#2330 High Throughput Clinical Testing of *RPGR* ORF15 in Patients with Inherited Retinal Dystrophies

John (PW) Chiang[1], Tina M. Lamey[2,3], Nicholas K. Wang[1], Jie Duan[1], Wei Zhou[4], Terri L. McLaren[2], Jennifer A. Thompson[2], Jonathan Ruddle[5], John N. De Roach[2,3]

[1]Molecular Vision Laboratory, [2]Australian Inherited Retinal Disease Registry and DNA Bank, [3]Centre for Ophthalmology and Visual Science, [4]Centrillion Technologies, [5]Royal Children's Hospital

Introduction

Retinitis pigmentosa (RP) is the most commonly diagnosed inherited retinal dystrophy[1]. Mutations in the highly repetitive, ORF15 region of *RPGR* account for roughly half of all X-linked RP cases[2], providing a key target for recently launched *RPGR* gene therapy trials[3]. Despite its significance, a robust and reliable high throughput method for the detection of ORF15 mutations has yet to be validated.

Here, we present the first clinically validated next-generation sequencing (NGS) method, complete with test accuracy and coverage data, for the detection of mutations in the difficult-to-sequence ORF15 region.

Methods and Materials

As part of a blind-test, 145 research samples previously tested by Sanger sequencing and 81 clinical samples (N=226) were evaluated using NGS of long-range PCR products fragmented with Illumina's Nextera library preparation kit initially, and Centrillion's OneTube technology as the final validated method. DNA fragments were analyzed using Agilent's DNA 1000 assay and sequencing was done on Illumina's MiSeq 2x150 or HiSeq2500 2x100. Data analysis and variant calling were performed using NextGENe by SoftGenetics. Duplication analysis was done using a custom, ORF15-specific, in-silico array covering 260,000 potential duplications.

Results

Using Nextera as the initial fragmentation method, 12 false-negatives, 1 false-positive, and 9 incorrectly called mutations were identified. 3 benign variants were also either incorrectly called or not detected. OneTube-NGS resulted in correct calls for 22 of the 25 previously discordant cases (Table 1) with the remaining 3 identified through secondary duplication analysis. In comparing fragmentation performance, OneTube library preparation yielded an improved average fragment length of 340bp compared to 600bp from Nextera.

Of the mutations identified, 65% are within the highly repetitive region of ORF15 (c.2184-3162) for which both Nextera and OneTube NGS show a relative lack of coverage. Controlling for average coverage of the entire exon (~35,000 reads), minimum coverage from OneTube NGS (~6800 reads) was significantly higher than from Nextera (~320 reads; Figure 1).

Discussion

Although many of the mutations missed by Nextera-NGS could be manually detected retrospectively, this blind-test highlights just how difficult it is to accurately and consistently identify mutations in ORF15 within a standardized pipeline. The increased sensitivity and specificity offered by OneTube-NGS can be attributed to the higher minimum coverages provided by more uniform fragmentation and confirms the importance of coverage depth in sequencing. While increased coverage resolved most of the incorrect callings, detection of large duplications within a region of such low sequence complexity required additional bioinformatics approaches (Figure 2). The final validated pipeline achieved complete concordance with Sanger results with a caveat of zygosity calling for duplications larger than ~50bp.

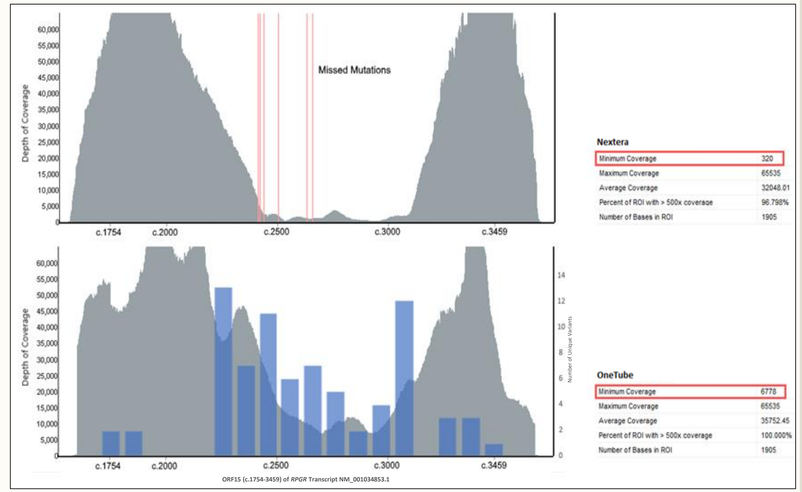

Figure 1. *RPGR* ORF15 mutation distribution and coverage curves from NGS of long-range PCR products fragmented with Nextera (top) and OneTube (bottom). Vertical red lines represent the position of missed mutations using Nextera (top). Blue bars represent the distribution of unique variants found (bottom; secondary y-axis). Note the considerable increase in minimum coverage using OneTube (red boxes). Coverage data from a representative sample.

Sample ID	Gender	Reason for testing	Sanger sequencing		Next-generation sequencing			
			Result	Zygosity	Nextera result	Zygosity	One-tube result	Zygosity
False-negative								
1343-2	F	Obligate carrier	c.2420_2435del	HET	Negative	N/A	c.2420_2435del	HET
1492-1	F	Possible carrier	c.2420_2435del	HET	Negative	N/A	Not tested†	N/A
1492-3	F	Obligate carrier	c.2420_2435del	HET	Negative	N/A	c.2420_2435del	HET
1700-1	F	Obligate carrier	c.2426_2427del	HET	Negative	N/A	c.2426_2427del	HET
1735-1	F	Obligate carrier	c.2501del	HET	Negative	N/A	c.2501del	HET
984-1	F	Obligate carrier	c.2635del	HET	Negative	N/A	c.2635del	HET
984-2	F	Obligate carrier	c.2635del	HET	Negative	N/A	c.2635del	HET
984-3	F	Obligate carrier	c.2635del	HET	Negative	N/A	c.2635del	HET
984-4	F	Obligate carrier	c.2635del	HET	Negative	N/A	c.2635del	HET
984-5	F	Obligate carrier	c.2635del	HET	Negative	N/A	c.2635del	HET
50-1	F	Obligate carrier	c.2426_2427del	HET	Negative	N/A	c.2426_2427del	HET
240-1	F	Obligate carrier	c.2426_2427del	HET	Negative	N/A	c.2426_2427del	HET
False-positive								
60-1	F	Possible carrier	Negative	N/A	c.2447del	HET	Negative	N/A
Mutations called incorrectly								
1343-1	M	Affected	c.2420_2435del	HEM	c.2424del	HET	c.2420_2435del	HEM
1492-2	M	Affected	c.2420_2435del	HEM	c.2423_2424del	HEM	c.2420_2435del	HEM
1049-1	M	Affected	c.2696_2715del	HEM	c.2714_2718del	HEM	c.2696_2715del	HEM
1049-1	M	Affected	c.2696_2715del	HEM	c.2714_2718del	HEM	c.2696_2715del	HEM
1049-2	F	Obligate carrier	c.2696_2715del	HET	c.2714_2718del	HET	c.2696_2715del	HET
1049-3	M	Affected	c.2696_2715del	HEM	c.2714_2718del	HET	Not tested†	N/A
1049-4	F	Possible carrier	c.2696_2715del	HET	c.2714_2715del	HET	c.2696_2715del	HET
1641-1	F	Obligate carrier	c.2362_2366del	HET	c.2358_2362del	HET	c.2362_2366del	HET
1789-1	M	Affected	c.2144_2216dup	HEM	c.2219_2220del	HET	c.2144_2216dup*	Cannot resolve for large duplications
Benign variants not detected or called incorrectly								
1049-1	M	Affected	c.2820_2840dup	HEM	c.2714_2718del	HET	c.2820_2840dup*	HEM
60-1	F	Possible carrier	c.2447_2661del	HET	c.2447del	HET	c.2447_2661del	HET
291-1	F	Possible carrier	c.2721_2744dup and c.2820_2840dup	HOM	Negative	N/A	c.2721_2744dup andc.2820_2840dup*	HOM

Table 1. Concordance in variant data between Sanger sequencing and NGS of *RPGR* ORF15 is significantly improved with OneTube fragmentation and secondary duplication analysis. *Duplication analysis. †DNA sample exhausted.

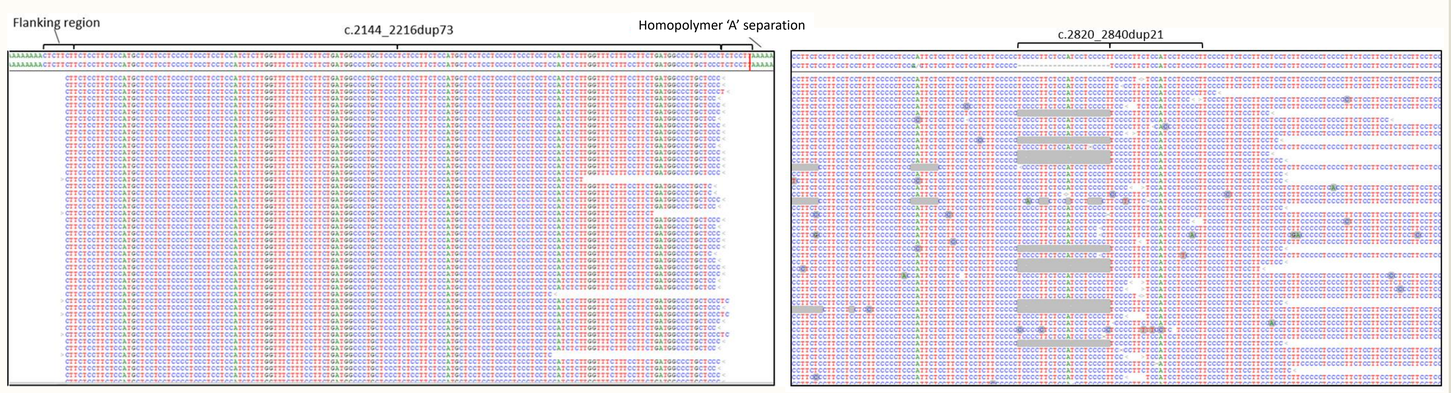

Figure 2. Duplication analyses. (Left) Duplication detection using alignment to an artificial reference sequence. Perfect alignment over this unique duplication junction indicates the presence of c.2144_2216dup in this sample. (Right) Duplication zygosity testing of a c.2820_2840dup heterozygous control. The wild-type allele appears as a 21bp deletion against the reference sequence for this duplication, while the allele containing c.2820_2840dup shows complete alignment.

Conclusions

This clinical validation highlights the efficacy of long-range PCR based NGS in conjunction with a new OneTube fragmentation technology for complete coverage of ORF15 in a standardized clinical pipeline with 100% mutation detection sensitivity and specificity. Through our blind testing of two unique methods, our results demonstrate both the weaknesses of previous NGS-based ORF15 sequencing methods, as well as the improvements that can be made. We suspect that many critical mutations within ORF15 may have been underreported previously and we hope that this advancement will lead the way for comprehensive, accurate, and practical implementation of NGS-based diagnosis for ORF15 and other similarly difficult-to-sequence regions within the genome.

Contact

John Chiang
Molecular Vision Laboratory, Hillsboro, OR
Email: jchiang@mvisionlab.com
Website: molecularvisionlab.com

Disclosures

John Chiang – Molecular Vision Laboratory I, E
Tina Lamey – None
Nicholas Wang – Molecular Vision Laboratory C
Jie Duan – Molecular Vision Laboratory E
Wei Zhou – Centrillion Technologies I, E
Terri McLaren – None
Jennifer Thompson – None
Jonathan Ruddle – None
John De Roach – None

References

1. Prokisch H, Hartig M, Hellinger R, et al. A population-based epidemiological and genetic study of X-linked retinitis pigmentosa. Invest Ophthalmol Vis Sci. 2007, 48:4012-4018.
2. Bader I. X-linked retinitis pigmentosa: RPGR mutations in most families with definite X linkage and clustering of mutations in a short sequence stretch of exon ORF15. Invest Ophthalmol Vis Sci. 2003,44:1458-1463.
3. Megaw RD, Soares DC, Wright AF. RPGR: Its role in photoreceptor physiology, human disease, and future therapies. Exp Eye Res. 2015, 138:32-41.