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A Multiplex PCR-Based Next-Generation Sequencing Approach Has Detected a Common Large Deletion in STS Gene in a Patient with X-Linked Ichthyosis

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Abstract

Several nuclear genes have been found to be linked to ichthyosis, and Next Generation Sequencing approach on panels of targeted genes has turned out to be particularly useful in analyzing diseases characterized by significant genetic and phenotypic heterogeneity. We developed a panel of 26 genes to be screened with the Ion Personal Genome Machine (PGM) for causative mutations relating to ichthyosis. Sequencing runs were obtained from a patient with ichthyosis using the Ion Torrent PGM and then processed with Ion Torrent Suite, Variant Caller, Coverage Analysis and wANNOVER tools. No causative mutations were found using Variant Caller and wANNOVER softwares, whereas the "Coverage Analysis" tool revealed a common large deletion in STS gene in a patient with X-linked ichthyosis. Identification of indels in Next Generation Sequencing (NGS) data is a veritable challenge. This study demonstrates the efficacy and effectiveness of using NGS approach to detect large deletions without resorting to specific algorithms for "indel" detection. Our results indicate that the NGS panel is a useful, rapid and cost-effective screening test for patients whose features are suggestive of a genetic etiology involving one of the genes embedded in the panel. It is an excellent alternative to Sanger sequencing as for costs, ease of analysis, and turnaround time.

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Keywords

X-Linked Ichthyosis, STS Gene, Next Generation Sequencing, Coverage Analysis

1. Introduction

Ichthyosis is a group of clinically and etiologically heterogeneous Mendelian skin disorders, characterized by dry or scaly and thickened skin [1] [2]. A list of several genes are known to be causative of ichthyosis [3], and this list keeps getting longer and longer, as more genes are being discovered. For each gene, many mutations have been recognized. However, epidemiology has been poorly described: $13.3/10^6$ liveborns in France [4], 5 - $10/10^5$ liveborns in the US [5]. First recognized by Wells and Kerr [6], the X-linked ichthyosis (XLI; MIM number 308100) stems from mutations in the steroid sulfatase (STS) gene, located on Xp22.3. This X-linked recessive skin disorder is generally restricted to males. The scales are dark, nigricant, not too large, primarily over the extensor surface. By contrast, the major folds of the body appear to be spared. Palms and soles are spared by hyperlinearity. STS is involved in the metabolism of cholesterol sulfate (CSO4), needed for developing a healthy stratum corneum.

XLI is the second most common type of ichthyosis, and appears as hyperkeratosis with skin barrier dysfunction. More than 85% of patients present with either a deletion involving the entire STS gene [7]-[9], or an intragenic deletion [8]. In addition, patients may show point mutations in the STS gene [7] [10]. PCR and Sanger sequencing in a gene-by-gene approach is time-consuming and expensive. Next Generation Sequencing (NGS) [11] [12], instead, represents a major drive in many research fields, providing a powerful and efficient way to study genetic diseases. These recent technologies allow us to sequence DNA and RNA much more quickly and inexpensively than the Sanger sequencing previously used. Methods have been developed that aim to achieve analysis of genome-specific regions, by detecting all genomic variations except for CNVs, for which statistical identification approaches are still rather limited.

In our study, we used a middle-throughput targeted NGS with a panel of 26 genes, and succeeded in identifying a mutation in the STS gene in a patient showing XLI.

2. Materials and Methods

Patient #3936-VR118A: Written informed consent was obtained from patient's parents. He was born after a 42-week uneventful pregnancy by Cesarean delivery due to the lack of physiologic progression. His birth weight and OFC fell within the 25^{th}-50^{th} centile range (3.450 kgs and 35 cms, respectively), while birth length fell into the 50^{th}-75^{th} centile range (52 cms). His skin was normal at birth. His weight and OFC at 3-month age (5.6 Kgs and 39.5 cms respectively) continued to increase within the 25^{th}-50^{th} centile range, while his length (60.5 cms) fell back into the 25^{th}-50^{th} centile range.

Clinical examination showed slight hypotonia and hyporeflexia, with good social participation, prominent forehead, deep-set eyes and nasal root, anteverted ears. His skin was widespreadly dry, with lamellar lesions mainly on the back and dorsal surface of the four limbs, with sparing of the folds. At the age of 15 months, his weight (9.5 Kgs), length (77 cms) and OFC (46.2 cms) fell back into the 10^{th}-25^{th} centile range, versus the genetic target falling within 25^{th} - 50^{th} centile range (173 cms); moreover, he presented with slight delay in toddling, speech language limited to 2 - 3 bisyllabic words; his skin was diffusely dry, with patches of wide hyperkeratosis pilaris on arms, legs and back, interspersed with whitish and brownish laminar desquamating areas. At the age of about 28 months, patient's auxologic and growth parameters were all falling within the 10^{th} - 25^{th} centile range (12 kgs, 87.5 cms, 48 cms, respectively), with hyperactive behavior, utterance of several words, often not easily understandable, lack of recognition of basic colors, and no bladder and bowel control. His skin was mildly dry on the back, arms, and legs.

Patient's gDNA sample was isolated from lymphocytes, checked for degradation on agarose gel and quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The primer design to create multiplex primer pools for Ion Sequencing Library was developed with Ion Ampliseq™ Designer technology (Thermo Fisher Scientific, Foster City, CA), which included 529 amplicons of 26 genes (ABCA12, ABHD5, ALOX12B, ALOXE3, AP1S1, CDSN, CSTA, CYP4F22, DSG1, DSP, FLG, GJB2, GJB3, GJB4, KRT1, KRT10, KRT2,

LOR, NIPAL4, POMP, SLC27A4, SLURP1, SPINK5, STS, TGM1, TGM5) [3] (Oji *et al.*, 2010). 10 ng of gDNA were amplified using the Ion AmpliSeq™ Library Kit 2.0 in a Bio-Rad C1000 (Bio-Rad, Hercules, CA, USA), then pooled together in equimolar concentrations using the Ion Library Equalizer Kit (Thermo Fisher Scientific). Diluted library (100 pM) was then amplified through emulsion PCR using the One Touch™ Instrument (Thermo Fisher Scientific) and enriched by the One Touch™ ES Instrument (Thermo Fisher Scientific) using the Ion Personal Genome Machine (PGM) Template OT2 200 KIT, following the manufacturer's instructions. Finally, sequencing was performed on the ION PGM (Thermo Fisher Scientific) with the Ion PGM 200 Sequencing Kit (Thermo Fisher Scientific), loading samples into a 314 chip. Data from the PGM runs were processed using an Ion Torrent Suite 5.0 (Thermo Fisher Scientific), the VariantCaller 5.0 (Thermo Fisher Scientific), the wANNOVER [13] and the Ion Reporter tools (Thermo Fisher Scientific). Generated sequence files were aligned to the genomic sequence (hg19). Following the sequence alignment and extraction of Single Nucleotide Polymorphism (SNP), all the variants were filtered using Reference SNP alleles at the dbSNP138 loci (http://www.ncbi.nlm.nih.gov/SNP). Coverage analysis was performed with the Ion Torrent Coverage Analysis 5.0 (Thermo Fisher Scientific). Data were exported from the plugin and analyzed with Excel sheet (Microsoft). DNA sequences were viewed with an Integrated Genomics Viewer (IGV) [14] [15] and, finally, the disease-causing variants were analyzed and confirmed by MLPA (SALSA MLPA probemix P160STS; MRC Holland, Amsterdam, the Nethetlands).

Table 1. Amplicon coverage summary results obtained from Coverage Analysis tool.

Chr	Gene	Number of amplicons	Mean of total reads
1	FLG	69	556
1	GJB3	5	178
1	GJB4	5	145
1	LOR	4	84
2	ABCA12	74	101
3	ABHD5	9	73
3	CSTA	3	93
5	NIPAL4	11	203
5	SPINK5	37	91
6	CDSN	12	202
6	DSP	60	112
7	AP1S1	7	75
8	SLURP1	3	325
9	SLC27A4	19	137
12	KRT1	17	144
12	KRT2	15	180
13	GJB2	5	196
13	POMP	8	113
14	TGM1	24	201
15	TGM5	20	220
17	ALOX12B	25	175
17	ALOXE3	24	148
17	KRT10	14	92
18	DSG1	26	67
19	CYP4F22	18	200
X	STS	15	0

3. Results and Discussion

We used the 26-gene panel, as developed in our laboratory, to genotype a patient who had met diagnostic criteria for ichthyosis. VariantCaller and wANNOVER/Ion Reporter tools analyses showed no causative mutations. Indeed, NGS technology, as known, enables the identification of missense, nonsense, splice-site as well as small insertions or deletions. Identification of indels in NGS data is a challenge, because indels are commonly analyzed using specific algorithms (Pindel, Genome Analysis Tool Kit's Unified Genotyper, HaplotypeCaller etc) [16]. In our patient, however, data from the PGM runs processed with Coverage Analysis 5.0 tool showed an alignment of reads in all panel genes, leading to a mean coverage of 186X across reads, except for the X-linked STS gene. This gene showed no PCR amplification (mean of total reads = 0, see **Table 1**) in any of the exons (15 amplicons), which is compatible with a hemizygous deletion of the whole STS gene. This deletion was further confirmed by MLPA and viewed in IGV tool.

Two different methodological approaches are usually required for the detection of point mutations and large deletions in XLI: sequencing analysis and array-CGH/MLPA respectively. Here, we show that our approach enables to detect both large deletions and point mutations. Indeed, complete/partial deletions of any X-linked gene can be easily detected, in hemizygous males, by the absence of target gene PCR-amplification (STS gene) and the presence of PCR-amplification of reference housekeeping genes (other genes within the panel; see **Table 1**). This method is particularly useful in patients negative for deletions (~15%) [7]-[9], for whom point mutation retesting analysis would be otherwise requested.

Further advantages of middle-throughput targeted NGS (multiple-gene panels versus gene-by-gene approach) include significant reduction in DNA sequencing costs (less expensive than Sanger approach) [17], enhanced efficiency of genetic testing, sparing of time, number of visits, and number of tests to be performed.

4. Conclusion

We used a middle-throughput targeted NGS with a panel of 26 genes, and succeeded in identifying a mutation in the STS gene in a patient showing XLI. When using ION torrent (Thermo Fisher Scientific) technology, it is strongly recommended to supervise the analysis of mutations in X-linked genes through the Coverage Analysis tool (Thermo Fisher Scientific), because large deletions in males might escape current identification if analyzed through wANNOVAR and/or IonReporter exclusively.

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