Liquid Biopsy Using Ascites and Pleural Effusion Supernatant for Genomic Profiling in Gastrointestinal and Lung Cancers

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Research Article

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Abstract

Background: Precision medicine highlights the importance of incorporating molecular genetic testing into standard clinical care. Next-generation sequencing (NGS) can detect cancer-specific gene mutations, and molecular targeted drugs can be designed to be effective for one or more specific gene mutations. For patients with special site metastasis, it is particularly important to use appropriate samples for genetic profiling test.

Methods: Tissues, plasma, ascites (ASC) supernatants, pleural effusion (PE) samples from gastrointestinal (GI) patients with peritoneal metastasis and lung cancer patients with pleural metastasis were collected for comprehensive genomic profiling. The sample were performed on next-generation sequencing (NGS) using 59 or 1021 cancer-relevant genes panel.

Results: The study enrolled 156 tissues, 188 plasma, 45 ascites supernatants, and 1 pleural effusion in 304 GI group and 446 pleural effusion supernatants, 122 tissues, 389 plasma, 45 pleural effusion sediments in 407 lung cancer group. The MSAF were significantly higher in ASC and PE supernatant than plasma ctDNA (50.18% ± 32.03% vs 12.31% ± 19.90%, p < 0.0001 and 33.74% ± 28.34% vs 6.28% ± 12.17%, p < 0.0001, respectively). ASC supernatant had a higher actionable mutation rate than plasma. ASC supernatant accounts for more actionable alterations than plasma ctDNA in 26 paired samples. PE supernatant had higher total actionable mutation rate than plasma (80.3% vs 48.4%, p < 0.05). PE supernatant had a higher frequency of uncommon variations than plasma no matter had distant organ metastasis.

Conclusion: ASC and PE supernatants could be better alternatives when tumor tissues are not available in the real world, especially in patients who have only peritoneal or pleural metastases.

Background

Precision medicine is a strategy designed to treat individual patients with the most suitable therapy at the most appropriate time based on the patient's biological and molecular features. It highlights the importance of incorporating molecular genetic testing into standard clinical care. Next-generation sequencing (NGS) can detect cancer-specific gene mutations, and molecular targeted drugs can be designed to be effective for one or more specific gene mutations. However, tumor tissues from advanced patients for molecular profiling are not always available. Thus, tumor-derived cell-free DNA (cfDNA) isolated from body fluids, including plasma, pleural effusions (PE), cerebrospinal fluids, saliva, and urine, are being investigated in cancer genomic profiling [1–4].

Gastrointestinal (GI) cancer and lung cancer have a high incidence and mortality rate in the world. Peritoneal metastasis appears to be the common pattern and is associated with a poor prognosis than other sites in gastric and colorectal cancer[5, 6]. Pleura is also the common metastasis site in lung cancer and worsen the survival[7]. Ascites (ASC) and pleura effusion (PE) were usually available in large quantities, by minimally invasive procedures, in patients with peritoneal and pleura involvement. ASC and
PE contain floating malignant cells as well as tumor cfDNA in the supernatant, and the cytological and
supernatants were used to detect genetic mutations using NGS in multiple types of tumors\cite{8–10}.
However, the performance of genomic profiling using ASC and PE in the real-world settings has not been
fully investigated.

In this study, we collected ASC, plasma, tissue, and PE samples from GI cancer who had peritoneal
metastasis and plasma, tissue, PE samples from lung cancer who had pleura metastasis to verify the
efficacy of ASC and PE in detecting genetic mutations. Paired samples from a subset of patients were
compared to systematically evaluate the concordance of genomic profiles from different sample types.

**Materials And Methods**

**Patients and samples**

In the GI cohort, 304 patients with peritoneal metastasis were prospectively enrolled. A total of 390
samples using next-generation sequencing (NGS), included 156 tissues, 188 plasma, 45 ascites
supernatants, and 1 pleural effusion, were used to analyze the efficacy of ascites in detecting genetic
mutations.

In the lung cancer cohort, 407 patients with pleural metastasis were prospectively enrolled. A total of
1002 samples using NGS, included 446 pleural effusion supernatants, 122 tissues, 389 plasma, 45
pleural effusion sediments, were used to analyze the efficacy of pleural effusion in detecting genetic
mutations.

**Next-generation sequencing**

ASC and PE were isolated for the extraction of cfDNA and seqзыkyдиментs used for genomic DNA
extraction. Circulating cell-free DNA (cfDNA) was isolated from plasma using the QIAamp Circulating
Nucleic Acid Kit (Qiagen, Valencia, CA). DNA from tumor tissues and sediments were extracted by the
QIAamp DNA mini kit (Qiagen, Valencia, CA). White blood cells DNA acted as the germline controls which
were extracted by the DNeasy Blood Kit (Qiagen, Valencia, CA)\cite{11}. Sequencing libraries were prepared
from cfDNA using KAPA DNA Library Preparation Kits (Kapa Biosystems, Wilmington, MA, USA), and
genomic DNA sequencing libraries were prepared with Illumina TruSeq DNA Library Preparation Kits
(Illumina, San Diego, CA). A total of 1392 libraries from 711 patients were hybridized to custom-designed
biotinylated oligonucleotide probes (Roche NimbleGen, Madison, WI, USA) on 59 genes or 1021 cancer-
related genes panel using the Illumina Nextseq CN 500 or Gene+Seq 2000 instrument\cite{12, 13}.

Sequencing data were analyzed using default parameters\cite{14}. The reads which removed adaptor
sequences and low-quality reads were aligned to the reference human genome (hg19) using Burrows-
Wheeler Aligner (BWA; version 0.7.12-r1039). Realignment and recalibration were performed by using
GATK (version 3.4-46-gbc02625)\cite{15}. Single nucleotide variants (SNV) were called using MuTect (version
1.1.4) and NChot, a software developed in-house to review hotspot variants. Small insertions and
deletions (InDel) were determined by GATK. Somatic copy number variations (CNV) were identified with
CONTRA (v2.0.8)[16]. The final candidate variants were all manually verified using Integrative Genomics Viewer. Targetable genomic alterations simultaneously detected by this assay included SNV, Indel, CNV and fusions.

Statistical Analysis

Fisher exact test was used to test the difference between any groups. P-value of <0.05 was defined as statistical significance. Statistical analyses were performed using GraphPad Prism 8.0.

Results

Study design and GI cancer patient characteristics

The study enrolled 304 GI cancer patients with peritoneal metastasis at diagnosis or during disease progression (Table 1). The median diagnosis age was 57 (range 20-93), and 145 (47.7%) were female. The major cancer type was colorectal cancer (CRC), and gastric cancer (GC), followed with appendix cancer, pancreatic cancer, small intestinal and ampullary carcinoma, and esophageal cancer. One hundred and sixty-nine (55.6%) patients had distant organ metastasis except peritoneal. Two hundred and five (67.4%) patients had previous system treatment. A total of 390 specimens were collected to analyze the efficacy of mutation detected ability among different samples in the real world, included 188 plasma, 156 tissue, 45 ascites supernatant, and 1 pleural effusion supernatant.

ASC supernatant in the real world

We retrospectively analyzed 304 GI cancer patients with peritoneal metastasis to further analyze the efficacy of ASC supernatant in the real world. Targeted NGS of 1021 or 59 cancer-related genes was applied to 398 specimens for genomic profiling. We compared the maximum somatic allele frequency (MSAF) in the different samples first. The MSAF were significantly higher in ASC supernatant (50.18% ± 32.03%) than plasma ctDNA (12.31% ± 19.90%, p < 0.0001) and tissues (27.41% ± 19.67%, p < 0.0001) (Figure 1A). ASC supernatant (100%, 45/45) had a higher frequency of somatic alterations detected than plasma (88.3%, 166/188) and tissues (98.7%, 154/156). Among 304 patients, 169 had distant organ metastasis while 125 had only peritoneal metastasis. We compared the MSAF of different samples of the distant organ metastasis patients to only peritoneal metastasis to explore the impact of the metastatic site on MSAF. The MSAF of plasma in patients of only peritoneal metastasis was significantly lower than that of distant organ metastasis (18.23% ± 23.66% vs 3.63% ± 5.95%, p < 0.0001). However, the MSAF were similar between distant organ metastasis and only peritoneal metastasis among ASC supernatant (53.65% ± 32.21% vs 51.95% ± 27.90%, p = 0.70) and tissue (29.44% ± 21.33% vs 25.19% ± 17.35%, p = 0.33) (Figure 1B). Actionable mutation rates of plasma, ASC supernatant, tissue was 55.86%, 83.33%, 75.29% in distant organ metastasis group and 41.67%, 61.54%, 64.29%, respectively. ASC supernatant had a higher actionable mutation rate than plasma in both two groups (Figure 1C & 1D). It was significant different in distant organ metastasis (83.33% vs 55.86%, p = 0.01) (Figure 1C). ASC supernatant had a higher or comparable actionable mutation rate than tissue.
ASC supernatant in paired samples among 26 patients

Twenty-six patients had more than one sample, 26 paired ASC supernatant and plasma ctDNA, 7 tissues, and 1 PE supernatant. All samples had detectable somatic alterations. In the different subtype groups, 69.2% (18/26) of ASC supernatant samples, 50% (13/26) of plasma ctDNA samples, 57.1% (4/7) of tissues, and 100% (1/1) PE supernatant samples had detectable actionable alterations (Table 2). ASC supernatant had a higher detectable rate in actionable alterations although there was no statistically significant difference. In the paired ASC supernatant and plasma ctDNA samples, 7 patients had actionable alterations detected only from ASC supernatant, 2 patients had actionable alterations detected only from plasma ctDNA. ASC supernatant accounts for more actionable alterations than plasma ctDNA. One CRC patient (P23) had paired ASC supernatant and PE supernatant when disease recurrence, and the actionable alterations in two samples had high consistency. Six patients had no distant organ metastasis, three patients (P01, P09, P10) had the same discovery in actionable alterations. However, actionable alterations were more detected from ASC supernatant compared with plasma ctDNA in the other three patients (P14, P15, P20). We then compared the MSAF of ASC supernatant and plasma ctDNA in the 26 patients. The MSAF in the ASC supernatant was significantly higher than plasma ctDNA (p=0.003) (Figure 2). We speculated that the higher MSAF may explicate the superior detecting efficacy of ASC supernatant compared to plasma ctDNA, especially in the patient who had no distant organ metastasis.

Study design and lung cancer patient characteristics

The study enrolled 407 lung patients with pleural metastasis at diagnosis or during disease progression (Table 3). The median diagnosis age was 60 (range 28-94), and 202 (49.6%) were female. The major histology subtype was adenocarcinoma (84.8%). One hundred and twenty-four (30.5%) patients had only pleural metastasis with stage M1a and 227 patients had the other organ metastasis with stage M1b/c. Three hundred and twenty-one (78.9%) patients had previous system treatment. A total of 1002 specimen were collected to analyze the efficacy of mutation detected ability among different samples in the real world, included 389 plasma, 122 tissue, 446 pleural effusion (PE) supernatant, and 45 pleural effusion sediment.

PE supernatant in the real world

We retrospectively analyzed 407 lung cancer patients with pleural metastasis to further analyze the efficacy of PE supernatant in the real world. Targeted NGS of 1021 or 59 cancer-related genes was applied to 1002 specimens for genomic profiling. Firstly, we compared MSAF in the different samples. The PE supernatant MSAF (33.74% ± 28.34%) was higher than plasma ctDNA (6.28% ± 12.17%, p < 0.0001), tissues (31.29% ± 23.31%, p = 0.88) and PE sediment (13.20 ± 16.64, p < 0.0001) (Figure 3A). Plasma MSAF were higher in M1b/c than M1a patients (8.18 ± 13.82 vs 2.33 ± 4.12, p = 0.0034) (Figure 3B). PE supernatant MSAF were higher in M1b/c than M1a patients too (37.35 ± 29.07 vs 30.71 ± 26.94, p = 0.04). Tissue MSAF and PE sediment MSAF were comparable in stage M1b/c and M1a patients, respectively.
Sequentially, we evaluated the targetable variations detected ability in different samples, including EGFR, ALK, BRAF V600E, KRAS, MET ex14 skipping, RET, ROS1, ERBB2, which had high evidence in lung cancer. Among stage M1a patients, PE supernatant, PE sediment, tissue had higher total actionable mutation rate than plasma, especially in PE supernatant (80.3% vs 48.4%, p < 0.05) and tissue (80.0% vs 48.4%, p < 0.05) (Figure 3C). EGFR had a higher mutation rate in the other three samples than plasma, especially in PE supernatant (37.4% vs 63.2%, p < 0.05). These results were observed in stage M1b/c patients too (Figure 3D). PE supernatant had comparable mutation detected ability with the tissue. EGFR uncommon mutation, BRAF V600E, MET ex14 skipping, RET, ROS1, ERBB2 were classified as uncommon variations. PE supernatant had a higher frequency of uncommon variations than plasma no matter had distant organ metastasis (Figure 3C & 3D).

**PE supernatant in paired samples among 139 patients**

One hundred and thirty-nine patients had simultaneously paired plasma and PE supernatant samples. About 89.2% (124) plasma and 93.5% (130) PE supernatant had mutation detected. PE supernatant had higher MSAF (31.47 ± 28.90 vs 6.23 ± 13.63, p < 0.0001) (Figure 4A) and more mutations detected (6.67 ± 5.12 vs 3.91 ± 4.60, p < 0.0001) (Figure 4B) than plasma ctDNA. PE supernatant had higher detectable rate of targetable alterations than plasma (79.1% vs 56.1%, p < 0.05) (Figure 4C). And it’s significantly different about EGFR mutation (Figure 4C). PE supernatant also had a higher frequency of uncommon variations than plasma (Figure 4C).

**Discussion**

Approximate 10%-40% of patients have peritoneal metastasis in gastric cancer and colorectal cancer[6, 17]. An approximate 15% of patients with lung cancer have pleura metastasis at the first presentation and 50% of patients develop a pleural effusion in the duration of disease.[18] ASC and PE are the common manifestations of peritoneal metastasis of digestive system tumors and pleural metastasis of lung cancer, respectively. Abdominal puncture and thoracentesis are widely used in patients with these body fluids. Content of ASC and PE are generally relatively large, which is average in a few thousand milliliters.

This is a large sample size study contained ASC, PE, tissue, and plasma. The result suggests that ASC and PE can provide important genomic information in different tumors. And they can act as potential and effective samples used for molecular analysis when tumor tissues are unavailable. ASC and PE supernatant had higher or comparable MSAF to tissue. Other reports have suggested that ASC and PE have a larger amount of DNA for genomic analysis compared to plasma[9, 10, 19]. In our study, we observed a significantly higher MSAF in ASC and PE supernatant than plasma based on a large sample size. This means that ASC and PE have more abundant tumor-derived DNA.

Target therapy has become an important strategy in the clinic and matched therapies provided longer survival[20, 21]. ASC and PE supernatant had a higher actionable mutation rate and more mutations detected than plasma in GI and lung cancers. We observed that ASC supernatant had more actionable mutations detected than plasma in 26 GI cancer patients who had paired samples. We also observed this
Phenomenon in limited paired samples of ASC supernatant and tissues. In a CRC patient (P23) who had simultaneous ASC and PE, the actionable variations were highly consistent; however, plasma and tissue had no actionable mutation detected.

With the development of drugs and the advancement of detection technology, patients have more opportunities to receive target therapy. Oncogenic fusions, such as $ALK$, $NTRK1/3$, and $RET$, were reported in gastrointestinal cancer[22, 23]. These variations are of great significance in-clinic treatment. CRC patients who had oncogenic fusion can benefit from target therapy based on previous case series[24, 25]. We observed $FGFR2$ fusion from ASC supernatant in gastric adenocarcinoma and $RET$ fusion from tissue in colon adenocarcinoma. FGFR inhibitors, erdafitinib and pemigatinib, were approved to treat $FGFR$ fusion urothelial carcinoma and cholangiocarcinoma, respectively[26, 27]. $RET$ inhibitors, selpercatinib and pralsetinib, were approved to treat $RET$ fusion non-small cell lung cancer etc[28, 29]. We also observed a higher uncommon actionable mutation detect rate in PE supernatant than plasma in lung cancer.

Plasma had lower MSAF and actionable mutation rate in the patient who had only peritoneal or pleural metastasis than widespread. The mutation detection ability of ASC or PE supernatant were comparable regardless of stage.

**Conclusion**

In a conclusion, cfDNA from ASC and PE supernatants provide more information for tumor genomic profiling than the plasma and PE sediment. ASC and PE supernatant had more advantages regardless of distant organ metastasis. Thus, ASC and PE supernatants could be better alternatives when tumor tissues are not available in the real world, especially in patients who have only peritoneal or pleural metastases.

**Abbreviations**

ASC, ascites; PE, pleural effusion; NGS, next generation sequencing; CRC, colorectal cancer; GC, gastric cancer; MSAF, maximum somatic allele frequency; cfDNA, cell-free DNA; SNV, single nucleotide variants; InDel, insertions and deletions; CNV, copy number variations.

**Declarations**

*Ethics approval and consent to participate*

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the ethics committee of Zhongshan Hospital, Xiamen University, and all patients provided informed written consents. All patients who used tissue and medical data in this study provided written informed consent in accordance with the Helsinki Declaration.
Consent for publication

All authors of this manuscript have read and approved the final version submitted. All authors agree the publication of this manuscript.

Availability of data and materials

The data and materials used and analyzed in the current study are available from the corresponding author on request.

Competing interests

The authors report no conflicts of interest in this work.

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Authors' Contributions

HTW, HNJ, WHY and MZ analyzed the patient data and wrote the manuscript. YFG and BKL collected clinical data of the patients. RRC and JYW interpreted the NGS data. HTW design the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 to 3 are only available as a download in the Supplemental Files section.

Figures

Figure 1

The mutation detect ability of different samples in gastrointestinal cancer. ASC supernatant had higher MSAF than plasma and tissue (A) and metastasis sites had influence on plasma (B); ASC supernatant had higher actionable mutation rate than plasma in distant organ metastasis group (C) and only peritoneal metastasis group (D).
Figure 2

MSAF in ASC supernatant and plasma in 26 paired samples.
Figure 3

The mutation detect ability of different samples in lung cancer. PE supernatant had higher MSAF than plasma, sediment, and tissue (A) and metastasis sites had influence on plasma and PE supernatant (B); PE supernatant had higher driver mutation rate than plasma in stage M1a group (C) and stage M1b/c group (D).
Figure 4

The mutation detect ability of PE supernatant and plasma in 139 lung cancer patients with paired samples. PE supernatant had higher MSAF (A) and more mutations (B) than plasma. PE supernatant had higher driver mutation detected rate than plasma (C).

Supplementary Files

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