of plasma samples from patient diagnosed at stages III-IV samples. FLT3 mutations were observed in 22.22% of samples which constitute a novel result in the field.

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Conclusions: Liquid biopsy using NGS is a valuable tool but still not sensitive or specific enough to provide clinical utility in pancreatic cancer patients.

PALABRAS CLAVE
Biopsia líquida;
KRAS;
Mutación;
Cáncer de páncreas;
Secuenciación de nueva generación;
Terapia dirigida

Evaluación de DNA circulante tumoral en paciente con Cáncer de páncreas empleando secuenciación de nueva generación

Resumen
Introducción: El cáncer de páncreas es uno de los cánceres más devastadores debido a la falta de métodos que permitan un diagnóstico temprano y la ausencia de opciones terapéuticas en enfermedad avanzada.

La técnica de secuenciación de nueva generación o Next Generation Sequencing (NGS) proporciona importantes resultados de alto rendimiento de información genética en muestras de DNA circulante tumoral (ctDNA); sin embargo, la utilidad clínica de la biopsia líquida en cáncer de páncreas no ha sido demostrado todavía.

El objetivo de este estudio fue evaluar si los resultados de un panel de secuenciación de nueva generación en muestras de plasma de pacientes con cáncer de páncreas podría tener un significado clínico.

Métodos: Empleando un panel comercial de NGS con 65 genes se evaluaron 27 muestras de plasma de pacientes con cáncer de páncreas recogidas entre diciembre del 2016 y enero del 2020 en 2 hospitales españoles. En el estudio se disponía de datos clínicos correspondientes a los pacientes. Se empleó el software VarSome Clinical para analizar resultados y establecer patogenicidad de las variantes.

Resultados: Se obtuvieron resultados evaluables en 18 de las 27 muestras de plasma. Se encontraron mutaciones patogénicas en los genes KRAS, BRCA2, FLT3 y HNF1A. El 50% de los pacientes diagnosticados en estadios II-IV presentaron alteraciones patogénicas en plasma. Se observaron mutaciones en FLT3 en el 22,22% de las muestras, lo cual es un resultado novedoso.

Conclusiones: La NGS en biopsia líquida es una herramienta valiosa pero todavía no sensible ni específica para proporcionar utilidad clínica en pacientes con cáncer de páncreas.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the tumors with poorest prognosis, with a 5-year survival rate of about 8%.[1] One of the reasons for the dismal prognosis is that eighty percent of patients are diagnosed at late stages when tumor is unresectable and therefore patients depend on systemic treatment.[2] First line adjuvant chemotherapy recommended in therapeutic guidelines based on gemcitabine combined with albumin-bound paclitaxel, FOLFIRINOX (regimen composed of folinic acid, 5-FU, irinotecan, and oxaliplatin) or PD-1/PD-L1 blockers still led to poor overall survival.[3]

Further improvements in the early detection of pancreatic cancer likely would occur if liquid biopsy tests were capable and reliable to detect the disease at early stages. Moreover, guided therapies could be used also in patients at late stage to improve response and quality of life. In the field of personalized medicine, circulating biomarkers in blood have been aim of research during the last decade but techniques must be optimized, validated and joint consensus must be reached. To rely on circulating tumor DNA (ctDNA), first, particular biomarkers should be differentially expressed in the pancreatic tumor, associated tissues or cells and then leak into blood. Second, we should have sensitive and specific techniques that could allow us to entrust these biomarkers in a clinical setting. Third, in order to be of clinical interest, these biomarkers should have direct or indirect associated targeted therapies. Overall, techniques that enable us to select actionable alterations are required to early diagnosis if possible and very importantly to guide treatment decisions leading to improved response in patients.[4]

Next-generation sequencing (NGS) has been increasingly being implemented for whole-genome sequencing (WGS), whole-exome sequencing (WES), transciptome sequencing, targeted region sequencing, epigenetic sequencing, etc. and it offers great potential for disease management, treatment, genetic counseling, and risk assessment.[5] The mechanisms underlying tumorigenesis of pancreatic cancer are still to be elucidated. NGS offers some advantages to the quantitative PCR (qPCR) or digital PCR (dPCR) as high DNA input permits high throughput analysis and screen for unknown variants (WGS & WES) and can identify structural variants and copy number variations at a global (unrestricted) level.[6]

NGS is currently being used for detection of somatic variants in molecular oncology but in pancreatic cancer, an expanded knowledge of biomarkers is needed particularly by using liquid biopsy as most of the patients are not resectable at diagnosis and biopsy samples are not available. It remains unclear how far information generated from NGS targeted gene panels is from clinical laboratory implementation.[6] Understanding the molecular pathogenesis of PDAC is essential to provide the rationale for the development of personalized diagnostic tests and design therapy in the near future. Despite numerous efforts done around the world, clinical utility of liquid biopsy to assess tumor DNA in pancreatic cancer has not been demonstrated yet.[7]

Our hypothesis is that NGS protocols have not been validated yet to be sensible and specific enough to be used in a clinical setting for patients with pancreatic cancer.

The purpose of this study was to evaluate whether results from NGS CleanPlex® OncoZoom® Cancer Hotspot panel on plasma samples from PDAC patients could have clinical significance. We sought to characterize ctDNA in plasma samples from PDAC evaluating if the techniques are sensitive enough to observe actionable alterations.

Materials and methods

Patient's samples

Between December 2016 and January 2020, 27 patients with PDAC were recruited from the Department of Gastroenterology, Hospital Clinico Valladolid, Spain and Hospital Donostia, San Sebastián, Spain. Patients with PDAC were independently diagnosed and the diagnoses were confirmed using clinical and histological data. Each patient's clinical cancer stage (TNM tumor–node–metastasis) stage was determined according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 8th edition.[4] Thirteen PDAC patients were presented with early-stage disease (I–II) and fourteen were in late stage (III–IV).

All participants signed written informed consents, and protocols were in accord with institutional guidelines and the Declaration of Helsinki and were approved by the Institutional Review Board of Basque Country and Universidad Isabel I Castilla.

After informed consent was obtained, plasma samples were collected and frozen at −80 °C until used for ctDNA extraction.

Data capture

Clinical, endoscopic, radiological and surgical data from patients were collected. These included demographic data, tumor location and staging, diagnosis technique and treatment collected from medical records.

Study data were collected and managed using REDCap electronic data capture tools hosted at Spanish Association of Gastroenterology-AEG[8] REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing (1) an intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for importing data from external sources.

Sample preparation and DNA extraction

Blood from patients diagnosed with PDAC and enrolled in EPIPANCREAS study was collected in EDTA tubes. Fourteen plasma samples were from patients from Hospital Clínico, Valladolid, Spain and ten plasma samples from patients diagnosed at Hospital Donostia, San Sebastián, Spain. Plasma was obtained through double centrifugation: 1500 × *g* 10 min and 2500 × *g* 15 min. Plasma samples were stored at −80 °C.

Before DNA extraction from plasma samples, visual evaluation to discard hemolyzed samples was performed. Circulating tumor DNA (ctDNA) was isolated from plasma using the Applied Biosystems™ MagMAZ™ Cell-Free DNA Isolation Kit (ThermoFisher Scientific) following the manufacture's protocol.

KingFisher™ Flex Magnetic Particle Processor with 96 Deep Well Head (ThermoFisher) was used for automatic ctDNA extraction.

Quality and quantity of nucleic acid was assessed by fluorimetric methods (PicoGreen).

Preparation of libraries

CleanPlex® OncoZoom® Cancer Hotspot Panel Kit (Paragon Genomics) was used to capture target regions according to the manufacturer's instruction. CleanPlex® OncoZoom® Cancer Hotspot Panel Kit is a multiplex assay for cancer profiling of somatic mutations across more than 2900 hotspot regions of 65 oncogenes and tumor suppressor genes. Oncogenes and tumor suppressor genes are: ABL1, AKT1, ALK, APC, ATM, BRAF, BRCA1, BRCA2, CDH1, CDKN2A, CSF1R, CTNNB1, DDR2, DNMT3A, EGFR, ERBB2, ERBB3, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, FNA11, GNA11, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MET, MLH1, MPL, MSH6, MTOR, NF1, NF2, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PIK3R1, PTCH1, PTEN, PTPN11, RB, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TERT, TP53, TSC1 and VHL.

The panel can theoretically detect mutations at 1% allele frequency (https://www.paragongenomics.com/targeted-sequencing/amplicon-sequencing/cleanplex-ngs-amplicon-sequencing/cleanplex_input_sensitivity/) although we set our filter in 1.0% to be more conservative in the detection of potentially significant mutations, especially those described as pathogenic.

Briefly, 10–20 ng of input DNA were used for PCR amplification made in presence of the multiplexed specific primers pairs which conform the panel. PCR products were cleaned for the removal of excess primers, nucleotides, salts and enzymes using CleanMag® Magnetic Beads (Paragon Genomics) and the CleanPlex Dual-Indexed PCR Primers for Illumina (Paragon Genomics) were used for barcoding and Illumina library preparation. The average size of library length had to be around ∼297 bp. Before sequencing, the libraries were normalized based on concentration measured by Bioanalyzer, pooled, washed and tritrated by Real Time qPCR to have a final concentration of 4 nM.

Table 1 Characteristics of cases of samples included in this study.

Characteristic	*N* = 18 PDAC (%)
Gender	
Male	7 (38.8%)
Female	11 (61.1%)
Age (years, mean, range)	74.5 ± 8.41, [55–87]
Race, ethnicity	
Caucasian	18 (100%)
AJCC stage	
I	5 (27.8%)
II	3 (16.7%)
III	2 (11.1%)
IV	8 (44.4%)

Sequencing and bioinformatics analysis

Libraries were sequenced in Illumina equipment's (NextSeq550 and MiSeq, using a 2 × 150 pair-end format) and FASTQ data were generated according to standard Illumina procedures. Following sequencing, reads were processed using PrinSeq to remove stretches with N, short reads or low quality reads. Processed fastq files were then loaded onto the VarSome Clinical platform (American College of Medical Genetics and Genomics)to find specific mutations. VarSome Clinical is a CE IVD and HIPAA-compliant platform to variant annotation and interpretation. Sequences were mapped against the human hg19 genome using the Paragon OncoZoom bed file and Varsome was used to analyze the possible impact of the variants found. To avoid background noise, we set a filter establishing an allelic balance of >1.0% to consider valid a variant.

Results

Patient characteristics

High on-target performance and high coverage to ensure efficient use of sequencing reads was gained in 18 out of the 27 (66.7%) samples. Reasons for failures in 9 samples (33.3%) included low DNA yield (not enough to achieve 20 ng) and poor DNA purity that did not render enough material to be sequenced and were discarded. Out of the 18 patients in which NGS results were evaluable, 7 (38.9%) were male and 11 (61.1%) with a mean age of 74.5 ± 8.41 years. 100% were Caucasian. Twelve (66.6%) patients were diagnosed at Hospital Clínico, Valladolid and six (33.3%) were diagnosed at Donosti Hospital, San Sebastián.

The patients were diagnosed at stage I (5 patients), stage II (3 patients), stage III (2 patients), and stage IV (8 patients), according to the TNM system. Patient characteristics are summarized in Table 1. Three patients (16.7%) had habits of alcohol consumption, and one patient (5.6%) had habits of cigarette smoking. A total of 8 (44.4%) patients were diagnosed with diabetes before PDAC diagnosis, one with Diabetes mellitus I and seven with Diabetes mellitus Type II. One patient had previously developed pancreatitis. All but three patients had CA19.9 elevated at diagnosis with average of 3567 U/mL.

Mutation status of the CleanPlex® OncoZoom® Cancer Hotspot Panel in PDAC

Although per instruction of use, library preparation could be performed by using just 10 ng of DNA, in our experience the recovery and quality of libraries obtained with this amount of DNA was not sufficient likely due to sample stability and final sequencing was performed by using 20 ng. The list of mutations detected by CleanPlex® OncoZoom® Cancer Hotspot Tumor can be found in Supplementary Fig. 1.

VarSome Clinical was used to analyze FASTQ data from OncoZoom Cancer Hotspot Panel leveraging massive cross-referenced knowledge base of the Varsome.com platforms. VarSome results were classified according to American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines. Filter applied was 1.0% allelic balance as well as pathogenic and likely pathogenic mutation classification. Summary of results after applying relevant filters has been shown in Table 1. Notably, main pathogenic mutations were identified in seven genes including the KRAS, FLT3, HNF1A, BRCA2, ALK, ABL1 and NF1 while mutations with uncertain or conflicting pathogenicity was found in PI3KR1, DNMT3, SMARCB1, EGFR, FGFR2, NOTCH1, ERBB44, PTEN and MLH1. No clear mutations were detected in the remaining 49 genes contained in the panel. Of the 18 PDAC samples, driver mutations seemed to be those found in KRAS, HNF1, BRCA2 and FLT3. Two (11.11%) patients harbored KRAS alterations clearly defined as pathogenic, one (5.55%) in HNF1A, two (11.11%) in BRCA2 and four (22.22%) in FLT3 (Table 2).

MH17 plasma ctDNA harbored a pathogenic missense mutation in oncogene KRAS occurring at exon 2, chr12:25398284 C⇒A. In this sample mutations in PIK3R1 (Vchr5:67593245 A⇒G) was found with uncertain or conflicting clinical significance. More likely the driver mutation in this PDAC patient was KRAS found in allelic frequency of 3.3%.

MH-28 plasma ctDNA presented a missense mutation in KRAS exon, at chr12:25380275 T⇒G classified as pathogenic and also a mutation in BRCA2 (chr13:32944557 C⇒T) classified as conflicting with allelic frequency of 4.02 and 3.05% respectively.

MH-6 plasma ctDNA harbored a likely pathogenic mutation in HNF1A (chr12:121437100 C⇒T) with 3% allelic frequency.

Four plasma samples MH-1, MH-3, MH6 and MH-10 showed ctDNA with a single nucleotide variant (SNV) in FLT3 (chr13:28602313 A⇒T; chr13:28602313 A⇒G), allelic frequency of around 3% in a −2 position which could be a critical position for splicing.

Sample MH-29 presented DNA with a frameshift deletion (chr13: 32913559 delA) in BRCA2 allelic frequency 1.2 and a deletion on NF1 (chr17:29553478 delC) with frequency of 1.6%.

Apart from these mutations clearly defined and previously described as pathogenic, other mutations were found within the category as ''conflicting or uncertain'' meaning conflicting interpretation of pathogenicity or uncertain

Table 2 Somatic mutations/deletion found in plasma samples.

Sample ID	Gender	Age	PDAC stage	Mutation (pathogenic)	Mutation (conflicting or uncertain)
MH-1	F	79	III	FLT3 3.7% 4161x	PI3KR1 4.6% 5022x
MH-3	M	65	II	FLT3 2.8% 5447x BRCA2 1.1x 20907x	ns
MH-5	F	85	IV	ns	ns
MH-6	F	76	IV	HNF1A 3.1% 5662x FLT3 3.0% 4759x	ns
MH-7	F	87	IV	ns	DNMT3A 7.5% 6129x
MH-8	F	55	II	ns	PIK3R1 4.5% 6202x FGFR2 1.2% 5519x
MH-9	F	85	IV	ns	ns
MH-10	M	67	I	FLT3 3.4% 5589x	ns
MH-12	M	74	I	BRCA2 1.0% 20898x	PI3KR1 4.5% 10088x SMARCB1 2.3% 5785x
MH-14	F	81	I	ns	ns
MH-15	F	72	I	ns	ns
MH-16	F	75	II	ns	ns
MH-17	M	67	IV	KRAS 3.3% 4454x	PI3KR1 3.8% 5576x EGFR 2.2% 11787x
MH-18	F	72	I	ns	ns
MH-19	M	84	IV	ns	ns
MH-21	M	66	III	ns	PIK3R1 3.5% 9641x FGFR2 1.5% 4605x
MH-28	F	75	IV	KRAS 4.2% 11479x BRCA2 3.05% 2349x ALK 2.5% 4728x ABL1 2.0% 8876x	NOTCH1 4.8% 1040x DNMT3A 12% 1971x ERBB4 3.5% 4636x PTEN 3.2% 5374x
MH-29	M	76	IV	BRCA2 1.2% 16252X NF1 1.6% 22822x	MLH1 1.9% 8620x

F, female; M, male; PDAC, pancreatic ductal adenocarcinoma; ns, non-significant; % is frequency (population frequency for the ethnicity specified); x is allelic balance (proportion of read in % that support the variant).

significance of the mutation. A dominant missense mutation (chr2:25466796 A⇒C) in DNMT3A was found in sample MH-7 with allelic frequency of 7.5% and in MH-28 with frequency of 12%.

In seven plasma samples, no pathogenic nor uncertain alterations were detected.

In this set of plasma samples, KRAS missense mutation was c.35G>T G12V (p.Gly12Val) in exon 2 of 6 position 46 of 122 in MH-17 and c.183A>C Q61H (p.Gln61His) in exon 3 of 6 position 72 of 179 in MH-28. The proto-oncogene KRAS is the most frequently mutated gene among driver oncogenes in PDAC. Within KRAS, $KRAS^{G12V}$ is one of the most frequent mutation together with $KRAS^{G12D}$, $KRAS^{G12R}$ and $KRAS^{Q61H}$. KRAS mutations in codon 12, 13 and 61 have largely diminished intrinsic GTPase activity (up to 40-80 fold lower than KRASwt).[9]

BRCA2 mutation at c8350C>T (pArg2784Trp), exon 19 of 27 and some deletions has been previously described frequently in PDAC, specially in familial pancreatic cancer.[10] Despite BRCA loss-of-function mutations are linked mostly to increased risk of ovarian and breast cancer, such mutations are also associated with increased risk of pancreatic cancer and 4–7% of patients with PDAC have germline BRCA mutations.[11]

HNF1A (Hepatocyte nuclear factor 1 alpha) found mutation was c.1531C>T (p.Gln511Ter) in exon8 of 10 position 30 of 122 in sample MH-6. This HNF1A variant creates a premature nonsense codon and therefore predicted to result in the loss of a functional protein.

FLT3 mutations found in four samples MH-1, MH-3, MH-6 and MH-10 c.2053T>A in intron 16 of 23 position 2 of 936 with frequencies varying from 2–8 to 3.7%. However, an

important proportion of samples in our serie (4 out of 15 samples) were found to harbor alterations in this gene.

DNMT3 mutation was c.1907T>G (p.Val636Gly) located in exon 16 of 23 position 56 of 85 is wide less frequent observed compared with KRAS.

Mutations in intronic regions were seen at various frequencies (>2.5%) in BRCA2, PI3KR1, NOTCH1, ERBB4 and PTEN. However, clinical significance has not been defined or clearly confirmed as pathogenic yet.

Discussion

Surgical resection is the main curative treatment for PDAC as roughly 30–40% of patients have locally advanced disease and another 40% have metastatic tumor at the time of diagnosis. Therefore, palliative chemotherapy remains the main treatment option for most patients.[14]

Despite efforts done in basic and clinical research, early diagnosis presents a challenge due to the lack of sensitive and specific biomarkers. Moreover, absence of biomarkers derive in lack of clinical meaningful clinical trials practicing personalized medicine.[15]

Genomic analysis performed through NGS is also known as ''high-throughput'' sequencing because facilitates millions of sequencing reactions to be run in parallel allowing simultaneous sequencing and detection of several samples at the same time.

In spite of numerous advantages of NGS compared to Sanger sequencing i.e. requires less time, offers more depth and is able to identify novel variants, it also has the disadvantage of read lengths are shorter and therefore error rates for individual read multiple times leading to consensus sequence.[16]

In fact during the last few years, the use of NGS has facilitated the potential utility of liquid biopsy evaluating circulating tumor cells (CTCs), ctDNA, miRNA, exosomes, etc. and although it has been looking promising it has not been translated into benefit to the patients. Earlier basic research results identified KRAS, TP53, CDKN2A, SMAD4 and BRCA2 as most frequent genes mutated in PDAC.[17,18] Still we wondered whether this molecular biology understanding of pancreatic cancer is still merely advance in knowledge or can be used in personalized medicine for early diagnosis or/and to develop appropriated target treatments.

In our study, all confirmed pathogenic mutations within KRAS and HNF1 as well as BRCA2 deletion were all found in stage IV patients. By using our panel, only FLT3 was found mutated in 2 early stage (I–II) patients and 2 late stage (III–IV) patients. Overall, in our study we found pathogenic mutations or deletion in 8 out of the 18 (44.44%) samples and 5 of the 8 (62.5%) corresponded to patients in state III-IV. The fact that 62.5% of pathogenic mutations are found in patients with advanced disease demonstrate that cDNA measurement is good estimator of tumor burden at the same time that liquid biopsy as per this specific panel and techniques used could not be sufficient to help in diagnosis of PDAC.

Despite NGS can account for some rare sequencing errors on nucleotide incorporation by DNA polymerases when detecting low abundance mutations, to be in the safe side we increased threshold to 1.0% allelic frequency. Moreover, we obtained an average sequencing depth of >1000× concluding then data obtained are reliable enough to provide criticism.

{Citation}It has been described that KRAS mutations in the G12, G13, and Q61 codons occur in >90% of PDACs.[17,19] However, these mutations occur in tumor tissue and not found in ctDNA in some cases. In agreement with many other publications, we only found KRAS mutations in plasma of 2 (13.3%) patients, being both of them stage IV. Thus, the absence of ctDNA detection relates to either the tumor not actively shedding ctDNA or the amount of ctDNA being below the limit of detection by NGS rather than a biologic discordance between tumor DNA and ctDNA as described in other solid tumors.[20] Distribution of KRAS mutations detected in plasma samples found by Le Calvez-Kelm et al.[21] demonstrated that G12D are the most common followed by G12V and G12R in around 21% of patients[21] although other authors found in up to 36%[22] of patients.

For early diagnostic purposes KRAS has been discarded as it could also potentially be mutated in pancreatitis as well as in some other inflammatory diseases and therefore could not be distinguished from PDAC. By contrast, KRAS was originally promising constituting a near perfect target for PDAC treatment as revealed for some publications, however KRAS interference is challenging and no reliable inhibitors have been described so far.[23] Pharmacological disruption has sometime shown efficacy *in vitro* and *in vivo* but not in clinical trials. Targeting approaches EGFR specific with erlotinib or multitargeted receptor tyrosine kinase sunitinib do not account for clinical benefit.[24]

BRCA mutations emerged as a target for the development of more effective therapies after alterations in germline BRCA and PALB2 were detected in 5–9% of PDAC patients. Theoretically, patients harboring these mutations should be more susceptible to cytotoxic agents that cause DNA damage. Moreover, PARP inhibitors emerged as effective non-cytotoxic approach to treat PDAC and recently one clinical trial has been complete to evaluate effectiveness of Olaparib in PDAC NCT02677038.[25] In our study, we found a somatic BRCA2 mutation in one patient also harboring KRAS mutation, still the clinical significance is undetermined. No familial history of pancreatic cancer or BRCA was reported for this patient.

FLT3 is a receptor tyrosine kinase important in cell proliferation and differentiation often associated with hematopoietic alterations. FLT3 has not been deeply explored in PDAC but surprisingly found in 26.6% of plasma from patients of our study equally distributed between early and late stages. FLT3 mutation analysis and targeting landscape has been traditionally linked to hematological diseases where are considered to confer a significantly poor outcome.[26] Midostaurin and gilteritinib are inhibitors recently approved for use in FLT-3 mutant acute myeloid leukemia (AML).[27] Ger et al.[28] associated FLT3 with PDAC as prognostic biomarker for immunotherapeutic response.

It has been hypothesized that aberrant DNA methyltranferase-3a is important for PDAC tumorigenesis although underlying molecular mechanism is not clear due to limited studies published. DNA methyltransferases (DNMT) are a family of enzymes that catalyse DNA methylation with either S-adenosyl-L-methionine or 5-methyl tetrahydrofolate as the methyl donor. Particularly DNMT3

may act as tumor suppressor gene[12] and its role associated to mutations commonly found in hematological malignancies. He et al. found a correlation of DNMT3 and GLI1 in pancreatic cancer.[13] Our data demonstrated that DNMT3 is mutated in plasma. Increased protein expression of DNMT3b was demonstrated in pancreatic cancer tissue and in vitro studies showed that knockdown of DNMT3b inhibits tumor progression.[29] Study by Gao et al. showed that knockdown of DNMT1 and DNMT3b expression significantly inhibited PDAC cell viability.[30] Scarce available literature data could indicate that epigenetic targeting of DNMT1 in pancreatic cancer is a pathway to be pre-clinically and clinically explored.

FGFR4 overexpression in pancreatic cancer mediated by an intronic enhancer activated by HNF1alpha.[31] HNF1A is a hepatic factor-transcription factor associated to increased risk of diabetes and multifactorial disorder of glucose and mostly related with hepatic metabolism. Whether this is a cause or a consequence of crosslink diabetes-pancreatic cancer is still to be explored. Recent genome-wide association study have implicated HNF1A as susceptibility gene for pancreatic cancer however the functional significance and molecular mechanism in PDAC remains unclear.[32] NHFA1 was found to be mutated in one stage IV patient within the study (6.6%).

In summary, in our experience ctDNA isolation regarding yield and quality can be challenging when performing NGS in plasma samples. Our success rate was 62.5%, which should be improved prior to this technique being expected in a clinical setting. Extraction methods should be standardized as its result is determinant for library preparation and further sequencing. Quality Check should be established in order to proceed with library preparation. Also, the amount of ctDNA (tumoral circulating) within cfDNA (total cell free DNA) is also quite variable, adding an additional hurdle for an efficient mutation detection and quantitation. NGS produces enormous amount of genetic information, however still difficult to interpret. VarSome provided us with some alterations found in introns which are still not well defined or classified as pathogenic. Nevertheless, it is known that point mutations (single nucleotide polymorphisms) in introns can introduce novel splice sites, activate novel promoters (which may direct sense or antisense transcription causing alterations in mRNA, miRNA or lncRNA expression), or introduce/eliminate enhancer activity, etc. which definitely can carry harmful effects in patients.

The study presented here has some limitations: (1) low number of patients evaluated in this study, therefore powerful statistical cannot be used; (2) patients were not followed up as to evaluate if biomarkers could predict recurrence or monitor response to treatment and evolution of the disease; (3) lack of use of molecular identifiers which cannot be reliable used in PCR based results which would allow a slightly best sensitivity due to noise reduction.

In this study mutation profiling using NGS has identified several alterations in plasma of some advanced cancer patients. However, concisely we do not have effective targeted therapies for most of the driver mutations detected in these PDAC. Still in a research setting, further studies will be required to determine if mutation are clinically relevant and targetable in this patient population.

In conclusion, liquid biopsy and NGS represents particularly interesting tool as minimally invasive and low-risk procedure to obtain genetic material from the tumor in blood. However currently liquid biopsy approached based on NGS panels are not sensible and specific enough for clinical utility. In NGS increasing sensitivity can decrease specificity and the right balance for clinical significance has to be found.

Beyond research purposes, it is required to improve techniques i.e. ultrasensitive techniques and new biomarkers, the joint use of different types of biomarkers.

It is required to standardize ctDNA extraction and validate NGS in order to determine targets for personalized medicine.

As reported by the ESMO Precision Medicine Working Group, currently there is not current indication for tumour multigene NGS. However there it is highly recommended to perform multigene sequencing in screening programs to provide access to innovative therapies with special relevant in pancreatic cancer as well as breast and hepatocellular carcinomas.[33]

Authors' contributions

Concept and design of the experiments: MHV, and LB. Laboratory work: RT. Data analysis and discussion: MHV, RR, RM, MCA, LB and LRR. Clinical information: LB, and LRR. Manuscript preparation: MHV, RM, RR, RT and MCA. All authors read and approved the final manuscript.

Conflict of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gastrohep.2021.12.011.

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