Establishment and application of a method of next generation sequencing of 285 genes in lung cancer based on Ion-Proton platform

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Background: The development of "precision medicine" needs a novel genetic screening and diagnostic technique for clinical detection. This study aims to establish a method for highly parallel multiplexed detection of genetic mutations in Chinese lung cancer samples through testing 285 genes by customized next generation sequencing (NGS) on Ion-Proton platform.

Methods: We reviewed the related literature and collected data of genomic alteration that occurred in lung cancer. We identified 285 target genes closely related to the pathogenesis, drug resistance, and metastasis of lung cancer. Targeted hybridization probes were designed using SureDesign software. The detection method was established by analyzing four cell lines and 13 lung cancer specimens which had been validated through Sanger sequencing. The sensitivity and specificity of the proposed method were preliminarily evaluated by comparisons with the Sanger sequencing and a LungCarta mutation-detection method.

Results: The proposed method was able to detect mutations of 285 genes in lung cancer cell lines and clinical lung cancer specimens. The reads, mapped reads, on target, mean depth and uniformity were 14.90 ± 4.37 ($\times 10^6$), $98.68\% \pm 0.61\%$, $60.49\% \pm 10.72\%$, 714.42 ± 264.13 and $90.51\% \pm 6.91\%$, respectively. The detected mutation result of cell lines was consistent with the observations on previously reported mutations, and the congruence rate was 100%. The proposed method can detect single nucleotide polymorphism (SNP), InDel, Fusion and copy number variation (CNV). The complete congruence rate of detected result of specimens between the proposed method and Sanger sequencing, LungCarta mutation-detection method, immunohistochemistry (IHC), real-time polymerase chain reaction (RT-PCR) method were all 100% regarding mutations in common genes like *EGFR*, *KRAS*, or fusions of *ALK, RET*, etc. In addition, NFE2L3_p.Ser511_Pro513del, ERBB2_E770delinsEAYVM, MET_S701N, PDGFRA_T674I, TP53_G245V, TP53_V274A, TP53_A276F, TP53_G334L, TP53_R337L and TP53_Y220C mutations were detected only through the proposed method. The proposed method can detect mutations from blood, this detection result was consistent with the cancer tissues of the same clinical lung cancer patient.

Conclusions: The proposed Ion-Proton technology-based NGS method can detect genetic mutations in Chinese lung cancer patients. Therefore, the proposed method could be used to detect mutations in other cancer tissues and plasma, which needs further validation.

Keywords: Lung cancer; next generation sequencing (NGS); Ion-Proton; mutation

Introduction

Clinical oncology has already entered the era of personalized medicine or precision medicine, which needs right drug for the right patient at right time especially in targetable cancers harboring driver genes like *EGFR*, *ALK* and *ROS1*, etc. (1,2). Since the proposal of "precision medicine" plan in 2015 by US president Obama, next generation sequencing (NGS) develops quickly and has been applied extensively as a novel genetic screening and diagnostic technique for clinical detection. Its genetic characteristics for disease detection have become an important part of modern precision medicine (3-5). NGS has become an effective and acceptable method for clinical gene detection (6,7). Although it is still in the early stage of clinical application for the diagnosis and treatment of tumors (8-10), its continuous innovation had accelerated the people's cognition on genetics markers and the molecular mechanisms of diseases. Genome alterations play a significant role in disease development and progression a management by predictive and biomarkers, identifying clinical decision making of targeted therapy. The initial sample of NGS can be genomic DNA (gDNA) or circulating free DNA (cfDNA). Liquid biopsy refers to targetable alterations or other biomarkers using circulating material like cfDNA, CTC and CSF, etc. (11,12). Given that many clinically relevant biomarkers continue to be identified, and targeted drugs are developed for personalized treatment, multigene mutation screening will be a requirement in routine clinical practice (13). Genetic mutations can be detected by Sanger sequencing, gene chips, super ARMS, digital polymerase chain reaction (PCR), FISH and immunohistochemistry (IHC). However, these method presents several limitations. For instance, all the methods can only detect genetic mutations that have been found of very few numbers of genes. A large number of genetic variants that may be related to the disease can not be detected. Therefore, a multigene detection method that is suitable for Chinese lung cancer patients should be established.

Briefly, the motivations of developing this new method are as follows: (I) NGS can simultaneously interrogate multiple gene mutation loci, which are screened for in-routine clinical practice. (II) Tissue specimens and nucleic acids are not enough to used for an analysis of multiple genes one after another. However, NGS method can reduces the amount of the clinical sample used. (III) Some novel molecular targets of Chinese lung cancer patients can be enrolled in the new method. (IV) The NGS method can detect a variety of genetic variants, such as single nucleotide polymorphism (SNP), InDel, Fusion and copy number variation (CNV).

We developed this customized NGS to detected a total of 285 cancer related genes and applied to cell lines and clinical specimens for the validation of its performance.

We present the following article in accordance with the STREGA reporting checklist (available at http://dx.doi.org/10.21037/tcr-19-2855).

Methods

Cancer cell lines, cancer tissues and patient selection

We used four NSCLC cell lines (i.e., H1650, H1975, A549 and H1299) that were selected from the cell bank of Guangdong Lung Cancer Institute (GLCI) for LoD (limit of detection) analysis of serial dilution of gDNA of these cells at 1:1, 1:5, 1:10 and 1:100. Twenty-four tumor tissues and 1 plasma was collected from the GLCI of Guangdong General Hospital between January 2015 and December 2016. All tissues samples were stored at –80 ℃ after being frozen in liquid nitrogen, were routinely assessed by pathologists to ensure that >20% tumor contend in each sample to be tested by NGS. The trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Guangdong Provincial People's Hospital [No. GDREC2015360H(R2), GDREC2016316H(R1)]. All patients were well informed and signed the informed consent.

Reagents and instruments

The following materials were used in this study: QIAGEN QlAampDNA Mini Kit (Qiagen, Valencia, Germany); QIAGEN QlAampBlood Mini Kit (Qiagen, Valencia, Germany); Ion Xpress Plus Fragment Library Kit (Life Technologies, New York, USA); Agencourt AMPure XP Kit (Beckman, USA); H2O (Sigma-Aldrich, USA); Thermo

NanoDrop 1000 (Thermo, MA, USA); SureSelect Target Enrichment System for Sequencing on Ion-Proton (Agilent Technologies, USA) ; SureSelect TE Reagent Kit, PTN (Agilent Technologies, USA); Herculase II Fusion DNA Polymerase kit (Agilent Technologies, USA); Ion OneTouch 2 System (Life Technologies, New York, USA); Qubit 2.0 (Life Technologies, New York, USA); Ion PI Template OT2 200 Kit v2 (Life Technologies, New York, USA); Ion PI Sequencing 200 Kit v2 (Life Technologies, New York, USA); ABI 3730xl Sequencing Machine (Life Technologies, New York, USA); and PCR machine (Life Technologies, New York, USA); QIAxcel Bioanalyzer (Qiagen, Valencia, Germany); ABI Ion-Proton Sequencing Machine (Life Technologies, New York, USA).

Design of a panel of targeted 285 genes

Selection of related driver genes in lung cancer
Search for the latest literatures by retrieving the PubMed database, comparing with the information of gene mutation in databases like DrugBank, COSMIC, dbSNP, OMIM, ClinVar, 5,000 exomes and 1,000 genomes. A total of 285 genes related to lung cancer pathogenesis, chemoradiotherapy, targeted drug resistance and metastasis were determined and included as a set for probe library design and target sequencing.

Probe Library design for hybrid selection
The targeted hybridization probes library of 285 genes were designed using SureDesign software web site based on genome hg19/GRCh37 (https://earray.chem.agilent.com/suredesign/index.htm). Runned the software, introduced the 285 gene list and adjusted the parameters such as databases, target region, region extension and allow synonyms. The total length of target capture sequence was 0.967 Mb. All the 4,700 exon loci of the 285 genes were included in the probe design kit. Each probe kit contains 16 tests as requested by ourselves. The designed gene hybridization probe was synthesized by the Agilent company.

gDNA purification and quantification

The gDNA was isolated from each tissue sample (tumor content more than 20%) with the QIAGEN QIAampDNA Mini Kit and the circulating DNA was isolated from 4 mL plasma with the QIAGEN QIAampBlood Mini Kit. DNA quantitative analysis was finished by using the Qubit analyzer.

NGS library construction

NGS libraries were prepared from plasma DNA and tumor and cell line gDNA. For patient plasma DNA, 112 ng DNA were used for library construction without additional fragmentation. For tumor, and cell line gDNA, 200–1,000 ng DNA was sheared prior to library construction with Ion Xpress™ Plus Fragment Library Kit for 150 bp fragments. The NGS libraries were constructed using the Ion Xpress Plus Fragment Library Kit (Life Technologies, New York, USA). The cleanup steps was using the Agencourt AMPure XP Kit (Beckman, USA). Ligation was performed for 15 min at 25 ℃ and 5 min at 72 ℃ using Ion Xpress Barcode Adapters. Single-step size selection was performed by adding 140 µL (1.4X) of Agencourt AMPure XP beads to enrich for ligated DNA fragments. The ligated fragments were then amplified using Herculase II Fusion DNA Polymerase (Agilent Technologies, USA) and 9–11 PCR cycles, depending on input DNA mass. Library purity and concentration was assessed by spectrophotometer (Qubit 2.0) and qPCR (KAPA Biosystems), respectively. Fragment length was determined on a QIAxcel Bioanalyzer using the QIAxcel DNA screening Kit (Qiagen).

Hybrid capture selection of target regions and NGS

SureSelect Target Enrichment System for Sequencing on Ion-Proton was used according to the manufacturer's protocol. One library was included in a single capture hybridization. Following hybrid selection, the captured DNA fragments were amplified with 9 to 11 cycles of PCR using 5× Herculase II Rxn Buffer and 2 µL SureSelect PTN PCR primer mix and 1 µL Herculase II Fusion DNA Polymerase in 50 µL reactions. Multiplexed libraries were mixed by 5 libraries with 20 µL of 12 pM and were sequenced using Ion PI Sequencing 200 Kit v2 runs on the Ion-Proton.

Data analysis and interpretation

Upon sequence completion, the sequencing data were mapped to the hg19 reference genome with the Suite software (Life Technologies, version 5.0.2), and mutations were Called with the Variant Caller software (Life Technologies, version 5.0.2.1), and the VCF files was produced at the same time. The VCF files was uploaded to the Ion Reporter cloud analysis platform (Life Technologies, https://ionreporter.lifetechnologies.com), and gene mutation informations were annotated by

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ABL1	BCOR	CSF1R	ERCC5	GATA2	KMT2B	MYCN	PIK3CA	SDHAF2	TBL1XR1
ABL2	BLM	CTCF	EXT1	GATA3	KRAS	MYD88	PIK3CG	SDHB	TBX3
ACVR1B	BMPR1A	CTNNB1	EXT2	GNA11	LIFR	NAV3	PIK3R1	SDHC	TET2
ACVR2A	BRAF	CUL3	EZH2	GNAQ	LMO1	NBN	PMS1	SDHD	TGFBR2
AIP	BRCA1	CYLD	FAM123B	GNAS	LRP1B	NCOA3	PMS2	SETBP1	TLR4
AJUBA	BRCA2	DAXX	FANCA	GPC3	LRRK2	NCOR1	POLQ	SETD2	TMEM127
AKT1	BRIP1	DDB2	FANCB	H3F3A	LTK	NF1	PPP2R1A	SF3B1	TNFAIP3
AKT2	BUB1B	DDR1	FANCC	H3F3C	MAP2K1	NF2	PRDM1	SIN3A	TP53
AKT3	CARD11	DDR2	FANCD2	HGF	MAP2K4	NFE2L2	PRF1	SKP2	TRAF7
ALK	CASP8	DICER1	FANCE	HIST1H1C	MAP3K1	NFE2L3	PRKAR1A	SLC38A3	TSC1
APC	CBFB	DIS3L2	FANCF	HIST1H2BD	MAPK8IP1	NKX2-1	PRX	SLX4	TSC2
AR	CBL	DNMT1	FANCG	HIST1H3B	MAX	NOTCH1	PTCH1	SMAD2	TSHR
ARHGAP35	CCND1	DNMT3A	FANCI	HLA-A	MDM2	NOTCH2	PTEN	SMAD4	TSHZ2
ARID1A	CDC73	EGFR	FANCL	HNF1A	MDM4	NPM1	PTPN11	SMARCA4	TSHZ3
ARID1B	CDH1	EGR3	FANCM	HRAS	MECOM	NRAS	PTPRD	SMARCB1	TYK2
ARID2	CDK12	EIF4A2	FBXW7	IDH1	MED12	NRG1	RAD21	SMC1A	U2AF1
ARID5B	CDK4	ELF3	FGFR1	IDH2	MEN1	NSD1	RAD51C	SMC3	USP9X
ASCL4	CDKN1A	EP300	FGFR2	IGF1R	MET	NTRK1	RAD51D	SMO	VEZF1
ASXL1	CDKN1B	EPCAM	FGFR3	IKZF1	MLH1	NTRK2	RASA1	SOCS1	VHL
ATM	CDKN1C	EPHA3	FGFR4	INHBA	MLL2	NTRK3	RB1	SOX17	WRN
ATR	CDKN2A	EPHA5	FH	JAK1	MLL3	PAK3	RECQL4	SOX2	WT1
ATRX	CDKN2C	EPHB6	FLCN	JAK2	MPL	PALB2	RET	SOX9	XPA
AXIN1	CEBPA	EPPK1	FLT3	JAK3	MSH2	PAX5	RHBDF2	SPOP	XPC
AXIN2	CEP57	ERBB2	FOXA1	KDM5C	MSH6	PBRM1	RNF43	SRC	ZMYND10
B2M	CHEK2	ERBB3	FOXA2	KDM6A	MST1R	PCBP1	ROS1	SRSF2	
B4GALT3	CIC	ERBB4	FOXL2	KDR	mTOR	PDGFRA	RPL22	STAG2	
BAP1	CREBBP	ERCC2	FOXP1	KEAP1	MUTYH	PDYN	RPL5	STK11	
BCL2	CRIPAK	ERCC3	FUBP1	KIT	MYC	PHF6	RUNX1	SUFU	
BCL2L11	CRLF2	ERCC4	GATA1	KLF4	MYCL1	PHOX2B	SBDS	TAF1	

Figure 1 A panel of 285 genes for the capture system.

the Ion Reporter with a series of filters. The variant filters include minimum quality, minimum coverage, maximum strand bias, maximum variant signal shift and so on. Integrative Genomics Viewer (IGV) software was applied by researchers to annotate the gene mutation information by comparing with the databases DrugBank, COSMIC, dbSNP, OMIM, ClinVar, 5,000 exomes and 1,000 genomes.

Results

Selection of 285 genes as a testing panel

We check the cancer gene census through literatures and

make a list of 285 genes including those common genes in lung cancer to be tested by NGS. We tested 4,700 regions in these 285 target genes, which were closely related to the pathogenesis, drug resistance, and metastasis of lung cancer and associated with relevant transduction pathways. The targeted 285 genes are listed in *Figure 1*.

NGS-related technical indicators

The proposed method was able to detect mutations of 285 genes in lung cancer cell lines and clinical lung cancer specimens. The reads, mapped reads, on target, mean depth and uniformity were 14.90±4.37 (×10^6), 98.68%±0.61%,
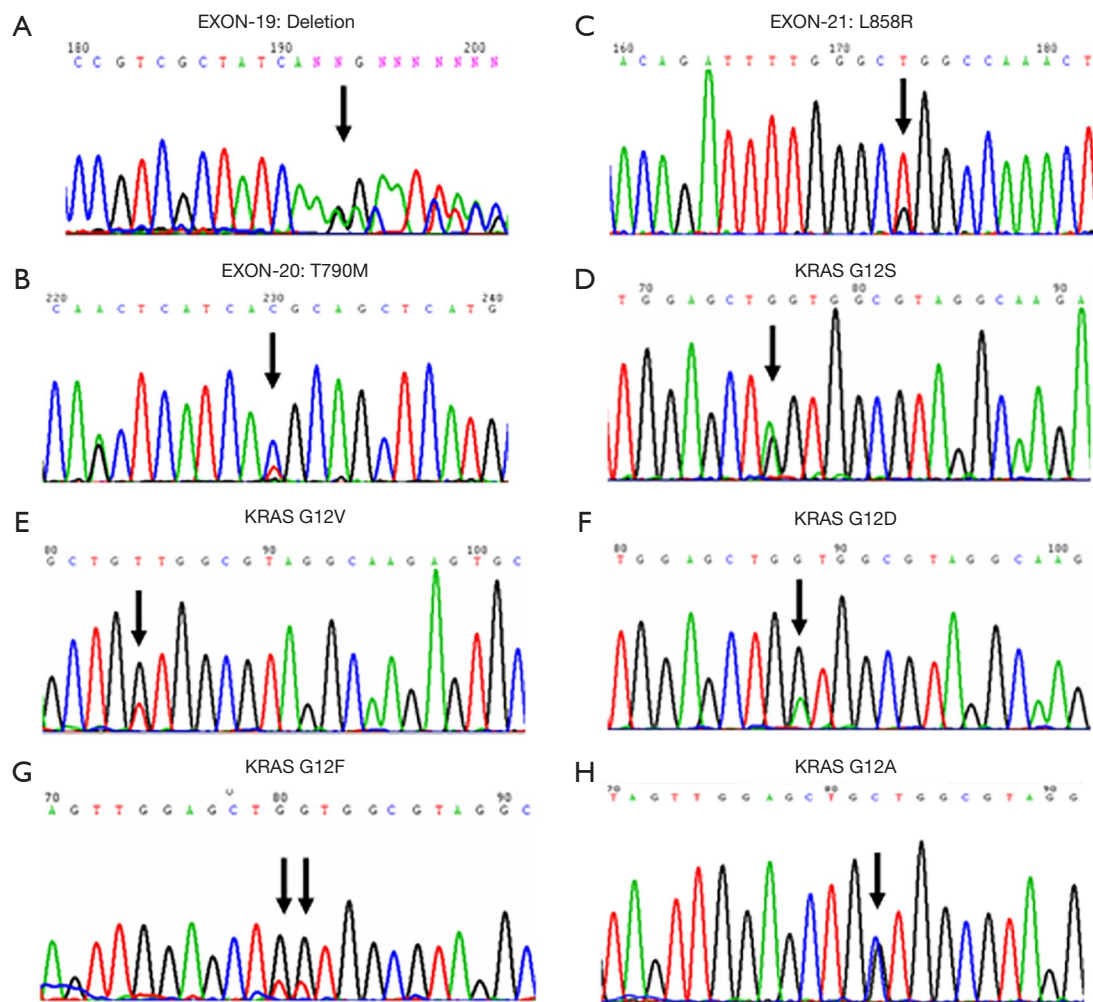
Figure 2 Chromatographs of Sanger sequencing of genes *EGFR* and *KRAS*. (A) *EGFR* exon-19 deletion; (B) *EGFR* T790M point mutation; (C) *EGFR* L858R point mutation; (D) *KRAS* G12S point mutation; (E) *KRAS* G12V point mutation; (F) *KRAS* G12D point mutation; (G) *KRAS*_G12F point mutation; (H) *KRAS*_G12A point mutation.

60.49%±10.72%, 714.42±264.13 and 90.51%±6.91%, respectively.

Establishment and validation of the customized NGS test in cell lines

The detection method used with the SureSelect target enrichment system for sequencing kit was established by analyzing four cell lines of lung cancer. The results from the newly established method were consistent with the previously reported mutations in cell lines. All the results were confirmed by Sanger sequencing, and the sequencing results are shown in *Figure 2*. The detailed mutation sites within genes and proteins are listed in *Table 1*. For the serial

dilution of gDNA of these four cell lines, mutations were successfully detected as shown in *Table S1*.

Comparison of the customized NGS test and Sanger sequencing in lung cancer specimens

A total of 13 lung cancer tissue specimens were detected by the established method and Sanger sequencing. This method was validated through comparison by the Sanger sequencing. The results show that established method can detected all the mutations analyzed by the Sanger sequencing. ERBB2_E770delinsEAYVM, MET_S701N, PDGFRA_T674I, TP53_G245V, TP53_V274A, TP53_A276F, TP53_G334L, TP53_R337L and TP53_Y220C

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mutations were detected only through the established method. All the mutations results are shown in *Table 2*. All the Sanger sequencing results and established method results are shown in *Figures 2,3*, respectively.

Comparison of the customized NGS test with LungCarta method in lung cancer patients

Five lung cancer tissue specimens were detected by the established method used the SureSelect target enrichment system for sequencing kit and LungCarta method. The results show that established method can detected all the mutations analyzed by the LungCarta method. The results

of the established method and the LungCarta method were shown in *Table 2*. However, a p.Ser511_Pro513del mutation in *NFE2L3* gene of K1736T was detected only through the established method (*Figure 4*).

Comparison of established method and IHC/real-time PCR (RT-PCR) method in lung cancer specimens

For ALK and RET fusion testing, five lung cancer tissue specimens were detected by the established method and IHC/RT-PCR method. The congruence rate of detect result of specimens between the established method and IHC method was 100%. The results are shown in *Table 2* (*Figure 5*).

Table 1 The main mutations in cell lines were detected by established method

Cell line	Documented by ATCC	Mutations detected by established method
H1650	EGFR_ Exon19 deletion	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)
H1975	EGFR_L858R; EGFR_T790M	EGFR_L858R (c.2573T>G); EGFR_T790M (c.2369C>T)
A549	KRAS mutation	KRAS_G12S (c.34G>A)
H1299	EGFR/ALK/KRAS negative	No mutation

Table 2 The main mutations in lung cancer tissues were detected by established method and validated using Sanger sequencing, LungCarta and IHC/RT-PCR

Sample ID	Mutations detected by established method	MAF, %	Mutations detected by Sanger method	Mutations detected by LungCarta	Fusion proteins or mRNA tested by IHC/RT-PCR method
1162	EGFR_L858R (c.2573T>G)	6.8	EGFR_L858R	–	–
	EGFR_T790M (c.2369C>T)	11.6			
29001	KRAS_G12V (c.35G>T)	9.6	EGFR_T790M	–	–
29003	KRAS_G12F (c.34_35delGGinsTT)	41.2	KRAS_G12V	–	–
	TP53_G245V (c.734G>T)	42.8			
29005	EGFR_L858R (c.2573T>G)	38.4	KRAS_G12F	–	–
	TP53_V274A (c.821T>C)	54.9			
29006	EGFR_L858R (c.2573T>G)	15.5	EGFR_L858R	–	–
	TP53_A276F (c.826_827delGCinsTT)	–			
	ERBB2_E770delinsEAYVM (c.2310_2311insGCATACGTGATG)	16.2			
29014	KRAS_G12A (c.35G>C)	47.8	EGFR_L858R	–	–
29015	EGFR_L858R (c.2573T>G)	16.9	KRAS_G12A	–	–
	EGFR_T790M (c.2369C>T)	19.1			

Table 2 (*continued*)

Table 2 (*continued*)

Sample ID	Mutations detected by established method	MAF, %	Mutations detected by Sanger method	Mutations detected by LungCarta	Fusion proteins or mRNA tested by IHC/RT-PCR method
29017	KRAS_G12D (c.35G>A)	29.4	EGFR_L858R	–	–
	MET_S701N (c.2102G>A)	9.6			
	PDGFRA_T674I (c.2021C>T)	8.5			
29018	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)	15.2	EGFR_T790M	–	–
	EGFR_T790M (c.2369C>T)	11.6			
29029	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)	18.2	KRAS_G12D	–	–
	TP53_G334L (c.1000_1001delGGinsTT)	25.6			
29035	EGFR_p.Ser752_Ile759del (c.2253_2276delATCTCCGAAAGCCAACAAGGAAAT)	49.7	EGFR_Exon19 del	–	–
29036	EGFR_L858R (c.2573T>G)	3.3	–	–	–
	TP53_R337L (c.1010G>T)	25.7			
29037	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)	41.8	EGFR_T790M	–	–
	EGFR_T790M (c.2369C>T)	11.4			
	TP53_Y220C (c.659A>G)	53.6			
22840	EGFR_L858R (c.2573T>G)	32.9	–	EGFR_L858R; EGFR_ A289V PIK3CA_ p.E542K	–
	EGFR_ A289V (c.866C>T)	38.6			
	PIK3CA_ p.E542K (c.1624G>A)	49.5			
K1736T	EGFR_L858R (c.2573T>G)	24.9	–	EGFR_L858R MET_N375S	–
	MET_N375S (c.1124A>G)	18.6			
	NFE2L3_p.Ser511_Pro513del (c.1529_1537delCTTCTGAAC)	12.3			
K1744T	EGFR_p.Glu746_Ala750del (c.2236_2250del15)	71.76	–	EGFR_Exon19 del	–
K1745T	No mutation	–	–	No mutation	–
K1746T	No mutation	–	–	No mutation	–
1215	EML4-ALK	11.04	–	–	ALK fusion (IHC)
33071	EML4-ALK	6.4	–	–	ALK fusion (IHC)
22968	EML4-ALK	5.7	–	–	ALK fusion (IHC)
17001	KIF5B-RET	7.7	–	–	KIF5B-RET (RT-PCR)
1146	KIF5B-RET	12.9	–	–	KIF5B-RET (RT-PCR)

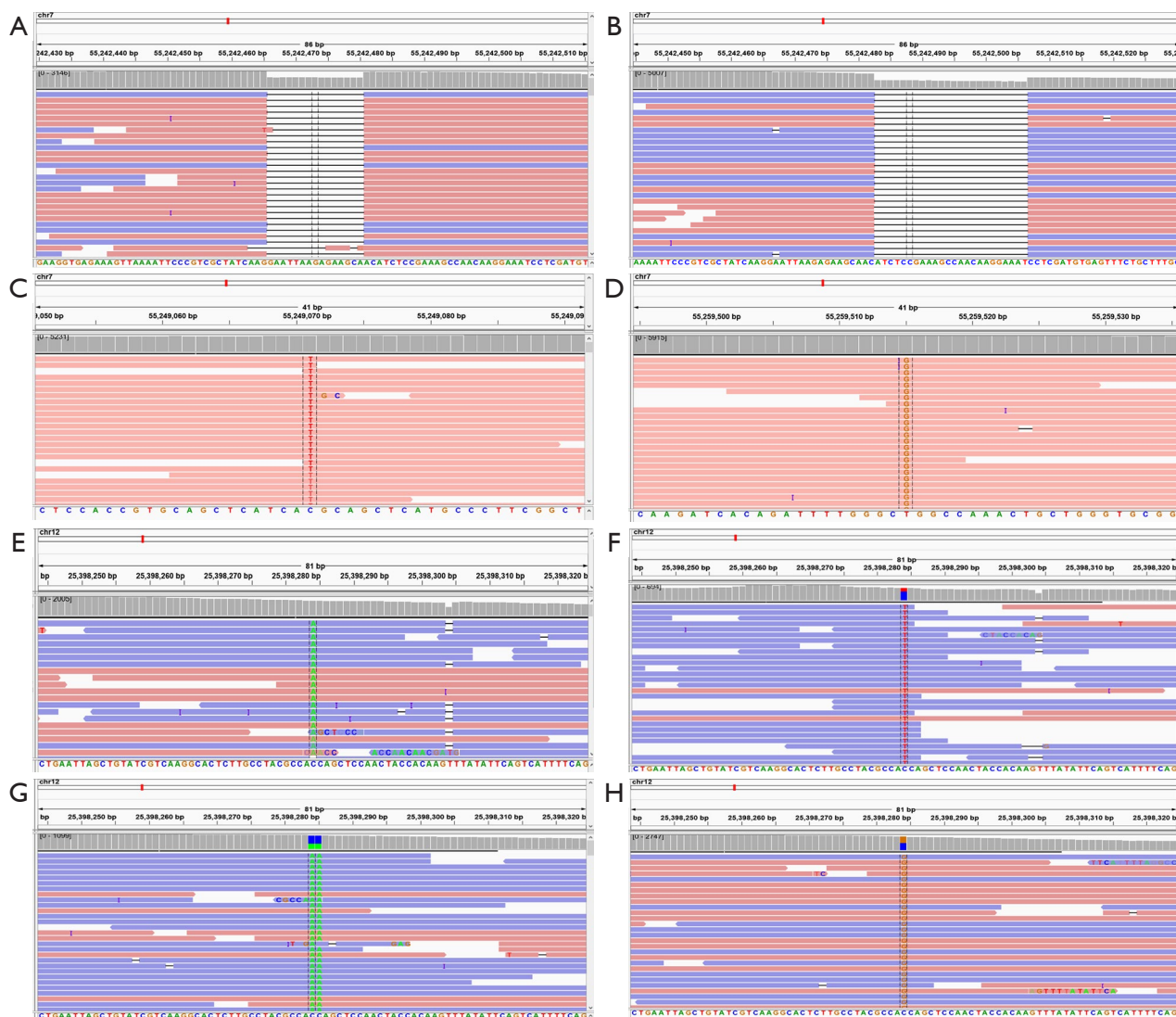MAF, minor allele frequency; IHC, immunohistochemistry; RT-PCR, real-time polymerase chain reaction.

Figure 3 IGV graphs of established method of *EGFR* and *KRAS*. (A) *EGFR* exon-19 deletion (p.Glu746_Ala750del); (B) *EGFR* exon-19 deletion (p.Ser752_Ile759del); (C) *EGFR* T790M point mutation. (D) *EGFR* L858R point mutation. (E) *KRAS* G12V point mutation; (F) *KRAS* G12D point mutation; (G) *KRAS*_G12F point mutation; (H) *KRAS*_G12A point mutation. IGV, Integrative Genomics Viewer.

NGS method detected mutations in cancer tissues and plasma in a representative patient

Cancer tissue and blood from a special patient were detected by the established method. The detected results showed that the mutation information of the patient's tissue samples were identical to those in the blood sample. EGFR_T790M and Glu746_Ala750 del (c.2236_2250delGAATTAAGAGAAGCA) and TP53_Y220C (c.659A>G) mutations were detected in cancer tissues and blood. The results are shown in *Table 3*.

Discussion

Single-gene detection methods for the molecular classification of lung cancer mainly include Quantitative PCR, Sanger sequencing, fluorescence *in situ* hybridization, amplification refractory mutation system, digital PCR and IHC. Multiplex genetic mutation-detection method include MassARRAY and gene chip. These methods can only detect the known gene mutations, and they are also expensive and time consuming. Many studies have shown tumors was complex, that tumors are not only associated with single
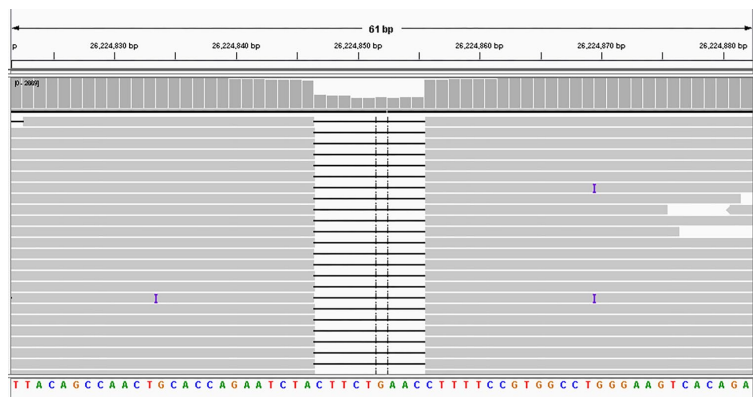
Figure 4 IGV graph of established method of NFE2L3 p.Ser511_Pro513del mutation. IGV, Integrative Genomics Viewer.
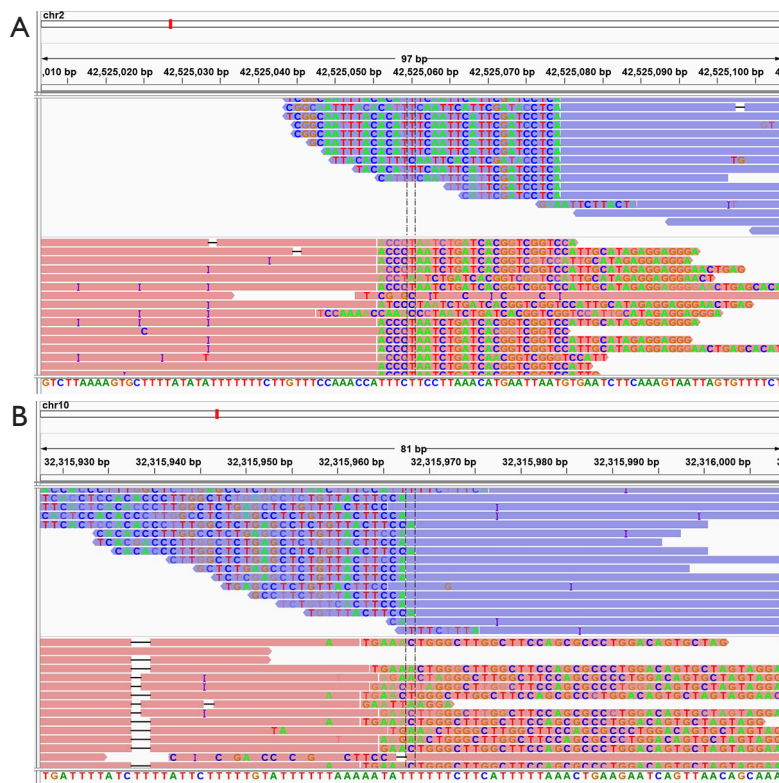

Figure 5 IGV graph of established method of EML4-ALK fusion and KIF5B-RET fusion. (A) EML4-ALK fusion; (B) KIF5B-RET fusion. IGV, Integrative Genomics Viewer.

driven gene mutations, but also associated with double or even multiple mutations (14). Considering the limitations of single-gene detection techniques and the complexity and unknowability of tumor driver genes, we need to establish a multiplex genetic mutation-detection method for lung cancer in clinical practice and translational medicine, Such as NGS. NGS has become an effective and acceptable method for clinical gene detection and has been an important molecular diagnostic tool for precision oncology (15,16).

According to the review of the related literature and data on lung cancer treatments, 285 genes were enrolled into

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Table 3 Mutations in cancer tissue and circulating plasma were detected by established method in a representative patient

Patient	Mutations	
	Cancer tissue (29037)	Plasma (29037-1)
29037	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)
	EGFR_T790M (c.2369C>T)	EGFR_T790M (c.2369C>T)
	TP53_ Y220C (c.659A>G)	TP53_ Y220C (c.659A>G)

the targeted panel. These genes not only include the eight genes which can using the related TKI of FDA-approved, such as EGFR, ALK, KRAS, ROS1, ERBB2, MET, RET and BRAF, but also include many tumor suppressors, such as *TP53* gene. So, the established method can not only detect common L858R mutations in EGFR gene, but also detect recent reports of C1156Y, L1196M, F1174L, and G1269S mutations in ALK gene, which was recently reported as the resistance mutations following crizotinib treatment in lung cancer (17,18). The study found established method could detect the mutations verified by Sanger sequencing, also could detect the rare mutation, such as the p.Ser511_Pro513del mutation in *NFE2L3* gene, that cannot be detected by the Sanger sequencing and MassARRAY platform. The results suggest that Compared to Sanger sequencing and MassARRAY, the established method has a high detection sensitivity and specificity. It is well known that the detection sensitivity of MassARRAY and Sanger were 1–5% (19) and 5–10% (20), respectively. So, the sensitivity of the established method should be less than 1%, that will be verified by the next phase of the experiment.

The proposed 285 genes-targeted NGS method demonstrates the following advantages: (I) the establishment NGS method can minimizes the weaknesses of traditional single-gene tests, such as time-consuming and tedious procedures and high cost. (II) Given the NGS library was built by a large number of fragmented DNA, theoretically, not only fresh tissue specimens but also formalin-fixed paraffin-embedded (FFPE) specimens, biopsy samples, pleural effusion and plasma could be used for effective detection, which needs further validation. (III) The establishment method can not only detect the known gene mutations, but also can detect the unknowability gene mutations.

Limitations of the study included relatively small sample size, absence of association analysis with clinical

therapy. Thus, was provided about how the results of this analysis might affect clinical outcomes. Our study did not systematically measure the turn-around time required for NGS experiments and to annotate, interpret and report NGS results. Future studies will be necessary to address these limitations.

In summary, we have successfully established a customized novel NGS detection method based on Ion-Proton platform, which are able to detect those actionable driver gene mutations, and may significantly reduce the costs of biomarker tests and thus demonstrates a promising potential for personalized treatment.

Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at available at http://dx.doi.org/10.21037/tcr-19-2855

Data Sharing Statement: Available at http://dx.doi. org/10.21037/tcr-19-2855

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.

org/10.21037/tcr-19-2855). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Guangdong Provincial People's Hospital [No. GDREC2015360H(R2), GDREC2016316H(R1)] and informed consent was taken from all the patients.

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Table S1 Sequencing results of serial dilutions of gDNA from cell lines by established method

Cell line	Serial dilution	Specific mutations	MAF, %
H1650	1:1	EGFR_p.Glu746_Ala750del (c.2236_2250delGAATTAAGAGAAGCA)	48.9
	1:10		6.3
	1:100		0.8
H1975	1:1	EGFR_L858R (c.2573T>G)/EGFR_T790M (c.2369C>T)	56.8/49.3
	1:10		6.8/5.9
	1:100		1.1/0.9
A549	1:1	KRAS_G12S (c.34G>A)	63.5
	1:10		8.2
	1:100		1.2
H1299	1:1	No mutation	–
	1:10		–
	1:100		–

gDNA, genomic DNA; MAF, minor allele frequency.