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作品名稱 **Novel Approach to Screening Mutations Causing Retinoblastoma, a Childhood Cancer of Retina**

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Abstract

Novel Approach to Screening Mutations Causing Retinoblastoma, a Childhood Cancer of Retina

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Retinoblastoma (RB) is a childhood retinal cancer caused by mutations in the *RB1* gene. Molecular diagnosis is crucial for early detection and treatment. Current DNA diagnostic screening requires substantial amounts of tumour and blood samples. However current screening methods face the challenges of limited DNA templates from minute retinal tumours and too much blood samples drawn from young patients. In addition, the starting DNA template amount and quality are important to ensure confident detection of disease-causing mutations. As the majority of *RB1* mutations are unique and distributed throughout the *RB1* gene with no real hot spots, the entire gene needs to be thoroughly analysed.

This investigation proposes to enrich DNA samples using a whole genome amplification (WGA) step prior to *RB1* mutation screening by *RB1* gene-specific PCR amplification as well as high resolution melt (HRM) analysis and sequencing. It also identifies *RB1* mutations in two RB patients and explores whether WGA and saliva products can be a source of DNA templates for *RB1* analysis. In addition, this study was conducted based on the hypotheses that *RB1* mutations were the underlying cause of the disease in the two patients, and that the products from WGA could be used specifically for *RB1* gene analysis to overcome the constraint of insufficient DNA samples.

Two anonymised genomic DNA samples from two unrelated RB patients and five normal healthy DNA samples were used in this project. WGA kits were compared according to three criteria, namely amplification yield, product fragment size and whether DNA is amplifiable. Prior to and after amplification, the optical density of two normal samples was measured to determine the increase in DNA yield. The amplicons were subjected to gel electrophoresis to determine the product fragment size. Exons 6, 14 and 25 of the original and amplified samples undergone PCR, and were examined again using gel electrophoresis to ascertain that the amplicons were amplifiable. Mutation analysis using HRM was carried out with pre-existing primers for all 27 exons and the

promoter of *RB1*. Samples from patients were analysed against 83 saliva DNAs extracted using Oragene•DNA (OG-500) Kit.

REPLI-g was observed to produce higher yield and products of reliable fragment size. Single distinct bands were also seen for exons amplified using REPLI-g, indicating that REPLI-g is more accurate and suitable in the amplification of DNA. Abnormal melt profiles were obtained for exon 6 in RB477 and exon 14 in RB572 for HRM. These exons were sequenced to determine the exact mutation. Exon 6 was found to have a splice-site mutation g.607+1G>T, while a point mutation, g.1363C>T (p.Arg455X) was identified in exon 14.

Both the uses of saliva as a non-invasive DNA source and the WGA approach for enriching DNA sample for application in *RB1* gene analysis have never been reported for RB. Although HRM analysis has been used for other diseases, this is its first instance applied in work on *RB1* gene. In short, this report offers novel and promising approaches which would contribute significantly to the molecular analysis of mutations in RB.

Project Summary

Novel Approach to Screening Mutations Causing Retinoblastoma, a Childhood Cancer of Retina

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1. Background and Objective of Research

Molecular diagnosis of retinoblastoma (RB) is important as early diagnosis allows for earlier treatment and better health outcomes for patients. Furthermore, siblings of patients with hereditary RB are more susceptible to RB and need to be screened and detected early. Diagnosis of hereditary RB is also important for the patient as the individual is susceptible to other secondary cancers, and vigilance is required. Mutation screenings are currently done using DNA extracted from tumour and blood samples. Since retinal tumours are often tiny, the DNA extracted can often be too little since both pathological and DNA analyses have to be carried out. It is also difficult to draw blood from young patients. Although polymerase chain reaction (PCR) can be used to amplify the specific *RB1* gene for study, the starting template amount and quality is important to ensure confident detection of disease-causing mutations. As the majority of *RB1* mutations are unique and distributed throughout the *RB1* gene with no real hot spots, the entire gene needs to be thoroughly analysed.

This report proposes to enrich DNA samples using a whole genome amplification (WGA) step prior to *RB1* mutation screening by *RB1* gene-specific PCR amplification as well as high resolution melt (HRM) analysis and sequencing. Based on literature review, two WGA approaches were selected, namely, random fragmentation following universal random amplification using the GenomePlex kit (Sigma, USA), and multiple displacement amplification using the REPLI-g Midi (Qiagen, USA) kit. The performances of these two kits were compared experimentally and the better kit chosen to amplify DNA samples.

To identify mutations in the *RB1* gene, mutation screening will be carried out using HRM analysis which will detect aberrant melt profiles from a PCR product carrying a mutation due to the different melting temperature of mutant DNA sequences from the wild-type sequences of a normal PCR product. This will be confirmed by DNA sequencing. Since HRM screening requires comparisons with normal samples, DNA was extracted from saliva samples recruited from volunteers from our school who do not have any known medical history of RB or eye disease.

This investigation aims to investigate whether WGA and saliva products can be a source of DNA template for *RB1* analysis, and also to identify *RB1* germ-line mutations in two hereditary RB patients with bilateral eyes affected. This study is also based on the hypotheses that the *RB1* mutations are the underlying cause of disease in the two patients, and that the products from the whole genome amplification (WGA) can be used specifically for *RB1* gene analysis to overcome the constraints of insufficient DNA quantities.

2. Research Methods and Materials

DNA Samples

Two anonymised genomic DNA sample from two unrelated patients diagnosed with RB were provided to us for this study. In addition, genomic DNA samples from five healthy subjects were also given as normal controls. These samples were provided by the Department of Paediatrics, National University of Singapore from subjects previously collected with informed consent and IRB approval. DNA from saliva samples were obtained from 110 volunteers from River Valley High School and this study also had the permission of the IRB of the school.

***Whole Genome Amplification* (*WGA*)**

Whole genome amplification of samples of both RB patients were carried out using the GenomePlex kit (Sigma, USA) and REPLI-g kit (Qiagen, USA).

WGA Kits Comparison

Amplification yield by OD analysis

For DNA quantification, optical density of each sample was measured using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA) at 230nm, 260nm and 280nm.

To analyse if WGA amplified DNAs were amplifiable by PCR

Both original genomic DNA and WGA amplified samples were amplified using PCR conditions.

Gel electrophoresis analysis

6µl of WGA amplified DNA were subjected to agarose gel electrophoresis at 100 volts for half an hour to determine the product fragment size while 6µl of PCR products were similarly analysed on 2% agarose gel.

Saliva Samples and DNA Extractions

Saliva samples of 2ml were collected from 110 subjects using the Oragene•DNA (OG-500) Kit (Genotek, USA). DNA was extracted from 110 saliva samples according to the procedures from the manufacturer's PD-PR-006 protocol (Genotek, USA).

High Resolution Melt (HRM)

Mutation screening was conducted using primers in the laboratory for all 27 exons and the promoter of *RB1*. The samples of the patients were analysed against 83 normal saliva DNA samples. PCR reactions were carried out using 96-well plate on CFX96 real-time PCR detection system (Bio-Rad, USA) under optimised conditions. Melt analysis was carried out on the post-PCR products in the same plate. Using the Bio-Rad Precision Melt Analysis Software (Bio-Rad, USA), melt curves were then generated and plotted into difference curves.

3. Interpretation of Data, Results and Findings

WGA kits comparison

Amplification yield

Two DNA samples (Cb6019 and Cb6035) were used for this study. It was found that Cb6019 amplified by REPLI-g increased by 26 folds as compared to DNA amplified by GenomePlex which only increased by 10 folds. DNA in Cb6035 had a 22-fold increase when amplified with REPLI-g and only a nine-fold increase with GenomePlex. For both normal samples, REPLI-g was observed to amplify a higher DNA yield and it was deduced that this would be a better WGA kit for our template enrichment approach.

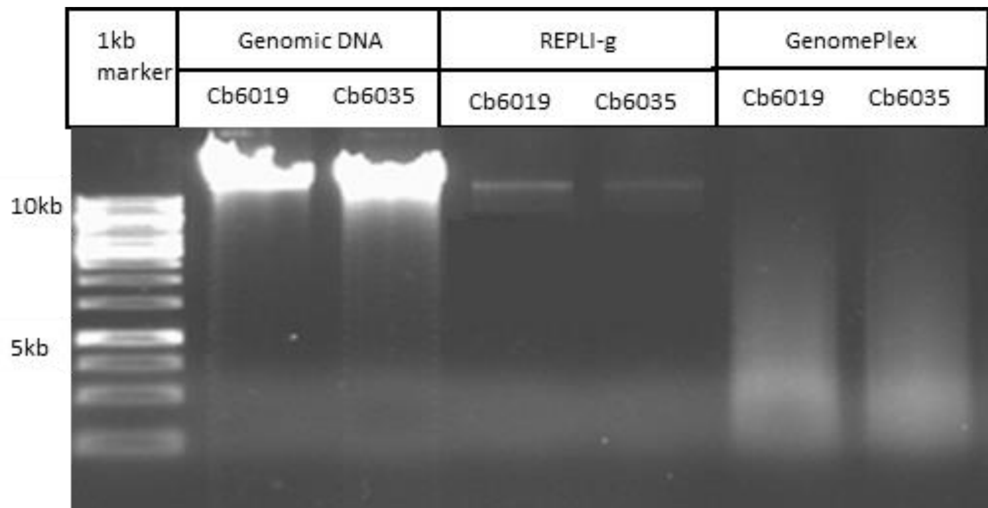

Figure 1: Gel electrophoresis of WGA amplified products

Product fragment size

Amplified products from REPLI-g showed bigger sized products than those from GenomePlex which had smears of smaller molecular sizes (Fig. 1).

Polymerase Chain Reaction (PCR)

WGA amplified DNAs from both kits could be amplified using primers for the *RB1* gene. Single distinct bands were observed for all exons amplified using REPLI-g. Multiple bands observed in

exon 25 of DNA amplified using GenomePlex could be due to multiple priming sites within the target sequence resulting from non-specific templates.

Our results suggest that REPLI-g amplified products have more uniform DNA yield of high-molecular-weight DNA. This is probably because of the methodology of WGA which uses alkaline denaturation of the templates.

Amplified Saliva Data

The optical density (260:280nm ratio) of all 110 saliva DNAs was measured.

Average of OD	Median of OD	Mode of OD	Highest OD	Lowest OD
1.79	1.80	1.80	2.01	1.57
Average Yield	Median Yield	Mode Yield	Highest Yield	Lowest Yield
6.41μg	5.56μg	5.14μg	29.3μg	3.68μg

Table 1: Average DNA quality and quantity parameters from 110 saliva extractions

High Resolution Melt Analysis

Aberrant melt profiles were observed in the two patient samples, namely for exon 6 in RB477 (Fig. 2) and exon 14 in RB572 (Fig. 3).

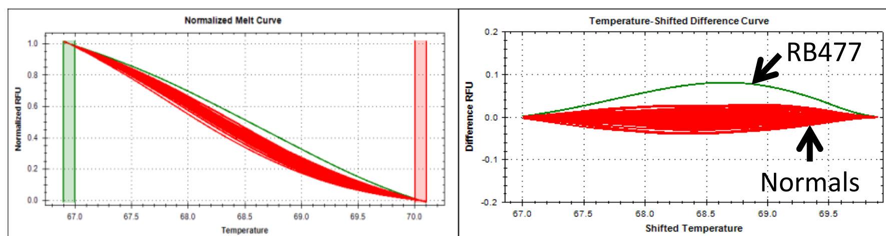

Figure 2: HRM analysis curves for exon 6 with RB477, RB 572 and 83 saliva samples

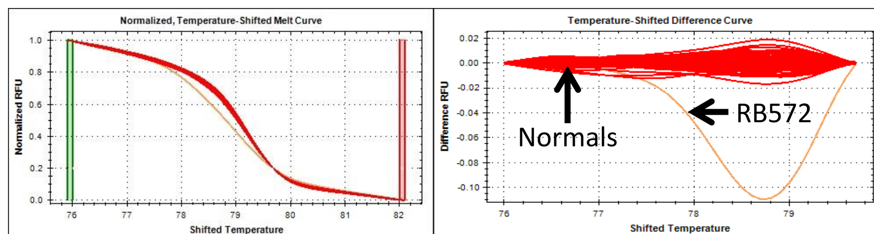

Figure 3: HRM analysis curves for exon 14 with RB477, RB 572 and 83 saliva samples

To confirm these mutations, exons 6 and 14 were further analysed against normal samples. Aberrant melt profiles were observed once again for exons 6 (Fig. 4) and 14 (Fig. 5). This confirmed that the aberrant curves detected in exons 6 and 14 were present only in the respective patients.

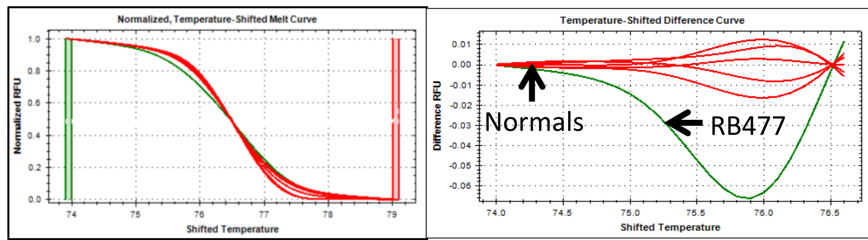

Figure 4: HRM analysis curves for exon 6 with RB 477 and 5 normal samples (NM113, NM115, NM118, NM121 and NM123)

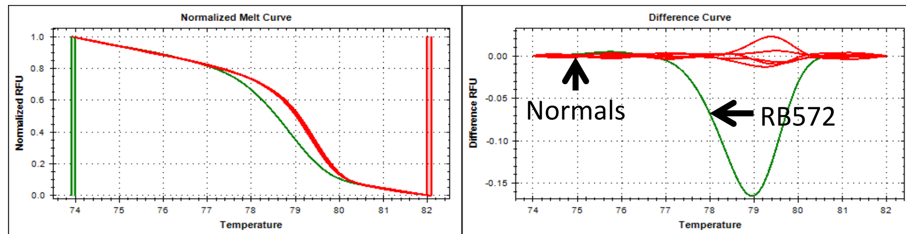

Figure 5: HRM analysis curves for exon 14 with RB 572 and 5 normal samples (NM113, NM115, NM118, NM121 and NM123)

DNA Sequencing

RB477 was found to carry a splice-site mutation c.607+1G>T in intron 6 splice site. The heterozygous G to T change in intron 6 alters the donor splice site and affects the ability of splice factors from recognising the splice site at the 5' end of intron 6, thus inhibiting proper splicing. This mutation possibly leads to exon skipping. An aberrant partially functional RB protein may be synthesised.

A nonsense mutation, c.1363C>T (p.Arg455X) was identified in exon 14 for RB572. The heterozygous C to T change caused an amino acid change from polar arginine to a stop codon affecting the A domain of the RB protein. The nonsense mutation causes a premature stop codon that is predicted to lead to a truncated non-functional protein.

4. Conclusions and Recommendations

Two *RB1* mutations c.607+1G>T and c.1363C>T were identified in this study and predicted to be pathogenic. Saliva has been shown to be a non-invasive DNA source for molecular analysis and that WGA can also be used to overcome limited DNA source from young patients. *RB1* gene sequences were successfully analysed from these sources. The uses of saliva DNA and WGA approach for enriching DNA source for *RB1* gene analysis has never been reported before for RB. Although HRM analysis has been used for other diseases, it has never been applied for *RB1* analysis. We believe that we have contributed towards novel approaches for mutation analysis of retinoblastoma patients. Future studies could include using saliva instead of blood samples from patients to screen for hereditary *RB1* mutations.

【評語】090021

1. This project screens the mutations causing Retinoblastoma.
2. By application DNA from ,the author found screen mutations is the DNA from sample.