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Silent genetic alterations identified by targeted next-generation sequencing in pheochromocytoma/paraganglioma: A clinicopathological correlations

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ABSTRACT

Aims: The goal of this pilot study was to develop a customized, cost-effective amplicon panel (Ampliseq) for target sequencing in a cohort of patients with sporadic phaeochromocytoma/paraganglioma.
Methods: Phaeochromocytoma/paragangliomas from 25 patients were analysed by targeted next-generation sequencing approach using an Ion Torrent PGM instrument. Primers for 15 target genes (*NF1, RET, VHL, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, MEN1, KIF1Bβ, EPAS1, CDKN2 & PHD2*) were designed using ion ampliseq designer. Ion Reporter software and Ingenuity® Variant Analysis™ software (www.ingenuity.com/variants) from Ingenuity Systems were used to analysis these results.
Results: Overall, 713 variants were identified. The variants identified from the Ion Reporter ranged from 64 to 161 per patient. Single nucleotide variants (SNV) were the most common. Further annotation with the help of Ingenuity variant analysis revealed 29 of these 713variants were deletions. Of these, six variants were non-pathogenic and four were likely to be pathogenic. The remaining 19 variants were of uncertain significance. The most frequently altered gene in the cohort was *KIF1B* followed by *NF1*. Novel *KIF1B* pathogenic variant c.3375 + 1G>A was identified. The mutation was noted in a patient with clinically confirmed neurofibromatosis. Chromosome 1 showed the presence of maximum number of variants.
Conclusions: Use of targeted next-generation sequencing is a sensitive method for the detecting genetic changes in patients with phaeochromocytoma/paraganglioma. The precise detection of these genetic changes helps in understanding the pathogenesis of these tumours.

1. Introduction

A neuroendocrine tumour that arises in the chromaffin cells in adrenal medulla is termed pheochromocytoma (PCC). Extra-adrenal tumours arising from chromaffin cells are paragangliomas (PGL) (Lam, 2015). The collective incidence of PCC and PGL is about one per 100,000–300,000 in the general population where PCC is the most frequent tumour and PGL are much rarer (0.5 per million) (Santos et al., 2014; Kirmani and Young, 1993). Currently, twenty-nine genes are known to be associated with pheochromocytoma and paraganglioma (*PHD2, VHL, SDHx, IDH, HIF2A, MDH2, FH, RET, NF1, KIF1Bβ, MAX, TMEM127, GDNF, H-RAS, K-RAS, GNAS, CDKN2A (p16), p53, BAP1, BRCA1&2, ATRX* and *KMT2D*) (Pillai et al., 2016). Overall, germline and somatic mutations in known PCC/PGL genes are present in 60% of tumours (Favier et al., 2015).

Advances in massive parallel sequencing technologies such as next-generation sequencing (NGS) have recently transformed the practice of DNA sequencing (Lenders et al., 2014; Toledo and Dahia, 2015). The new technique allows simultaneous sequencing of multiple genes in a single run at a much lower cost than conventional DNA-sequencing techniques (Toledo and Dahia, 2015). Thus, in recent years, a few studies have showed profiles of genetic changes in PCC/PGL using NGS (Burnichon et al., 2016). There is, however, a lack of studies on populations in the Asia-Pacific region. In this context, differential genetic and environmental backgrounds may affect which genes are most likely to be involved in pathogenesis of the tumours in the region.

The goal of this pilot study was to develop a customized, cost-effective amplicon panel (Ampliseq) for the complete and accurate sequencing of fifteen among the twenty-nine genes listed using NGS in patients with PCC/PCL from Asia-Pacific region. In addition, the study intends to

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obtain proof of concept of the sensitivity, specificity, and accuracy of this type of NGS procedure as well as to refine the analysis pipeline to achieve cost-effective results from the perspective of a routine laboratory set-up.

2. Materials and methods

2.1. Samples collection

The samples for this study were prospectively recruited from patients with phaeochromocytoma/paraganglioma resected by the authors in Australia (VL) and Hong Kong (CYL). All the 5 patients from Australia are of European decent (4 born in Australia and 1 born in New Zealand) whereas 20 patients from Hong Kong are ethnic Chinese. Ethical approval for this study was obtained from the Griffith University Human Research Ethics Committee (GU Ref Nos: MED/19/08/HREC and MSC/17/10/HREC). After clinical and pathological review, 25 tissues (22 formalin fixed paraffin embedded and 3 fresh frozen) were used in this study for DNA extraction. They were obtained from 25 patients (14 females; 11 males) diagnosed with PCC/PGLs (22PCC/3PGL). The mean age of the patients with PCC/PGL was 42(range 18–75 years).

2.2. DNA extraction from archived/fresh frozen tissues

The selected tissues were sectioned by microtome and stained for haematoxylin and eosin. The presence of tumour was confirmed. The non-tumour tissue in the section was identified and removed from further sections. They were then sectioned in 7 μm slices. The genomic DNA was extracted from these tissues using the QIAGEN Blood and Tissue kit (Qiagen, Redwood, CA, USA). Purification steps were done as previously reported (Gopalan et al., 2013). The DNA sample quantity and the purity of the nucleic acid samples were assessed using a Qubit 2.0 flourometer (Thermo Fisher Scientific, Freemont, CA, USA).

2.3. Ampliseq panel design and ion torrent PGM sequencing

A custom panel was designed with the help of the ampliseq designer online tool (https://www.ampliseq.com), which was employed to generate optimized primer designs compatible for formalin fixed paraffin embedded tissues. In the current study, primers were designed for fifteen genes (*NF1, RET, VHL, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, MEN1, KIF1Bβ, EPAS1, CDKN1B* and *PHD2*) present in the human reference genome (hg19). These genes were selected from the National Centre for biotechnology information (NCBI) databases of USA and those described in pathogenesis of pheochromocytoma and paraganglioma(Lam, 2015; Pillai et al., 2016). This design allowed analysis of 227 exons by the targeted sequencing of 806 amplicons.

The primer pairs were divided into two pools to optimize the coverage and multiplex polymerase chain reaction (PCR) conditions. The overall coverage of the design region was 98%. The amplicon-sequencing analyses benefited from the use of the Ion Ampliseq technology (Thermo Fisher Scientific) which uses very low input genomic DNA for a simple and fast library construction method for the affordable sequencing of specific human genes or genomic regions. Amplicon library preparation was performed with the Ion Ampliseq Library kit v2.0 using approximately 10 ng of DNA as advised by the manufacturer. The PCR cycling conditions were as follows: initial denaturation: 99 °C for 2 min, cycling: 21 cycles of 99 °C, 15 s and 60 °C, 4 min. PCR products were partially digested using FuPa reagent as instructed, followed by the ligation of barcoded sequencing adapters (Ion Xpress Barcode Adapters 1–16 kit; Life Technologies, Carlsbad, CA, USA). The samples were barcoded to allow the pooling of multiple patients DNA on the same sequencing chip. The final library was purified using Agencourt AM Pure XP magnetic beads (Beckman Coulter, Brea, CA, USA) and quantified using qPCR (Ion Library Quantitation kit) on a Step One qPCR machine (both from Life Technologies). The individual libraries were diluted to a final concentration of 100 pM and ten libraries were pooled and processed to library amplification on Ion Spheres using an Ion PGM Template OT2 200 kit. Unenriched libraries were quality-controlled using Ion Sphere quality control measurement on a Qubit instrument. Following library enrichment (Ion OneTouch ES), the library was processed for sequencing using the Ion Torrent Hi-Q sequencing chemistry and the barcoded libraries were loaded onto a single 316-v2 chip following the Ion PGM Hi-Q Sequencing Kit v2 manual.

2.4. Data analysis

The data from the sequencing runs were analysed using the Torrent Suite v4.0.2 analysis pipeline. Single-nucleotide polymorphisms (SNP), insertions, and deletions were identified across the targeted subset of the reference genome (hg19) using the analysis plug-in Torrent Variant Caller (v4.0-r76860), with the parameter settings optimized for germline high frequency variants and minimal false positive calls. The output variant call format (VCF) file was then annotated through Ion Reporter and with Ingenuity Variant Analysis software (www.ingenuity.com/variants) from Ingenuity systems (Qiagen, Hilden, Germany). SIFT (Sorting Intolerant From Tolerant) which uses sequence homology to predict whether an amino acid substitution will affect protein function and PolyPhen-2 (Polymorphism Phenotyping version 2) which is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations were used to classify the variants.

An allele frequency calculation - 1000 Genomes Frequency - was used to determine the frequency of the variants in the population.

3. Results

3.1. Genetic alterations

The technical assessments of the current NGS study are detailed in supplementary file. In this study, NGS was designed for archived paraffin embedded issues. Fifteen PCC/PGL susceptibility genes covered by 806 amplicons were used. Overall, 713 variants were identified in the ten patients; these variants identified from the Ion Reporter ranged from 13 to 162 per patient. Single nucleotide variants (SNV) were the most common genetic variants. Additional annotation of the 713 variants with the help of Ingenuity variant analysis revealed 257 variants of which 156 were common variants. Further annotation of the 156 variants revealed 29 different uncommon DNA variants in twelve genes (Table 1). Of these, four were classified as pathogenic or probably pathogenic, one in *KIF1B*, two in *NF1* and one in *SDHD* with the help of Ingenuity software. No genetic variants were observed in *SDHB*, *MAX* and *VHL* genes in PCC/PGL.

Among the 29 variants, 12 were noted to be present in chromosome1, while chromosome 17 showed the presence of seven variants. The most frequently altered gene in the current study cohort was *KIF1B* followed by *NF1*.

3.2. KIF1B mutation analysis in pheochromocytoma/paraganglioma

Among the 25 PCC/PGLs, three PCCs harboured *KIF1B* genetic alterations. In total, eight genetic variants were found in *KIF1B.* Of these, seven were missense variants and one was predicted to be pathogenic. Among the seven missense variants, six were of uncertain significance and one was non-pathogenic. The pathogenic variant was c.3375 + 1G>A. This variant decreased the splicing efficiency and its likely effect was altered exonic insertion leading to a frame shift effect. In addition, this variant was not found in the dbSNP databases nor could it be found in a PubMed search to best of our knowledge, making it a novel mutation.