Biomarker Discovery

Application of a Highly Sensitive Method for Early Detection of Cancer

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This application note describes the use of matched biological tissue and plasma sample sets to detect predictive biomarkers in colorectal cancer. Using DNA extracted from these samples in conjunction with a special next-generation sequencing (NGS) technology, the common KRAS G12D and BRAF V600E variants were detected in patients with stage 1 colorectal cancer.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide, with over 1 million new cases occurring each year. Unfortunately, the 5-year survival rate is relatively poor for later stage CRC patients, and resistance to drug treatment remains an important factor in the poor prognosis.

Among the hallmarks of CRC are mutations in certain genes that are involved in cellular signaling pathways which promote cell survival and proliferation. These genes include APC, BRAF, CTNNB1, EGFR, KRAS, NRAS, and PIK3CA. Particularly well known is the occurrence of KRAS mutations in the early stages of approximately 50% of all CRC cases.

KRAS mutants have been correlated with resistance to commercial drugs that target EGFR; in fact, using anti-EGFR therapy on these patients shortens the survival rate. The BRAF V600E mutation has also been linked to poor prognosis. It is therefore critical to obtain a patient's mutational profile based on a set of biomarker genes, particularly in the early stages of oncogenesis. This information may then be used for the subsequent application of companion diagnostics, where the effective use of a drug is analyzed for each patient.

BioChain Institute, Inc. offers matched biological sample sets to facilitate such biomarker research and is currently at the forefront of profiling CRC biomarker genes from both formalin-fixed paraffin-embedded (FFPE) tissue obtained through surgery and from the same donor's plasma.

In this study, five donors exhibiting various stages of colorectal cancer were analyzed for biomarkers. The findings revealed the ability to detect the variants in each sample, even in the plasma-derived cfDNA itself. Among the detected hotspots are the very frequently mutated G12 in the KRAS oncogene and the aforementioned BRAF V600E substitution. The ability to detect such mutants in cfDNA demonstrates the utility of liquid biopsy as both diagnostic and prognostic tools in oncogenesis.

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Methods

Sample Procurement

FFPE tissues and plasma from five donors exhibiting various stages of colorectal cancer were provided by BioChain (Table 1). The FFPE tissues were obtained through surgery and consist of both the primary tumor and adjacent normal regions for comparison purposes. Blood samples were drawn from each patient into EDTA Vacutainer tubes (BD) using standard phlebotomy procedures. The blood was centrifuged at 1,500 g for 15 minutes and the supernatant (plasma) was collected. The resulting plasma was centrifuged again at 2,000 g for 15 minutes to ensure removal of residual white blood cells and the supernatant was collected.

FFPE DNA Extraction

FFPE DNA was extracted from each FFPE tissue using BioChain's AnaPrep FFPE DNA Extraction kit. Briefly, 4 x 10 um tissue sections of each donor were de-paraffinized and allowed to digest at 56oC for 2 hours in the presence of proteinase K and a proprietary lysis buffer. The released nucleic acids were then de-crosslinked at 90oC for 1 hour and centrifuged through a filter column to remove any residual debris. Finally, the resulting flow-through was loaded onto the AnaPrep 12 instrument (BioChain) for automated FFPE DNA extraction using pre-loaded reagent cartridges and a pre-programmed protocol.

Cell-free DNA Extraction

cfDNA was extracted from 5 ml of plasma using BioChain's cfPure V2 Cell Free DNA Extraction kit according to the instructions provided in the user manual. The purified cfDNA samples were then run on a BioAnalyzer 2100 instrument (Agilent) using the High Sensitivity DNA Kit (Agilent). The resulting chromatogram was visually inspected for the presence of a cfDNA peak (Figure 1).

Mutational Profiling

Both the extracted FFPE DNA and cfDNA were analyzed using DiaCarta's XNA-Based OptiSeq™ Lung and Colorectal Cancer Panel. This assay provides high sensitivity for the detection of 17 hotspots in 7 genes (APC, BRAF, CTNNB1, EGFR, KRAS, NRAS, and PIK3CA) that are frequently mutated in colorectal cancer (Table 2). A cutoff was set such that each mutation is determined to be a positive detection if the number of reads is greater than 10.

Donor ID	Tumor Type	Tumor Stage
H50154T	CRC	II
H50157T	CRC	I
H50158T	CRC	I
H50165T	CRC	I
H50166T	CRC	III

Table 1. Five donors exhibiting various stages of colorectal cancer (I, II, and III) were selected for this study.

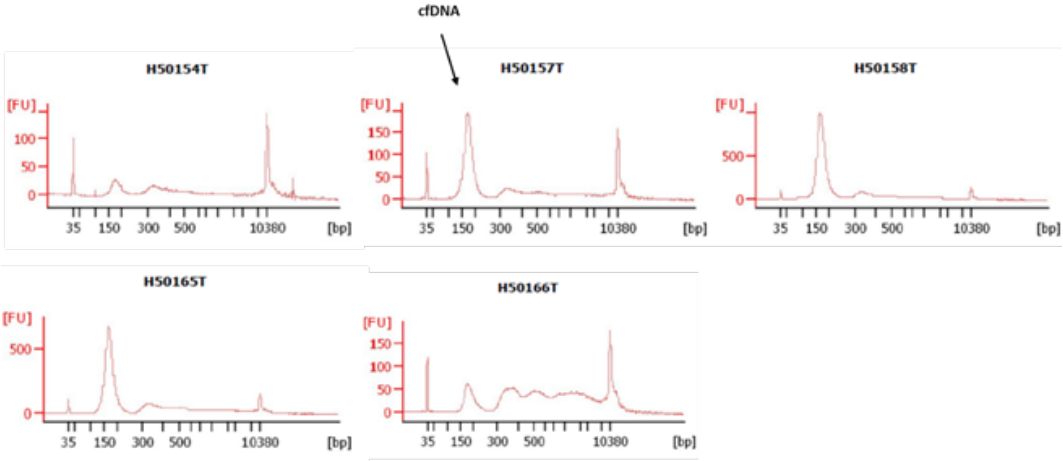

Figure 1. cfDNA was extracted from the plasma of 5 CRC donors (see Table 1) using BioChain's cfPure V2 Cell Free DNA Extraction kit. An Agilent BioAnalyzer run using the extracted samples shows that the kit is able to successfully extract cfDNA (represented in the image as a peak of around 170 bp) from each plasma source.

Hotspot Number	Gene Name	Hotspot Covered	Mutation Type
1	NRAS	A59	SNV
2	NRAS	Q61	SNV
3	NRAS	G12	SNV
4	NRAS	G13	SNV
5	CTNNB1	S45	Deletion
6	PIK3CA	H1047	SNV
7	APC	E1309	Deletion
8	EGFR	G719	SNV
9	EGFR	E746-A750	Deletion
10	EGFR	T790	SNV
11	EGFR	L858	SNV
12	BRAF	V600	SNV
13	KRAS	A146	SNV
14	KRAS	A59	SNV
15	KRAS	Q61	SNV
16	KRAS	G12	SNV
17	KRAS	G13	SNV

Table 2. DiaCarta's XNA-Based OptiSeq™ Lung and Colorectal Cancer Panel detects 17 hotspots in 7 genes frequently mutated in colorectal cancer (table from DiaCarta).

Results

KRAS Mutant Detection

Using the OptiSeq™ panel on the extracted FFPE DNA samples revealed the presence of the KRAS G12D hotspot mutation with relatively high read counts in four out of the five CRC donors (Table 3). Interestingly, the mutation was not detected in any of the samples derived from adjacent normal tissue, confirming the specificity of this variant in driving tumorigenesis. Furthermore, this variant was not detected in the cfDNA samples, which suggests that only utilizing liquid biopsy is not sufficient to detect tumor drivers in patients; the tissues where the cancer originated are still critical for diagnostic applications.

BRAF Mutant Detection

Using the OptiSeq™ panel on the extracted FFPE DNA samples revealed the presence of the BRAF V600E mutation in both primary tumor and adjacent normal tissues from all five donors (Table 4). Interestingly, this variant was also detected in the cfDNA derived from plasma samples for all five donors (Table 4). The detection of BRAF V600E in patients with cancer as early as stage 1 highlights the utility of this gene panel in early cancer diagnostics.

Donor ID	Hotspot	Sample Type	Number of Reads
H50154T	KRAS G12D	Primary Tumor Tissue	1529
H50157T	KRAS G12D	Primary Tumor Tissue	1114
H50158T	KRAS G12D	Primary Tumor Tissue	Not Detected
H50165T	KRAS G12D	Primary Tumor Tissue	792
H50166T	KRAS G12D	Primary Tumor Tissue	855

Table 3. The KRAS G12D hotspot mutation was detected in FFPE DNA extracted from primary tumor tissues in four out of the five donors that were analyzed (highlighted in yellow). This variant was not detected in FFPE DNA from adjacent normal tissue or cfDNA derived from plasma. A positive detection of the mutation was defined as those where the number of reads was greater than 10.

Donor ID	Hotspot	Sample Type	Number of Reads
H50154T	BRAF V600E	Primary Tumor Tissue	15
H50154T	BRAF V600E	Adjacent Normal Tissue	28
H50157T	BRAF V600E	Primary Tumor Tissue	11
H50157T	BRAF V600E	Adjacent Normal Tissue	21
H50158T	BRAF V600E	Primary Tumor Tissue	19
H50158T	BRAF V600E	Adjacent Normal Tissue	22
H50165T	BRAF V600E	Primary Tumor Tissue	21
H50165T	BRAF V600E	Adjacent Normal Tissue	20
H50166T	BRAF V600E	Primary Tumor Tissue	15
H50166T	BRAF V600E	Adjacent Normal Tissue	11
H50154T	BRAF V600E	Plasma	18
H50157T	BRAF V600E	Plasma	24
H50158T	BRAF V600E	Plasma	36
H50165T	BRAF V600E	Plasma	61
H50166T	BRAF V600E	Plasma	62

Table 4. The BRAF V600E variant could be detected not only in FFPE DNA extracted from primary tumor and adjacent normal tissues but in circulating cell-free DNA obtained from plasma (highlighted in green). A positive detection of the mutation was defined as those where the number of reads was greater than 10.

Conclusions

BioChain Institute, Inc. has successfully demonstrated the utility of the OptiSeq™ panel for the detection of hotspot mutations in colorectal cancer. In this study, five donors with various stages of CRC were selected. FFPE DNA and cfDNA were extracted from these donors' archived tissue and plasma, respectively. Using the OptiSeq™ panel technology, the KRAS G12D variant was detected in four of the donors and its specificity was confirmed by its presence only in tumor tissues.

Interestingly, the BRAF V600E mutant was found not only in primary tumor and adjacent normal tissues from these donors, but in plasma as well. The mutation was discovered in patients with cancer as early as stage 1. This finding has strong implications for the concurrent use of liquid biopsy along with this panel in early cancer detection. Furthermore, the simultaneous presence of BRAF V600E with the absence of KRAS G12D in one of the donors establishes the need for such technology in the application of companion diagnostics.

In the end, the results of any cancer detection panel will only be as good as the samples that are used as input. Therefore, a reliable source of biological material is crucial for optimal purity and accuracy of downstream analysis. BioChain has been a leading provider of high-quality cancer tissue and matched plasma sets, along with efficient nucleic acid extraction kits that provide consistency from lot to lot. Using the best starting material, the technology summarized in this application note may then be applied to facilitate novel biomarker discovery in oncogenic research.

References

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