Original Article

Use of Liquid Biopsy to Identify Actionable Mutations in Patients with Recurrent or Advanced Colorectal Cancer in Clinical Practice: A Single Institution Experience

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Key Words
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Purpose. Patients with recurrent or late stage colorectal cancer (CRC) could develop multiple lesions. Tissue biopsy is not always ideal for genetic testing because the tissue can be scarce and difficult to access. We conducted a retrospective study of circulating tumor DNA (ctDNA) next-generation sequencing analysis in patients with recurrent or advanced CRC. The purpose of the study was to explore the utility of ctDNA tests in providing clinical actionable information in colorectal cancer.
Methods. Twenty patients, ten men and ten women, were included. Each patient's peripheral blood was collected and centrifuged to extract both plasma cell-free DNA and buffy coat white blood cell (WBC) DNA. Both plasma cfDNA and WBC gDNA were sequenced using hybrid capture – based NGS panel (74 genes). The sequencing results were then compared and analyzed to report ctDNA mutation by filtering out the false positive variants that could be produced by clonal hematopoiesis.
Results. *TP53* was the most frequently mutated gene (15/20) in these patients. Among the patients, thirteen had concordant *KRAS* hotspots or *BRAF* V600E status compared with the original tissue pathology results at diagnosis. Sixteen patients (16/20) had detectable mutations which could lead to next therapy option or treatment efficacy monitoring purpose.
Conclusion. Simple blood ctDNA tests could reflect the tumor genome profile and provide clinicians with an alternative means of exploring the next possible regimen or evaluating the treatment efficacy.

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In Taiwan, colorectal cancer (CRC) is the most common cancer in men and the second most common in women.[1] Despite ongoing efforts for early preventive screening of the disease, 20%-30% of patients are diagnosed as having CRC at an advanced stage, and relapse occurs in 40%-50% of patients diagnosed in earlystages.[2] Tumor molecular profiling is a crucial tool to determine whether patients are suitable for targeted therapies, such as anti-epidermal growth factor receptor (EGFR) antibodies (cetuximab or panitumumab), and to assess whether they have acquired resistant mutations during the treatment course.

Tumor tissue biopsy is the standard method for cancer diagnosis. Tumor biopsies provide samples for pathological assessment and molecular profiling. However, they also have clinical shortcomings. Conducting repeat biopsies to monitor disease progression, which is critical for patients with late-stage CRC, is dif-

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ficult because of the substantial trauma they incur and poor patient compliance. Tissue biopsy may also be unable to reflect the heterogeneous nature of the disease, especially when the patient has multiple lesions.

Cell-free DNA (cfDNA) in the blood of patients with mCRC includes various levels of circulating tumor DNA (ctDNA) released by cancer cells. This tumor DNA could be used to provide prognoses and predictions of therapeutic sensitivity or resistance. With the improvements in next-generation sequencing (NGS) technology, surveying clinically relevant genes using circulating tumor DNA (ctDNA) extracted with a simple blood draw is becoming a promising tool for detecting molecular changes in metastatic solid tumors.

Studies are increasingly describing potential uses for circulating tumor DNA (ctDNA) in the care of patients with CRC. Because of the rapid development of this area of research, the Colon and Rectal – Anal Task Forces of the United States National Cancer Institute (NCI) convened a panel of multidisciplinary experts to summarize current data on the utility of ctDNA in the management of CRC and provide guidance for promoting the efficient development and integration of this technology into clinical care.[3] The panel focused on four key areas in which ctDNA could change clinical practice: detecting minimal residual disease, managing patients with rectal cancer, monitoring responses to therapy, and tracking clonal dynamics in response to targeted therapies and other systemic treatments.

In this retrospective study, we employed a commercially available ctDNA testing panel to explore the next possible treatment opportunity for twenty patients with recurrent or progressive mCRC.

Materials and Methods

Patients

For this retrospective study, we reviewed the medical records of patients with recurrent or metastatic CRC (mCRC) treated at Cheng-Hsin General Hospital over a period of 5 years (May 2016 to Apr 2021) who had received the molecular assessment of their disease using ctDNA. All patients with standard tissue molecular test results (including *RAS* exon2 and *BRAF* exon15) at diagnosis as the comparison reference. All patients were looking for next line potential treatment and were unable or unwilling to undergo tissue biopsy to obtain additional tumor tissue for molecular testing. The final test results were analyzed to evaluate the utility of the ctDNA NGS panel in assisting treatment decision and the management plan in patients with recurrent or advanced CRC. Patients' originally staging at diagnosis were between IIIB-IVC. All aspects of this study were approved by the institutional review board (IRB) approval No. (882)110-28, of Cheng-Hsin General Hospital.

ctDNA

A 20 mL sample of peripheral blood was collected from each patient. Genetic analysis was performed with CellMax Life (Sunnyvale, CA, United States) using the 74 genes LBx ctDNA NGS panel. CellMax Life is a US-based lab accredited by the College of American Pathologists (CAP) and Clinical Laboratory Improvement Amendments (CLIA). In brief, cell-free DNA (cfDNA) from plasma and genomic DNA (gDNA) from buffy coat white blood cells were sequenced in parallel to filter out non-tumor-derived variants. CellMax Life sequencing technology is based on NGS technology (based on hybrid capture) with a single molecule analytical sensitivity and 100% specificity (0.2% VAF). CellMax Life sequencing detects various types of cancer-related gene alterations (clinically actionable mutations), including single nucleotide variants, insertions or deletions, gene fusions or rearrangements, and copy number variations.[4] All clinically relevant alterations were reported in accordance with the joint guidelines of the CAP, Association for Molecular Pathology, and American College of Medical Genetics and Genomics.[5]

Results

Patients' tumor characteristics and gene mutation status before blood collection

Twenty patients (originally staging at Dx between

IIIB-IVC) were included in the analysis. The median age was 59 years (range, 43-73 years); ten (50%) of the patients were men, and ten (50%) were women. Fifteen patients (P01, P03, P04, P05, P06, P10-P14, P16-P20) were under systemic therapies, five patients (P02, P07, P08, P09, P15) were diagnosed with relapse and just finished surgery or had not received any treatment yet (P08). All patients were looking for available therapies.

Patients' original standard tissue genotyping of *KRAS*, *NRAS*, and *BRAF* had revealed *KRAS* mutation in five patient (25%, codon 12&13) and *BRAF* codon V600E mutation in two patient (10%). All twenty patients displayed moderate EGFR expression in their tissue immunohistochemistry staining results. No high microsatellite instability was noted in these patients.

The demographic characteristics and treatment evaluations of each patient are presented in Table 1. The European Society for Medical Oncology guidelines indicate that bevacizumab combined with folinic acid-fluorouracil-irinotecan therapy is the first-line treatment for patients with advanced CRC with *RAS* gene mutations.

Tissue biopsy were not performed considering the disease status or willingness of these patients. Therefore, a comprehensive circulating tumor DNA test was performed to explore the next possible treatment target or evaluate the residual disease. The tested gene list is provided in Table 2.

Circulating tumor DNA gene testing results for twenty patients

The genotyping results are detailed in Table 1. High microsatellite instability was not detected in any of the patients, which is consistent with the original pathological status at diagnosis. Seven of the twenty patients (35%) were determined to have *APC* mutations, and fifteen out of twenty patients (75%) had *TP53* mutations. Patient 5 and 16 (P05 & P16) displayed concordant *BRAF* V600E mutation with the tissue test at diagnosis. P09, P14, and P19 were detected with the same *KRAS* mutations (G12D or G12S). The overall concordance rate between the liquid biopsy and patients' original tissue genotyping results of *KRAS* and *BRAF* hotspot mutations at diagnosis are 85% and 95% as summarized in Table 3.

Regarding de novo mutations in patients treated using guideline-recommended therapies, the *KRAS* G12V mutation was identified in P04 and the *BRAF* V600E mutation was observed in P07. Neither mutation was observed in the original tissue genotyping results. For P17 and P20, the original *KRAS* G13D and G12V were not detected in the liquid biopsy test. *BRAF* I582V mutation was detected in P06; however, its clinical significance remains unknown. Mutation in genes (*PIK3CA* and *ERBB2*) which are not included in the current standard molecular test of colorectal cancer at diagnosis but have potential targeted therapies were also observed in three patients (P05, P10 and P11).

Discussion

Liquid biopsy is a minimally invasive approach to obtain circulating materials that originated from tumor cells using body fluid samples (mainly peripheral blood). Liquid biopsy has emerged as an approach to orient the care of patients with CRC because of its ability to detect tumor-derived nucleic acids and characterize tumor-specific genomic abnormalities. The proportion of patients with CRC in whom ctDNA can be detected ranges from 50% in patients with non-metastatic disease to nearly 90% in patients with metastatic disease.[6]

The current standard of care for advanced CRC involves testing tumor tissues for three biomarkers: expanded *RAS* mutations (negative predictor of benefit from anti-EGFR antibodies), *BRAF* V600E (negative prognostic marker and positive predictive marker for *BRAF* V600E-targeted therapies), and microsatellite instability (MSI) status (which has prognostic and predictive value regarding responsiveness to immune-checkpoint inhibitors and is used as a screening tool for Lynch syndrome).[7] However, several factors may affect the availability of tissue in the advanced CRC patients including patients' condition and willingness. Therefore, ctDNA-based testing could provide a minimally invasive solution for molecular diagnosis or

Table 1. Demographic, clinicopathological characteristics and liquid biopsy results of twenty patients

Case No.	Gender	Tumor type	Location	Staging at Dx	Gene mutation at Dx	Regimen before liquid biopsy	Gene mutation detected by liquid biopsy (VAF%)	MSI status by liquid biopsy
P01	F	Colorectal adenocarcinoma	Left	Stage IVA	None	FOLFIRI+Bevacizumab	*APC* Q1294fs (40.08); *APC* S1356X (6.419); *TP53* P153fs (37.58); *SMAD4* G386V (46.18)	MSI-H not detected
P02	M	Colorectal adenocarcinoma	Left	Stage IVC	None	Surgery only	*APC* S1275fs (20.44); *TP53* R273H (52.05)	MSI-H not detected
P03	M	Colorectal adenocarcinoma	Left	Stage IVA	None	FOLFIRI+Bevacizumab	*TP53* R273H (0.2)	MSI-H not detected
P04	F	Colorectal adenocarcinoma	Right	Stage IIIB	None	Irinotecan+Capecitabine + Bevacizumab	*KRAS* G12V (0.34); *APC* E1397X (0.17); *TP53* C238Y (0.67)	MSI-H not detected
P05	F	Colorectal adenocarcinoma	Right	Stage IVC	*BRAF* V600E	FOLFIRI+Bevacizumab	*BRAF* V600E (0.76); *PIK3CA* H1047R (0.59); *MAP2K1* P124L (0.84); *PTEN* T319X (0.61); *TP53* L275P (0.84); *HNF1A* R263C (1.19)	MSI-H not detected
P06	F	Colorectal adenocarcinoma	Right	Stage IVB	None	FOLFIRI+Bevacizumab	*APC* E578X (2.18); *TP53* V272M (5.09); *BRAF* I582V (2.52)	MSI-H not detected
P07	M	Colorectal adenocarcinoma	Right	Stage IVC	None	Surgery only	*BRAF* V600E (26.96); *TP53* H179del (28.77)	MSI-H not detected
P08	F	Colorectal adenocarcinoma	Right	Stage IIIC	None	None	*APC* P1440fs (1.91); *TP53* R248Q (1.89)	MSI-H not detected
P09	F	Colorectal adenocarcinoma	Right	Stage IVC	*KRAS* G12D	Surgery only	*KRAS* G12D (0.46); *APC* P1453fs (0.4); *TP53* C135G (0.42)	MSI-H not detected
P10	M	Colorectal adenocarcinoma	Left	Stage IVC	None	FOLFIRI	*ERBB2* amp; *TP53* E180X (0.49)	MSI-H not detected
P11	M	Colorectal adenocarcinoma	Right	Stage IVA	None	FOLFIRI+Bevacizumab	*ERBB2* amp; *TP53* I195T (24.1)	MSI-H not detected
P12	M	Colorectal adenocarcinoma	Left	Stage IIIB	None	FOLFIRI+Bevacizumab	*FLT3* amp; *APC* Q1152X (10.49); *TP53* G245S (11.29); *SMAD4* R361C (11.94)	MSI-H not detected
P13	M	Colorectal adenocarcinoma	Left	Stage IVA	None	FOLFIRI+Cetuximab	None	MSI-H not detected
P14	M	Colorectal adenocarcinoma	Left	Stage IVB	*KRAS* G12S	FOLFIRI+Bevacizumab	*KRAS* G12S (20.77); *FLT3* amp; *CTNNB1* T41A (25.56); *TP53* R175H (20.76)	MSI-H not detected
P15	F	Colorectal adenocarcinoma	Right	Stage IVB	None	Surgery only	None	MSI-H not detected
P16	M	Colorectal adenocarcinoma	Right	Stage IVB	*BRAF* V600E	FOLFIRI+Bevacizumab	*BRAF* V600E (0.54); *TP53* c.783-2A>T (0.78)	MSI-H not detected
P17	M	Colorectal adenocarcinoma	Right	Stage IIIB	*KRAS* G13D	FOLFIRI+Bevacizumab	None	MSI-H not detected
P18	F	Colorectal adenocarcinoma	Right	Stage IVB	None	FOLFIRI+Bevacizumab	None	MSI-H not detected
P19	F	Colorectal adenocarcinoma	Left	Stage IIIB	*KRAS* G12D	FOLFIRI+Bevacizumab	*KRAS* G12D (1.05); *TP53* R282W (1.12)	MSI-H not detected
P20	F	Colorectal adenocarcinoma	Right	Stage IVC	*KRAS* G12V	FOLFIRI+Bevacizumab	*CTCF* R29P (0.8)	MSI-H not detected

Dx: diagnosis: ; FOLFIRI: folinic acid + fluorouracil + irinotecan; VAF: variant allele frequency; MSI-H: microsatellite instability-High; amp: amplification; fs: frameshift mutation; X: nonsense mutation; del: in-frame deletion.

Table 2. ctDNA NGS panel gene list

ABL1	*AKT1*	*ALK*[2]	*APC*	*AR*[1]	*ARID1A*	*ATM*	*BRAF1*	*BRCA1*[1]	*BRCA3*[1]
CDH1	*CDKN2A*	*CSF1R*	*CTCF*	*CTNNB1*	*DNMT3A*	*EGFR*[1]	*EP300*	*EPHA3*	*EPHA5*
ERBB2[1]	*ERBB4*	*EZH2*	*FBXW7*	*FGFR1*[1]	*FGFR2*[1]	*FGFR3*[1,2]	*FLT3*	*GATA3*	*GNA11*
GNAQ	*GNAS*	*HNF1A*	*HRAS*	*IDH1*	*IDH2*	*JAK2*	*JAK3*	*KDR*	*KEAP1*
K1T[1]	*KRAS*[1]	*LIFR*	*MAP2K1*	*MAP3K1*	*MET*[1]	*MLH1*	*MPL*	*MTOR*	*NAV3*
NFE2L2	*NOTCH1*	*NPM1*	*NRAS*	*NTRK1*[2]	*PDGFRA*[1]	*PIK3CA*[1]	*PIK3R1*	*PTCH1*	*PTEN*
PTPN11	*RB1*	*RUNX1*	*ROS1*[2]	*SETD2*	*SMAD2*	*SMAD4*	*SMARCA4*	*SMARCB1*	*SMO*
SRC	*STK11*	*TP53*	*VHL*	Microsatellite instability – High[3]					

[1] Copy Number Variation (CNV) covered.

[2] Fusion covered.

[3] *NR-21, NR-24, BAT-25, BAT-26* and *MONO-27* were analyzed. Sample with two or more unstable sites are considered as MSI-H.

Table 3. The overall concordance of *KRAS* and *BRAF* hotspot mutations between original tissue molecular test at diagnosis and the liquid biopsy

	P01		P02		P03		P04		P05		P06		P07		P08		P09	
	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L
KRAS	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
BRAF	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-

	P10		P11		P12		P13		P14		P15		P16		P17		P18	
	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L
KRAS	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-
BRAF	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-

	P19		P20		Concordance
	T	L	T	L	
KRAS	+	+	+	-	85% (17/20)
BRAF	-	-	-	-	95% (19/20)

T: tissue; L: liquid. The table only compares the concordance between standard molecular test at diagnosis (hotspots in *RAS* exon2 and *BRAF* exon15) and the corresponding liquid biopsy result of each patient.

monitoring in patients whose cancer tissue is unobtainable.

The overall concordance rate of *KRAS* and *BRAF* hotspot mutations are high (85% and 95%, Table 3). Seventeen patients displayed consistent *RAS* status compared with the original tissue test results at diagnosis, with fourteen patients displaying *RAS* wild type and three (P09, P14, P19) had the same *KRAS* mutations. Two patient (P05, P16) also had concordant *BRAF* V600E mutation results in tissue-based and ctDNA-based tests. These high concordance rates were comparable to those reported in other studies discussing concordance between ctDNA and standard-of-care tumor tissue – based *RAS* testing.[8,9] Notably, P04 was originally tested wildtype for the *KRAS*, however, we observed *KRAS* G12V mutation in the ctDNA test result which could indicate resistance to anti-EGFR therapies. *KRAS* mutation in liquid biopsy could reportedly be a false positive signal caused by the lymphocytes because of the clonal hematopoiesis (CH) effect.[10] To minimize the non-tumor-derived signal noise, the ctDNA test we used employs two simultaneous NGS sequencing for one sample (plasma cell-free DNA and white blood cell gDNA). Therefore, we can confidently rule out the *KRAS* G12V mutation identified in P04 was a CH-related variant.

CH has been acknowledged as a crucial source of non-tumor-derived variants detected in the blood, and its effect could be considerably enhanced through the use of a greater sequencing depth and more sensitive ctDNA testing. Increasing evidence suggests that CH mutations can be detected in genes that are commonly

mutated in solid tumors, including *KRAS*, *GNAS*, *NRAS*, and *PIK3CA*.[10-13] One of the key recommendations of the NCI Colon and Rectal – Anal Task Forces whitepaper on selecting a ctDNA test is to consider whether the test simultaneously sequences the genomic DNA from white blood cells.[3] Clonal hematopoiesis can have a huge effect when the liquid biopsy is applied for minimal residual disease evaluation after treatment in patients with cancer.[14]

APC and *TP53* mutations occurred in 35% and 75% of our cohort, respectively. Somatic mutations in *APC* were reported in 34%-70% of patients, and *TP53* mutations were observed in 80% of patients with sporadic advanced CRC.[15-18] The ratios observed in the present study were similar to those reported. Another previous colorectal cancer NGS study in Taiwan using primary resected tumor tissues from stage I-IV patients (19/32 were stage I & II) showed mutation ratio of *APC* and *TP53* to be 59.38% and 50%.[19] Notably, P04 was discovered to have *KRAS* G12V, which was not present at diagnosis, and P07 exhibited a novel *BRAF* V600E mutation. Guidelines has recommended anti-EGFR therapy should be avoided if patient has *KRAS* resistant mutations and BRAF inhibitors could be considered while *BRAF* V600E mutation presents.[20] Therefore the test findings provided important guidance for physicians to avoid potential drug resistance or consider next available treatment option.

Two patients (P10 and P11) had *ERBB2/HER2* amplification in this study. *HER2* amplification occurs in 2% to 3% of patients with metastatic colorectal cancer (CRC).[23-25] It has been shown in recent studies that *HER2* amplification is associated with relative resistance to anti-epidermal growth factor receptor (EGFR) targeting in *RAS* wild-type CRC.[26-28] Various clinical studies has demonstrated the combinations of trastuzumab + lapatinib, trastuzumab + pertuzumab, and trastuzumab-deruxtecan are associated with significant activity in *HER2*-amplified *RAS* wild-type CRC, leading to their endorsement in the NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) for Colon Cancer as a treatment option for this patient population.[30-32]

Both P05 and P07 were detected with *BRAF* V600E by the liquid biopsy. However, P07 expired within 2 months after liquid biopsy test. P05 has remained in stable disease for over 6 months. The difference in mortality may be related to the higher tumor burden observed in P07 (*BRAF* V600E, 26.96%; *TP53* H179del, 28.77%) compared with that observed in P05 (*BRAF* V600E, 0.76%; *TP53* L275P, 0.84%). A study reported that ctDNA levels are closely related to cancer stage and tumor burden.[19] A study analyzing ctDNA in patients with CRC at different stages revealed that the ctDNA concentration in stage I patients was significantly lower than that in stage IV patients and that ctDNA concentration was positively correlated with tumor size.[20]

Many clinical trials report that the primary tumor location plays an important prognostic role in CRC, particularly in patients with wild-type RAS who were treated with anti-EGFR antibodies.[33,34] Other predictive biomarker candidates for anti-EGFR therapy include microsatellite instability and *BRAF* V600E and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations. The prevalence of these biomarkers was also reported to be different between patients with right-sided and left-sided primary tumors.[35,36] Due to the limited numbers of our study, comparing the presence of *BRAF* and *PIK3CA* mutations between left/right sidedness was not statistically meaningful. However, in our cohort, all the *BRAF* and *PIK3CA* mutations were found in patients (P05, P06, P07, P16) with right sided colon cancer which was consistent with previous studies that these mutations tend to present more frequently in right colon cancer.[37,38]

Though the limited numbers of the size, our findings still support the importance of applying comprehensive molecular genotyping to evaluate treatment options for CRC patients which can provide potential guidance to the following treatment strategy. Unfortunately under the current National Health Insurance system in Taiwan, although novel *BRAF* V600E and *ERBB2/HER2* amplification were identified in patients (P07, P10, P11) with available targeted drugs, since patients have to pay out of own pockets, they finally decided not to take the relevant drugs.

Nontheless, our report echoes the value of liquid biopsy emphasized in the consensus statement of the

NCI Colon and Rectal – Anal Task Forces. This statement reports that one key utility of the ctDNA test is the analysis of circulating tumor DNA, which may provide crucial information for adjusting the treatment strategy and reveal the patient's tumor burden.[3]

Liquid biopsy has been clinically proven feasible. There were only twenty patients included in this study, however, to our knowledge, studies in Taiwan discussing liquid biopsy NGS panel utility in advanced CRC patients to explore treatment options and evaluate patients' tumor burden were rare. Therefore sharing our experience would provide important value for the physicians to evaluate the benefit of incorporating liquid biopsy NGS testing into daily practice.

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原　著

臨床使用液態切片檢測找尋復發或晚期大腸直腸癌患者具臨床治療參考意義基因突變 – 單一機構經驗

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目的　復發或第四期的大腸直腸癌患者常具有多處腫瘤轉移，而此時若需要進行組織採檢以進行基因檢測可能會受限於患者組織取得不易或可取得的組織稀少以致無法進行。我們以回顧性的方式分析了二十位大腸直腸癌患者的循環腫瘤 DNA 次世代定序檢測結果。本研究的目的是探討循環腫瘤 DNA 檢測對於提供有大腸癌治療有意義臨床資訊的臨床應用性。

方法　分析包含二十位患者。每位患者皆抽血並離心，分別萃取上層血漿的游離懸浮 DNA 以及白血球細胞核內的 DNA，接著使用雜交體捕捉技術平台各自同時定序 74 個臨床癌症標靶治療相關基因。最後，兩邊 DNA 定序結果同時進行比對分析以去除來自克隆性造血所造成的非腫瘤相關基因突變的干擾。

結果　*TP53* 基因突變出現在十五位患者中 (15/20)。所有二十位患者中，十三位的 KRAS 熱點基因突變與 BRAF V600E 基因突變狀態與確診時的標準臨床組織檢測一致。十六位患者於檢測中找到可能作為下一線的治療選擇或作為後續評估治療效果的基因突變。

結論　簡單的抽血檢測循環腫瘤 DNA 可以顯示患者目前體內腫瘤的基因變異狀態並提供臨床醫師另一個檢測工具來找尋可能的治療標的或是評估現行治療的效果。

關鍵詞　大腸直腸癌、循環腫瘤 DNA、次世代定序、克隆性造血。