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科研費
KAKENHI

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研究課題名（和文）Next generation sequencing of circulating tumor DNA to monitor treatment response to nivolumab in advanced gastric cancer

研究課題名（英文）Next generation sequencing of circulating tumor DNA to monitor treatment response to nivolumab in advanced gastric cancer

研究代表者

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研究成果の概要（和文）：本研究の目的は、免疫療法応答性モニタリングにおけるctDNAの臨床的有用性を評価することである。
ローパス全ゲノムシーケンシングにより、血中cfDNAの腫瘍割合を反映したゲノム全体のコピー数変動（CNA）の全体像が明らかになった。我々の結果では、血中cfDNAで高い変異アレル頻度（VAF)10%以上のサンプルや、VAF10%以下の変異の多いサンプルでは、腫瘍分画との高い相関関係を示した。モニタリング期間でcfDNA中のCNAパターンの変化は、免疫療法の応答性を反映する可能性が示唆された。NGSによる500遺伝子パネルは、血中の腫瘍変異負荷の評価を可能にした。

研究成果の学術的意義や社会的意義
Next generation sequencing of cell-free DNA including low-pass whole genome sequencing for genome-wide copy number alteration assessment and large panel targeted sequencing for blood tumor mutational burden evaluation might be useful for immunotherapy responsiveness monitoring.

研究成果の概要（英文）：The current proposal aimed to evaluate the clinical utilities of circulating tumor DNA (ctDNA) in immunotherapy responsiveness monitoring.
Low-pass whole genome sequencing revealed genome-wide copy number alterations (CNA) landscape reflecting tumor fraction in cfDNA. Our findings revealed higher tumor fraction correlating with high variant allele frequency (VAF) more than 10%, or with samples containing multiple mutations with a VAF less than 10% from blood cfDNA. Changes on CNA patterns in cfDNA during monitoring might reflect immunotherapy responsiveness.
Large 500 genes targeted panel for NGS allows the evaluation of blood tumor mutational burden. However, subset of mutations with VAF below the limit of detection (LOD) 0.5% were not detected. Several factors, including TMB evaluation, gene coverage, and LOD, should be considered when utilizing targeted sequencing for ctDNA detection in monitoring immunotherapy responsiveness.

研究分野：cancer genetics

キーワード：liquid biopsy　ctDNA　cell-free DNA　NGS　immunotherapy　tumor mutational burden

※科研費による研究は、研究者の自覚と責任において実施するものです。そのため、研究の実施や研究成果の公表等については、国の要請等に基づくものではなく、その研究成果に関する見解や責任は、研究者個人に帰属します。

(1) Scientific background

The clinical applications of circulating tumor DNA (ctDNA) analyses are well established, with valuable insights gained in disease biology, treatment response and mechanisms of treatment resistance. ctDNA are short cell-free DNA (cfDNA) fragments found in the bloodstream that originate from tumors or cancer cells. ctDNA acts as a surrogate, with genetic alterations revealing the tumor genomic profile of a patient. ctDNA assessment from peripheral blood offers a promising and non-invasive approach to monitor real-time dynamic changes of genomic features from the tumor. Our lab has established approaches and conducted research studies for ctDNA detection and surveillance that include the detection of minimal residual disease of various solid tumors and treatment responsiveness of targeted therapy.[1-5] The following are the main findings from our previous research studies, which are very much aligned with other previous publications, in assessing the clinical utilities of ctDNA using genomic profiling:

A) ctDNA mutations are in high concordance with tumor tissue.
B) ctDNA detect disease progression/recurrence earlier than radiological imaging.
C) ctDNA show better dynamic range compare to routine tumor marker in assessing tumor burden changes.
D) ctDNA can capture tumor intra- and inter-heterogeneity derived from different tumor clones.
E) ctDNA detect mechanisms of acquired resistance of targeted treatment, outperforming tissue biopsy.

Immunotherapy has demonstrated survival benefit and hence has been emerged to be one of the standard-of-care for various solid tumor. Most patients with solid tumor who receive immunotherapy today are on one of two kinds of checkpoint inhibitors: PD1/PDL-1 or CTLA-4 inhibitors. Although the survival benefit is unquestionable, not all the patients benefit from this therapy. Thus, emerging strategies and continuing research focus on (1) finding ways to predict/monitoring immunotherapy responses, (2) sourcing solutions for resistance and (3) investigating the characteristics of cancer cells evade or suppress immune responses against immunotherapy. Unlike targeted therapy, treatment responsiveness monitoring of immunotherapy appears to be more challenging owing to the lack of monitoring biomarkers. Up-to-date, PD-1/PD-L1 expression and tumor mutation burden (TMB) that reflect neoantigen load were identified to be important predictive biomarkers evaluated before the initiation of immunotherapy. These biomarkers are found to be associated immunotherapy responsiveness. Low-pass whole-genome sequencing (LP-WGS) that is capable of evaluating tumor fractions and identifying genome-wide copy number alterations (CNA) coupled with ultradeep targeted sequencing integrated molecular barcode that could detect mutation and copy number variations up to limit of detection at 0.1% in cfDNA might provide solutions to monitor treatment responsiveness of immunotherapy.

(2) Research objectives

To evaluate the feasibility of using cfDNA to evaluate treatment response of immunotherapy in advanced cancer patients with the following approaches:

(1) Establishment of LP-WGS for the evaluation of tumor fraction and

(2) Evaluation of ultradeep targeted sequencing for mutation profiling to investigate the possibility to detect resistance/non-responsive mutation when patients encounter progression disease.

(3) Design of a customized panel for immunotherapy responsiveness monitoring

This research grant will support the proof-of-concept of this approach before it could be implemented in the clinical studies.

(3) Research Method

3.1 Cell-free DNA extraction

cfDNA from the plasma was isolated using MagMax cell free total nucleic acid isolation kit. In brief, bead binding buffer, proteinase K, Magnetic Bead Suspension were added to 6-8ml of plasma sample. The circulating cell-free DNA were bound to the magnetic beads before eluted with 18µl of elution buffer. All the cfDNA and stored at -20ºC.

3.2 Low-pass whole-genome sequencing (LP-WGS)

A total of 5-10ng of cfDNA were used to prepare sequencing libraries by the ThruPLEX Tag-Seq Kit and purified by Agencourt AMPure XP beads. Libraries were quantified using KAPA Library Quantification Kit and the size of the library were confirmed by TapeStation. Sequencing libraries were pooled at equal amount and WGS were performed at 0.5x on Illumina MiSeq 2x300 bp paired-end sequencing. For analysis, after aligning to human reference genome, CNVs were called using Hidden Markov Model (HMM)-copy suite. Tumor fraction of cfDNA were estimated using ichorCNA software. The signal detected were normalize genome-wide using 27 healthy donors included in the ichorCNA software. Tumor fraction could be used as an estimation of tumor mutation burden to evaluate immunotherapy responsiveness.

3.3 Ultra-deep targeted sequencing

3.3.1 Oncomine Precision Assay (OPA)

cfDNA were analyzed using a fully automated Ion Torrent™ Genexus™ Integrated Sequencer (Genexus) coupled with Oncomine Precision Assay (OPA) that requires as little as 20ng of DNA from tumor tissues or cell-free DNA to assess a total of 1,773 mutations and indels, 981 fusions and 15 copy number variations on 55 genes. This amplicon-based NGS technology has integrated molecular barcode system that are known to reduce error and could accurately detects low-frequency variants from 8-20ng of cfDNA. Sequencing data was mapped and analyzed using on-instrument Genexus software.

3.3.2 TruSight Oncology 500 ctDNA assay (TSO500 ctDNA)

The panel size of TSO500 ctDNA is about 1.94 Mb DNA that covers 523 genes including 59 for CNVs, 23 genes for fusions and 76 loci of microsatellite sites. Importantly, this panel allows the evaluation of tumor mutational burden (TMB) that is an important factor for immunotherapy responsiveness. Sequencing libraries were prepared using TSO500 ctDNA kit. In brief, end repair and A-tailing were performed with the input of 30ng of cfDNA. Subsequently, adapters were ligated and products were purified before performing index PCR. For enrichment, two rounds of hybridization-capture were performed before PCR amplification and purification of the enriched library. Libraries were quantified and normalized. Libraries were pooled and sequencing was performed using 2x151bp with 400 million reads per samples for 35,000x coverage on Illumina NovaSeq. Subsequent analyses were performed using the default DRAGEN TSO500 ctDNA analysis software on the Illumina DRAGEN v3 server.

3.4 Design of customized panel for immunotherapy responsiveness monitoring

A relative smaller panel (100-150 genes) were designed based on common cancer mutated genes and literature review of biomarkers that are relevant to immunotherapy responses.

(4) Results

4.1 Low-Pass whole genome sequencing

LP-WGS was firstly conducted using a positive control that harbored *EGFR* CNV. The coverage of the positive control is 0.15X and tumor fraction was estimated as 0.4622 with LP-WGS (Figure 1). Copy number alteration was observed at chr7p which coincide with the genomic coordination of *EGFR*. LP-WGS

was conducted with additional nine cfDNA with variant allele frequency (VAF, data retrieved from ultra-deep targeted sequencing) ranged from 3%-40%. The average coverage of LP-WGS of these nine samples was 0.14X (range 0.124-0.158). The tumor fraction ranged from 0-42.84, with higher tumor fraction correlated with higher variant allele frequency (VAF>10%) or samples having multiple mutations with <10%.

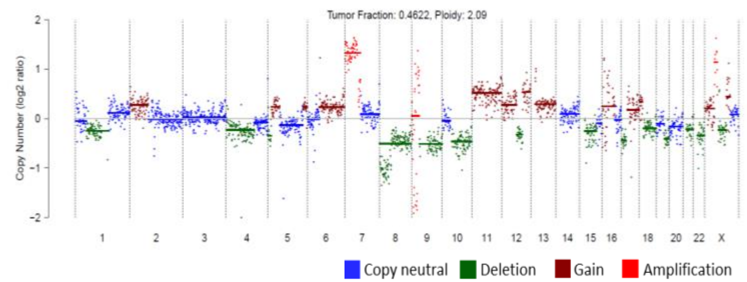

Figure 1: Low-pass whole genome sequencing result of positive control with EGFR CNV.

4.2 Ultra-deep targeted sequencing

Two different panels, OPA (50 genes) and TSO500 ctDNA assay (523 genes), were used to evaluate the genomic profile of cfDNA; The recommended limit of detection (LOD) of the two panels are at VAF 0.2% and 0.5%, respectively. Genomic alterations using 23 cfDNA detected from these panels showed to be high concordant with R2-value at 0.9889 (Figure 2). Genomic alterations at VAF<0.2 detected from OPA were not detected by TSO500 ctDNA assay (below its LOD). Nevertheless, TSO500 ctDNA detected 15/23 cfDNA to be TMB-H with threshold of ≥10 mut/Mb from blood cfDNA. Although TMB is an important indicator for immunotherapy, ctDNA detection remained challenging considering the higher limit of detection at 0.5% with limited input of cell-free DNA from cancer patients compare to smaller size panel, especially at minimal disease residual setting. In addition, OPA panel has limited immunotherapy-related alterations that restricted its utilities in responsiveness monitoring among patients who received immunotherapy.

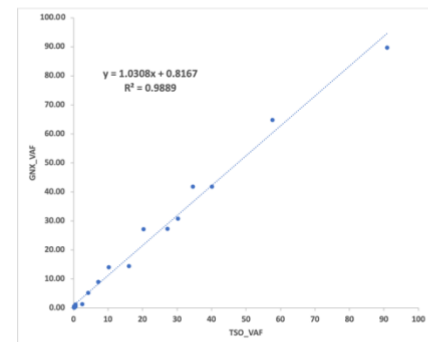

Figure 2: Concordance between 2 panels

4.3 Design of customized panel that are suitable for immunotherapy responsiveness monitoring

Study design and panel selection are important factors for liquid biopsy study. After evaluating various approaches that could be potential tools for immunotherapy monitoring, a customized panel covering 143-panel including genes related to immunotherapy responsiveness retrieved from the literature review were designed.

Conclusion

LP-WGS analysis provides genome-wide CNA landscape reflecting the tumor fraction in blood cfDNA. Changes on CNA patterns in cfDNA during disease monitoring might reflect immunotherapy responsiveness. Several factors include evaluation of TMB, gene coverage and LOD need to be taken into consideration for ctDNA detection with ultra-deep targeted sequencing to monitor immunotherapy responsiveness.

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Frontiers in Oncology	1055968(1)~(14)
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.3389/fonc.2022.1055968	有
オープンアクセス	国際共著
オープンアクセスとしている（また、その予定である）	該当する

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International Journal of Molecular Sciences	11521～11521
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10.3390/ijms231911521	有
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10.3390/cancers14133275	有
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10.21037/tlcr-21-981	有
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掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/cas.15304	査読の有無 有
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2．論文標題 Ultradeep targeted sequencing of circulating tumor DNA in plasma of early and advanced breast cancer	5．発行年 2020年
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〔図書〕　計0件

〔産業財産権〕

〔その他〕

－

6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関