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SHORT COMMUNICATION

Molecular profiling of genetic alterations in selected non-small cell lung cancer on formalin-fixed paraffin-embedded tissue specimens

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ABSTRACT

Lung cancer arises as a result of multiple genetic alterations and environmental influences such as cigarette smoking, radiation and air pollution. Molecular classification of these alterations may help in the development of individualised anticancer therapies. In this study, Ion Torrent technology was used to sequence genomic material extracted from formalin-fixed paraffin-embedded (FFPE) tumours. Multiple genetic variants were identified in each tumour sample. About 65% of the identified mutations occurred in the epidermal growth factor receptor (EGFR) and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) genes. Results from this study demonstrate the feasibility of using FFPE material in next-generation sequencing (NGS). In conclusion, specific key mutations associated with human cancers are identified.

***Keywords**: NGS; Ion Torrent; FFPE; mutations; lung cancer*

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Introduction

Multiple genetic alterations have been identified in the tumorigenesis of lung cancer[1]. Studies have shown that cancers carry multiple genetic and epigenetic changes, indicating activation of oncogenes and inactivation of tumour suppressor genes during the process of tumorigenesis leading to the development of lung cancer[2]. Treatment options vary for non-small-cell lung cancer (NSCLC) harbouring different mutations; therefore, the characterization of these genetic alterations is important in the development of personalised therapy of NSCLC. Information of genetic alterations in human cancers has made it possible to classify tumours according to the driver mutations they harbour with regard to the molecular pathways activated or inactivated by these genetic alterations, and serve as predictive biomarkers, thus influencing the type of therapeutic agents to be used in management[3].

In recent years, a revolution in sequencing technologies had taken place. Next-generation sequencing (NGS) has now been widely applied in various research settings such as whole genome sequencing, targeted deoxyribonucleic acid (DNA) sequencing, ribonucleic acid (RNA) sequencing, and epigenomics[4]. Major discoveries from NGS have reported to provide better insights into the complexities of the NSCLC genome. For example, lung tumours have been shown to harbour multiple genetic and epigenetic aberrations (>20 per tumour)[5].

This paper describes the use of Ion Torrent (Life Technologies) NGS platform on selected formalin-fixed paraffin-embedded (FFPE) tumour from NSCLC patients and the results obtained from NGS analysis.

Material and Methods

A total of nine NSCLC patients' samples with known epidermal growth factor receptor (EGFR) mutation status were selected for this study. The results of EGFR mutation testing from other methods—Rotor Gene (Qiagen), Cobas (Roche) and direct sequencing—were collected (**Table 1**). This study was approved by Sime Darby independent ethics committee (IRB reference number: 201102.3), by Universiti Putra Malaysia ethics committee (Reference number: UPM/FPSK/PADS/T7-MJKEtikaPer/F01 (JSB_Aug(11)03)) and National Institute of Health, Malaysia.

Genomic DNA was extracted from FFPE tissue sections using QIAamp DNA FFPE Tissue Kit (Qiagen) according to manufacturer's instructions.

Table 1. Distribution of genetic alterations identified.

No	Gene	No. of samples with mutation	Chromosome	Position	Coding DNA sequence	Amino acid changes	Type of variant
1	AKT1	3	14	105246407	c.175+18C>T	Intron (non-coding)	Intronic variant
2	APC	8	5	112175770	c.4479G>A	p.Thr1493=	Synonymous
3	CTNNB1	1	3	41266097	c.94G>A	p.Asp32Asn	Missense
4	EGFR	1	7	55242464	c.2235_2249del15	p.E746_A750delELREA	Deletion
5	EGFR	2	7	55242465	c.2235_2244del10	p.Lys745_Ala750del	Deletion
6	EGFR	1	7	55242466	c.2236_2238del3	p.Glu746del	Deletion
7	EGFR	1	7	55242470	c.2240_2250del11	p.Leu747_Pro753delinsSer	Deletion
8	EGFR	1	7	55242478	c.2248G>C	p.Ala750Pro	Missense
9	EGFR	1	7	55242479	c.2249C>G	p.Ala750Gly	Missense
10	EGFR	1	7	55242481	c.2251A>G	p.Thr751Ala	Missense
11	EGFR	1	7	55242483	c.2253A>T	p.Thr751=	Synonymous
12	EGFR	1	7	55242490	c.2260A>G	p.Lys754Glu	Missense
13	EGFR	6	7	55249063	c.2361G>A	p.Gln787=	Synonymous
14	EGFR	3	7	55259515	c.2573T>G	p.Leu858Arg	Missense
15	FGFR2	1	10	123274774	c.1144T>C	p.Cys382Arg	Missense
16	FGFR3	9	4	1807894	c.1617G>A	p.Thr539=	Synonymous
17	KDR	7	4	55972974	c.1416A>T	p.Gln472His	Missense
18	KIT	1	4	55593464	c.1621A>C	p.Met541Leu	Missense
19	MET	2	7	116339672	c.534C>T	p.Ser178=	Synonymous
20	MET	2	7	116340262	c.1124A>G	p.Asn375Ser	Missense
21	PIK3CA	1	3	178952085	c.3140A>T	p.His1047Leu	Missense
22	PIK3CA	1	3	178952088	c.3140A>T	p.His1047Leu	Missense
23	PIK3CA	1	3	178952090	c.3145G>T	p.Gly1049Cys	Missense
24	PIK3CA	1	3	178952091	c.3146G>C	p.Gly1049Ala	Missense
25	PIK3CA	4	3	178938877	c.2119G>A	p.Glu707Lys	Missense
26	PIK3CA	1	3	178936093	c.1635G>T	p.Glu545Asp	Missense
27	PDGFRA	9	4	55141055	c.1701A>G	p.Pro567=	Synonymous
28	PDGFRA	3	4	55152040	c.2472C>T	p.Val824=	Synonymous
29	RET	7	10	43613843	c.2307G>T	p.Leu769=	Synonymous
30	SMO	2	7	128845088	c.582A>G	p.Glu194=	Synonymous
31	TP53	1	17	7578535	c.395T>C	p.Leu132=	Synonymous
32	TP53	1	17	7577538	c.743G>A	p.Arg248Gln	Missense
Total	14 genes	85					

The extracted DNA was quantified using Qubit dsDNA BR Assay Kit (Life Technologies, CA, USA). Samples were sequenced using the Ion AmpliSeq™ Cancer Panel (Life Technologies), which was designed to detect 739 COSMIC mutations from 46 cancer-related genes, according to manufacturer's protocols. Briefly, 10 ng of genomic DNA were used to perform polymerase chain reaction (PCR) amplification with the following cycling conditions: one cycle of 99 °C for 2 min, 17 cycles of 99 °C for 15 s, 60 °C for 4 min, and a hold at 4 °C. Barcoded amplicon libraries were constructed using the Ion Xpress™ Barcode (Life Technologies) and PCR amplified using Platinum® PCR SuperMix High Fidelity and Library Amplification Primer Mix (Life Technologies). The cycling condition were as follows: one cycle of 98 °C for 2 min, 5 cycles of 98 °C for 15 s, 60 °C for 1 min, and a hold at 4 °C. PCR clean-up was then carried out using the Agencourt® AMPure® XP Reagent (Beckman Coulter, CA, USA). Purified library was subjected to a bioanalyzer HS assay to assess its quantity and quality. The amplicon libraries were then subjected for template preparation using the Ion OneTouch™ System (Life Technologies), followed by sequencing on the Personal Genome Machine (PGM) sequencer using Ion 316 chip (Life Technologies).

Data were analyzed using the Torrent Suite 3.0 software, and variants were called using the Variant Caller plug-in. All variant calls were reviewed manually using Integrative Genomics Viewer (version 2.3)[6] and was compared to the 1000 Genomes Project database[7]. For the analysis, only missense and somatic mutations were selected. Synonymous mutations that do not change the amino acid residues were excluded.

Results and Discussion

In the nine NSCLCs tumours, 85 alterations in 14 genes were identified (**Table 1**). At least five alterations were found to be present in all of the tumours (with a range of 5–10 alterations). Multiple genes were altered across the nine samples: *EGFR* (9), *PDGFRA* (9), *FGFR3* (8), *RET* (8), *APC* (8), *KDR* (7), *PIK3CA* (4), *AKT1* (3), *TP53* (2), *MET* (2) and *SMO* (2). *FGFR2*, *CTNNB1* and *KIT* were each altered in one tumor sample (**Figure 1**). Of the 85 gene alterations, there were 29 missense mutations, 48 synonymous mutations, 5 deletions and 3 intronic variants. Most of the mutations were found in the *EGFR* gene (12 of 34, or 35.3%) followed by *PIK3CA* (9 of 34, or 26.5%), *KDR* (7 of 34, or 20.6%), *MET* (2 of 34, or 5.9%), *FGFR2* (1 of 34, or 2.9%), *TP53* (1 of 34, or 2.9%), *CTNNB1* (1 of 34, or 2.9%) and *KIT* (1 of 34, or 2.9%).

Both *PIK3CA* and *EGFR* are components of the epidermal growth factor receptor signaling pathway- one of the key pathways that regulate growth, survival, proliferation, and differentiation in mammalian cells[8]. Major signalling pathways include the Ras/mitogen-activated protein kinase and PI3K/Akt pathways which link *EGFR* activation to cell proliferation and survival[9]. Genes in the EGFR signalling pathway are frequently found to be mutated in NSCLC and very seldom in other tumours. The prevalence of *EGFR* mutations is high among East Asians, with mutation rates ranging from 20% to 60%[10–14].

Besides *EGFR*, *PIK3CA* was reported as one of the most commonly mutated oncogenes found in human cancer, although the mutation rates are relatively low in NSCLC[9]. *PIK3CA* is an oncogene responsible for activating signalling cascades involved in cell growth, survival, proliferation, motility and morphology[15]. The *AKT* gene functions as a central node in cell signaling that regulates several processes, including cell survival, proliferation, and protein synthesis. Activating the *AKT* gene would result in cancer cell survival. Alterations in the *AKT1* gene decrease signaling through the pathway, and hence the reduction in cell survival signals.

Genetic variations in the genes encoding these molecules may regulate signaling through this pathway and result in variation in the development of toxicity or clinical outcomes following platinum-based therapy. A study by Pu *et al.*[16] showed that patients with single nucleotide polymorphism (SNP) in *PIK3CA* have a significantly increased risk of toxicity and that SNPs in *AKT1* would result in decreased risk for distant disease progression. In this study, sequence variants were found in both the *PIK3CA* and *AKT* gene. Aside from the increased risk of chemotherapy toxicity, genetic alterations in *PIK3CA* were reported to be the cause of over-activation of the PI3K-AKT pathway, which stimulates the malignant transformation of lung epithelial cells[16].

Other less frequently mutated genetic variations in this study include *KDR* mutation, which was reported to be associated with favourable overall survival in lung cancer[17], and *MET* mutation (N375S), which shows high rate of occurrence among East Asians and its ability to confer resistance to *MET* therapeutic inhibitor[18]. Besides, the fibroblast growth factors receptor *FGFR2*, a tumour suppressor gene which is responsible in the regulation of cellular growth, differentiation and angiogenesis, was reported to be amplified in

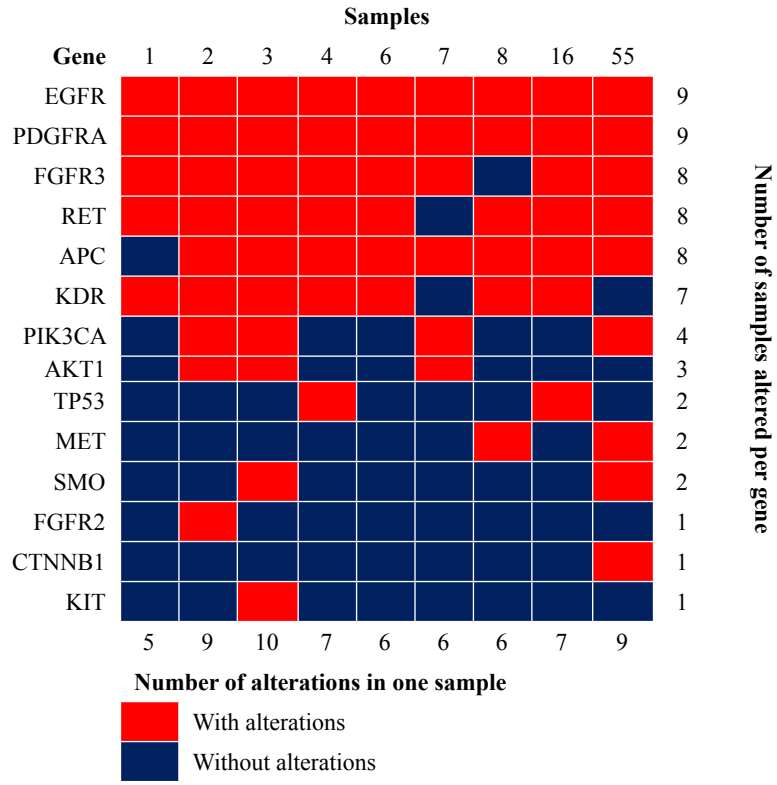

Figure 1. Genetic alterations observed across genes in nine NSCLC tumours. Tumours with and without alterations in the indicated genes are labelled in red and blue in the corresponding columns, respectively. The columns in the table denote samples, and the rows denote genes.

some breast cancer and its SNPs were found to be associated with an increased risk to the disease. Mutations in the *FGFR2* gene were also described in gastric cancer, lung cancer, breast cancer, ovarian cancer, and endometrial cancer[19,20]. Also, the *TP53*, a tumour suppressor gene which plays a role in regulating cell division and preventing tumour formation[21], was detected in this study. Nearly 50% of human cancers were reported to have inactivating mutation in the *TP53* gene[22]. A study by Kosaka *et al*. revealed that the incidence of *TP53* mutations is not associated with *EGFR* mutations[23].

In addition to *FGFR2*, mutations in *CTNNB1* and *KIT* were each found in a tumour sample in this study. The *CTNNB1* (β-catenin) gene is a component of the Wnt signalling pathway. Mutation in *CTNNB1* was found to be rare in lung cancer and was reported to be involved in the development of a subset of human lung cancer[24,25]. Meanwhile, mutations of *KIT* have been widely described in human cancers such as gastrointestinal stromal tumour (GIST) and melanoma. Tumour samples harbouring activating *KIT* mutations were reported to achieve significant response to *KIT* inhibitor imatinib (Gleevec®, Novartis)[26,27].

Conclusions

In summary, genetic alterations were successfully identified in genomic samples extracted from FFPE tumours. By profiling these genetic alterations, it is hoped that the information may contribute to the knowledge of the molecular basis of lung cancer, leading to the identification of potential therapeutic targets, and ultimately assist in physician's clinical decision-making.

Nevertheless, there were some limitations that need to be addressed in this study. These include the small study population of nine patients, which reduces the power of statistical tests. Also, this study involved results of *EGFR* mutation testing using other platforms (Rotor Gene, Cobas and direct sequencing). However, no definitive conclusion was drawn here because this study was not designed to compare between the testing methods. Additionally, the Ion AmpliSeq™ Cancer Panel was designed to detect mutations that occur within the "hotspot" regions, which is a drawback, in which mutations that falls out the region may go undetected.

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Authors' contributions

YK Cheah conceived and designed the project. TSY Ng performed the experiments, analyzed the data, and drafted the paper. YK Cheah and P Rajadurai gave critical evaluation of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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