Ultra-deep next-generation sequencing: a reliable method for the molecular diagnosis of McCune Albright syndrome

Mallet-Moták, Delphine[1]; Roucher Boulez Florence[1,3]; Nicolino, Marc[2]; Morel, Yves[1,3]

[1] Molecular Endocrinology and Rares Diseases, Centre de Biologie et de Pathologie Est, Hospices Civils de Lyon, Bron, France; [2] Pediatric Endocrinology, Hôpital Femme Mère Enfant, Hospices Civils de Lyon, Bron, France; [3] Université Claude Bernard Lyon 1, Lyon, France

INTRODUCTION AND OBJECTIVES

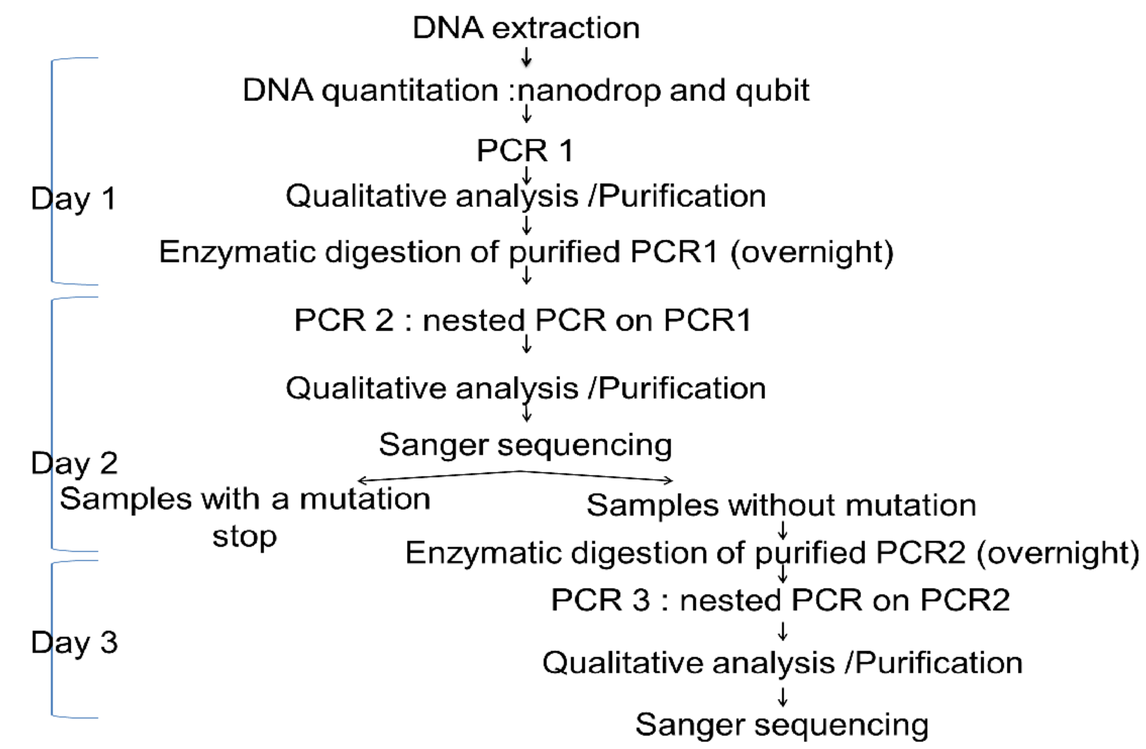
McCune Albright syndrome is a rare disease characterized by the clinical triad of fibrous dysplasia of bone, café-au-lait skin lesions, and precocious puberty. It results from mosaic gain of function mutations of the GNAS gene, coding for the Gsα protein. Two recurrent mutations have been described, p.R201C (chr20:57484420C>T) and p.R201H (chr20:57484421G>A). The proportion of cells bearing the mutation may be low, so the method used to detect the mutation should be very sensitive. Conventional Sanger sequencing is not sufficient.

Molecular diagnosis is performed in our lab since 2006, using the reference method, selective enrichment with nested PCR and enzymatic digestion. This technique is quite reliable and costless, but the major problems are contamination and time-wait between the different steps. This is why we tested the ability to detect GNAS mutations with ultra-deep next generation sequencing on our Ion PGM sequencer.

METHODS

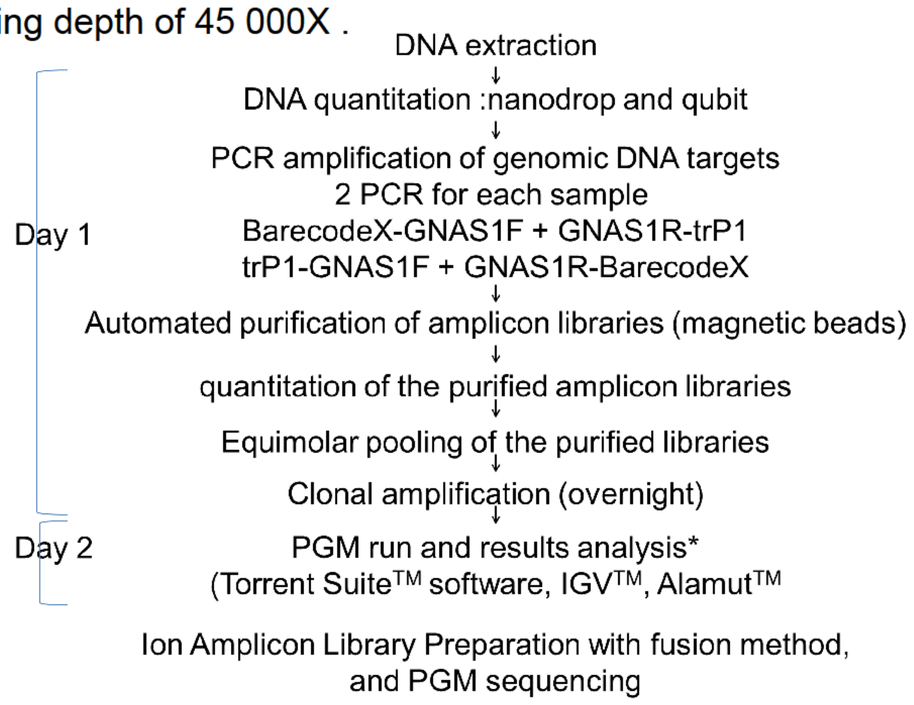
SELECTIVE ENRICHMENT METHOD

11 patients were studied at the same time. The protocol is described below. Briefly, a forward primer containing a mismatch is designed. This mismatch, followed by wild type DNA, contains a restriction site for the enzyme EagI. So, wild type DNA is sensitive to EagI digestion. In contrast, the two mutations modify the sequence and render the DNA resistant to EagI. Successive steps of PCR, enzymatic digestion and nested PCR allow selective enrichment of the mutant allele (fig. 1).



ULTRA-DEEP NEXT GENERATION SEQUENCING

We chose to study the same number of samples with the NGS technique, including a positive control for the two mutations and a negative control without mutation. 2 pairs of primers were designed for each sampled, according to the Ion Amplicon Library Preparation (Fusion Method) User Guide, allowing bi-directional sequencing (fig. 2). The size of the targeted fragment was 100 bp. The samples were multiplexed on an Ion 314 chip. We routinely obtain about 50 Mb of data on 314 chip, giving an average sequencing depth of 45 000X .



* Variant calling was performed with the Torrent Variant Caller, and PGM somatic low stringency parameters, allowing the detection of mutations with mutant allele frequency < 20 %. A hotspot file targeted to the two mutations was also used.

RESULTS AND DISCUSSION

23 known samples (13 mutated and 10 not mutated), previously analyzed with the selective enrichment method, were studied with the NGS technique. All previously identified mutations were confirmed, and the mutated allele frequency was estimated by the Torrent Variant Caller for the 13 mutated samples.

To date, 7 series have been performed with the NGS technique. Regarding the **negative control**, for the 2 mutation's positions the number of time each nucleotide was seen in all the reads was counted in order to evaluate the rate of sequencing errors. The mean mutant allele frequencies observed were: : 0.112 % (C>T) for p.R201C and 0.051 % (G>A) for p.R201H.

10 samples without mutation identified by the selective enrichment technique were also studied by NGS. No mutation was identified for these samples, confirming the previous results and showing the absence of contamination with the mutant alleles, even with high depths of sequencing (mean 32 000X, median 29 000X).

Sample 9 was known to be mutated. Ultra deep sequencing allowed us to detect the mutation with an allele frequency at 0.3 %, which is below the Torrent Variant Caller detection limit, even with custom parameters. The mutation was detected by comparing the number of reads with a G and an A at position using IGV and Alamut. The mutant allele frequency was superior to the limit fixed by the negative control, so the mutation could be called. However, the error rate of the NGS technique on Ion Torrent platforms is estimated around 1 %. Therefore, this was used as a threshold for the detection of the mutation in unknown samples.

Sample	Mutation detected by selective enrichment / step	Mutation detected by NGS / estimated allele frequency
Sample 3 - bone	p.R201H / PCR2	p.R201H / 9.3 %
Sample 4 - blood	p.R201C / PCR2	p.R201C / 12-15 %
Sample 6 - normal liver	ND	p.R201H / 12 %
Sample 6 - affected liver	p.R201H / PCR1	p.R201H / 41 %
Sample 7 - bone	p.R201H / PCR2	p.R201H / 2-3 %
Sample 8 - blood	p.R201C / PCR2	p.R201C / 1.1 %
Sample 9 - blood	p.R201H / PCR3	p.R201H / 0.3 %*
Sample 10 - blood	p.R201C / PCR2	p.R201C / 8 %
Sample 11 - blood	p.R201C / PCR2	p.R201C / 2.1 %
Sample 15 - blood	p.R201C / PCR1	p.R201C / 7.9 %
Sample 16 - blood	p.R201C / PCR2	p.R201C / 5.1 %
Sample 17 - blood	p.R201C / PCR2	p.R201C / 3.1 %
Sample 18 - bone	p.R201H / PCR1	p.R201H / 9.3 %

* Below the detection limit of the Torrent Variant Caller (1 %).

CONCLUSION

These data suggest that ultra deep sequencing, using the primer fusion technique on an Ion PGM platform, is a reliable method to detect GNAS somatic mutations in patients with McCune Albright syndrome. To date, when a mutation is identified in a patient, the selective enrichment method is still used to confirm the result.

ESPE 2015 536--P2
Miscellaneous
Delphine Mallet-Motak
DOI: 10.3252/pso.eu.54espe.2015
Poster presented at: 54th Annual Meeting 1-3 October ESPE 2015 BARCELONA European Society for Paediatric Endocrinology
PosterSessionOnline.com