Application of liquid biopsy-based targeted capture sequencing analysis to improve the precision treatment of non-small cell lung cancer by tyrosine kinase inhibitors

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Appendix S1-Methods

Testing *EGFR* variants in cfDNA by qPCR

Cobas *EGFR* Mutation Test v2 (Roche Diagnostics, QC, Canada) a real-time qPCR-based assay, was used for testing *EGFR* variants in plasma ctDNA from NSCLC patients. It is an in vitro diagnostic product that detects known mutations in the exons 18, 19, 20, and 21 of *EGFR* through PCR amplification. The Cobas cfDNA Sample Preparation Kit (Roche Diagnostics) was used for the extraction of cfDNA from 2 mL plasma samples. Briefly, plasma samples were treated with a mixture of protease K and lysis/binding buffer for releasing cfDNA. After binding to the glass fiber filter, cfDNA was purified through the serial of washing steps by using the provided washing buffer. Plasma cfDNA was then collected into the provided elusion buffer. The extracted plasma cfDNA was used for *EGFR* testing by the Cobas EGFR Mutation Test kit v2 (Roche Diagnostics). Three qPCR amplification detection systems were prepared for separately testing different *EGFR* variants present in one plasma cfDNA sample. Following the manufacturer's instructions, the qPCR detection of target *EGFR* variants in cfDNA was performed on the Cobas z 480 analyzer (Roche Diagnostics).

Testing pan-cancer gene mutations in cfDNA by NGS

AVENIO ctDNA expanded kit (Roche Diagnostics) that includes the reagents for sequencing test and the post-sequencing analysis software was used for testing somatic mutations in the cfDNA of NSCLC patients. The AVENIO ctDNA expanded kit (Roche Diagnostics) is a hybridization capture sequencing-based 77 genes pan-cancer assay, and target tumour biomarker genes covered by the panel are listed in table S1.

AVENIO cfDNA Isolation Kit (Roche Diagnostics) was used to extract cfDNA from plasma according to the user's manual. With a high pure extender assembly (HPEA)

unit, the cfDNA extraction kit was used to isolate cfDNA from 4 mL (2-5 mL) plasma samples. The quality of extracted plasma cfDNA samples was checked before library preparation. Agilent High Sensitivity DNA Analysis Kit (Agilent Technologies, CA, USA) was used to determine the length distributions of different cfDNA fragments for assessing the genomic DNA contamination. The concentration of cfDNA was also tested by using the Qubit® dsDNA HS Assay Kit on the Qubit 3.0 fluorometer (ThermoFisher Scientific, CA, USA).

After cfDNA extraction, the AVENIO ctDNA Expanded Kit (Roche Diagnostics) was used to prepare the sequencing library based on a hybridization capture-based technology. Briefly, with 10-50 ng input of cfDNA, the first step was adapter ligation. After post-ligation cleanup, PCR was performed to amplify the adapter-ligated cfDNA template. Hybridization was performed by using biotin-labeled probes to specifically capture the target genes fragments which were then enriched through binding to streptavidin beads. The expanded panel included in this kit was used for identifying and characterizing 77 genes (e.g. *BRAF*, *EGFR*, *PIK3CA*, *et al*.) that are associated with solid tumors (Table S1). After post-hybridization washes, a second PCR was performed to amplify the enriched library. The quality of the enriched library was assessed again by the Agilent High Sensitivity DNA Analysis Kit (Agilent Technologies) and the Qubit® dsDNA HS Assay Kit on the Qubit 3.0 fluorometer (ThermoFisher Scientific). With 50 ng input cfDNA, the typical concentration of enriched library is expected from 0.5 ng/μL to 30 ng/μL. The ideal peak size of the library is approximately 300 bp in the Bioanalyzer detection profile.

Prepared libraries were sequenced on the NextSeq 500 500/550 High Output Kit V2 (300 cycles) on Illumina NextSeq sequencing platform according to manufacturer's instructions, (Illumina, CA, USA). A 15% PhiX Sequencing Control V3 (Illumina) pike-in sequencing control was included. AVENIO ctDNA Analysis Software (Roche Diagnostics) was used for sequencing data analysis and generating diagnostic reports. Specifically, the AVENIO ctDNA Analysis Software uses integrated digital error suppression (iDES) strategies, combining molecular bar codes within silico error suppression techniques to call variants (1). According to the manufacture's introduction, the limit of detection of this kit has been estimated to reach down to 0.1%, and it has also been reported that the sequencing kit can detect mutant allele fraction down to 0.02% (2), which is consistent with the results from this study. By using the high-quality control process that is the iDES strategies, combining molecular bar codes within silico error suppression techniques, there is no cut-off value available for different called gene variants. The mutant allele fraction that is lower than 0.1% can still be reported with high confidence. The run metrics of this study are shown in figure S2 and table S2.

Testing *EGFR* mutations in cfDNA by MassArray

The MassARRAY detection system is based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technology, It is a sensitive method for testing low allele frequency biomarker gene variants (3). A clinically validated UltraSEEK Lung Cancer Panel (Agena Bioscience, CA, USA) was the reference method in this study.

QIAamp Circulating Nucleic Acid Kit (Qiagen, CA, USA) was used for plasma cfDNA extraction. Briefly, purified cfDNA from 4 mL of plasma samples were prepared

through four steps (lysis, binding, washing, and elution) of extraction procedures. The Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific) was used for testing the concentration of extracted plasma cfDNA. Using 10 ng input cfDNA, a multiplex PCR was performed to amplify target genes in cfDNA, and followed by a mutation-specific single based extension reaction for the MassArray UltraSEEK Lung Cancer Panel (Agena Bioscience), according to the manufacturer's instructions. The extension products were enriched through a streptavidin-biotin interaction-based hybridization capture process and then transferred to a SpectroCHIP Array (Agena Bioscience) for the following detection through the MassArray Analyzer (Agena Bioscience). Generally, the enriched extension products (DNA sequences) were ionized and accelerated into a flight tube towards a detector. The extended probes were separated based on the differences of time-of-flight needed, and a mass spectrum for each sample was generated with relative intensity on the y-axis and mass/charge on the x-axis. The acquired data were further processed by the MassARRAY Typer (Agena Bioscience) software for generating a detailed detection report of the detected gene variants.

Appendix S2-Figures and tables

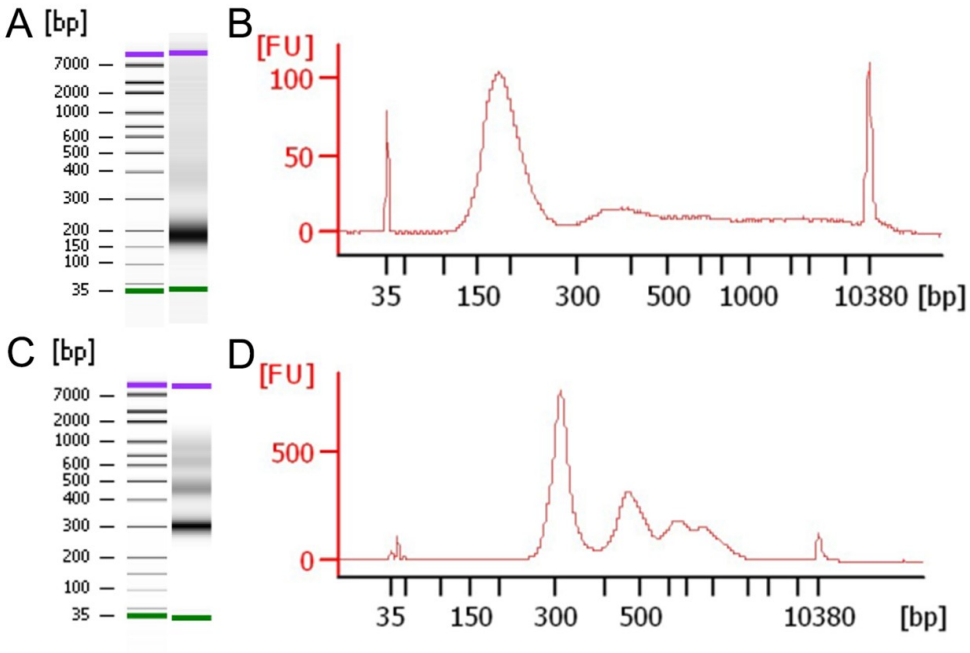

Figure S1. Electrophoresis files of typical good quality cfDNA (A, B) and enriched library (C, D) tested by the high-sensitivity DNA assay of Agilent Bioanalyzer.

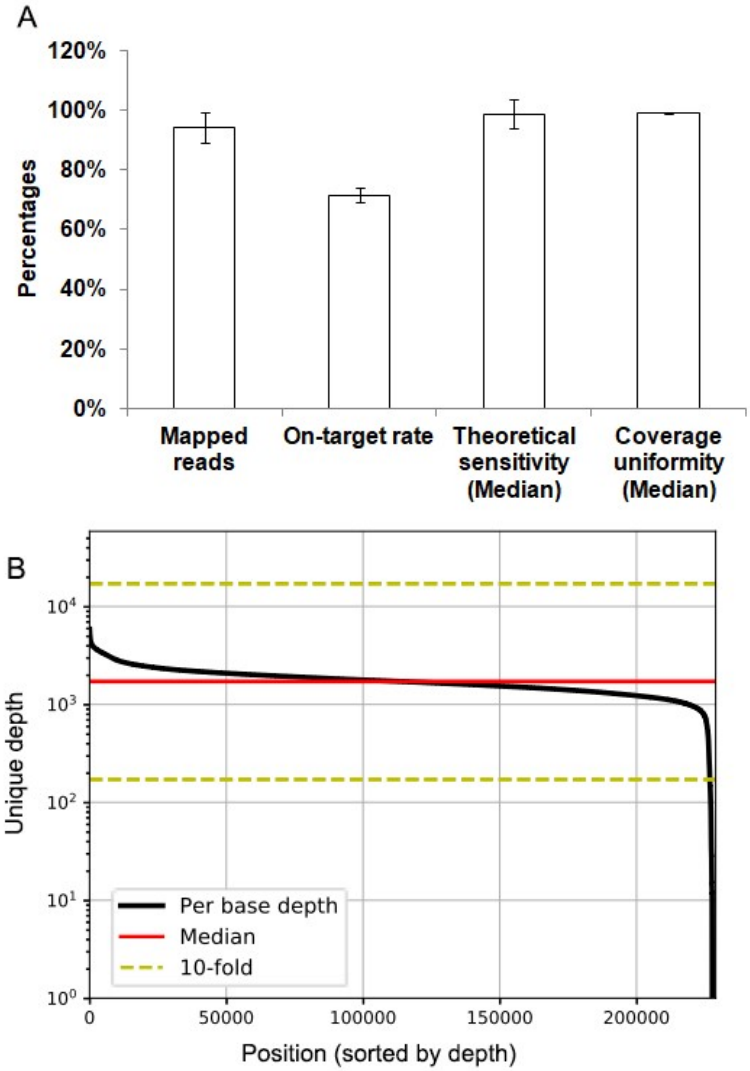

Figure S2. Performance of testing tumour biomarkers in cfDNA by NGS-based Avenio expanded panel. A: Average of the total mapped reads, on-target rate, theoretical sensitivity and coverage uniformity of the NGS assay for testing plasma cfDNA samples. B: Typical coverage uniformity for each base of the cfDNA tested by the NGS assay.

Table S1. Target tumour biomarker genes covered by the Avenio Expand Panel

List of target genes
ABL1 AKT1 AKT2 ALK APC AR ARAF BRAF BRCA1 BRCA2 CCND1 CCND2 CCND3 CD274 CDK4 CDK6 CDKN2A CSF1R CTNNB1 DDR2 DPYD EGFR ERBB2 ESR1 EZH2 FBXW7 FGFR1 FGFR2 FGFR3 FLT1 FLT3 FLT4 GATA3 GNA11 GNAQ GNAS IDH1 IDH2 JAK2 JAK3 KDR KEAP1 KIT KRAS MAP2K1 MAP2K2 MET MLH1 MSH2 MSH6 MTOR NF2 NFE2L2 NRAS NTRK1 PDCD1LG2 PDGFRA PDGFRB PIK3CA PIK3R1 PMS2 PTCH1 PTEN RAF1 RB1 RET RNF43 ROS1 SMAD4 SMO STK11 TERT TP53 TSC1 TSC2 UGT1A1 VHL

Table S2. The metrics of testing tumour biomarker genes in cfDNA by the Avenio panel

	Number of read pairs (M)	Sequencing depth (Median)	Unique depth (Median)	Error rate	Fragment length (bp) (Median)
Average	25	9004	2870	1.14E-05	175
Standard Deviation	5	1543	1094	5.33E-06	6

Table S3. Results of testing *EGFR* from plasma cfDNA by NGS, MassARRAY and qPCR methods

Mutations	Variants	NGS-based Avenio Expanded Panel	MassARRAY-based UltraSEEK Lung Panel	qPCR-based Cobas *EGFR* Mutation Test
EGFR sensitizing mutations	Exon 18 p.G719A	4.2% (1/24)	4.3% (1/23)	8.3% (1/12)
	Exno 20 p.S768I	4.2% (1/24)	4.3% (1/23)	8.3% (1/12)
	Exon 21 p.L858R	20.8% (5/24)	17.4% (4/23)	16.7% (2/12)
	Exon 21 p.L861Q	8.3% (2/24)	8.7% (2/23)	8.3% (1/12)
	Exon 19 Dels	33.3% (8/24)	26.1% (6/23)	41.7% (5/12)
TKIs resistant *EGFR* mutation	Exon 20 p.T790M	33.3% (8/24)	34.8% (8/23)	25.0% (3/12)

Table S4. List of *EGFR* variants showed mismatched testing results by different assays

Mutations	Variants	NGS-based Avenio Expanded Panel		MassARRAY-based UltraSEEK Lung Panel	qPCR-based Cobas *EGFR* Mutation Test
		VAF	CNV (Copies/mL)	Test result	Test result
EGFR sensitizing mutations	Exon 21 p.L858R	0.17%	1.79	Negative	Positive
	Exon 19 Dels	21.00%	1590	Inconclusive	NA
	Exon 19 Dels	0.11%	89.1	Negative	NA
	Exon 19 Dels	0.00%	0	Negative	Positive
TKIs resistant *EGFR* mutation	Exon 20 p.T790M	0.42%	4.45	Negative	Negative
	Exon 20 p.T790M	0.00%	0	Positive	NA

References

1. Newman AM, Lovejoy AF, Klass DM, Kurtz DM, Chabon JJ, Scherer F, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol. 2016;34(5):547-55.

2. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014;20(5):548-54.

3. Sutton BC, Birse RT, Maggert K, Ray T, Hobbs J, Ezenekwe A, et al. Assessment of common somatic mutations of EGFR, KRAS, BRAF, NRAS in pulmonary non-small cell carcinoma using iPLEX(R) HS, a new highly sensitive assay for the MassARRAY(R) System. PLoS One. 2017;12(9):e0183715.