GENETIC DIAGNOSIS OF CONGENITAL PRIMARY ADRENAL INSUFFICIENCY BY MASSIVE PARALLEL SEQUENCING

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INTRODUCTION AND OBJECTIVES

Congenital Primary Adrenal Insufficiency (PAI) can occur as three types: isolated glucocorticoid or mineralocorticoid deficiency, or global adrenal insufficiency, with combined mineralo and glucocorticoid deficiency. Once the most frequent genes (*CYP21A2*, *ABCD1*…) have been discarded by biological tests, many other genes may be involved in each type, and one gene may be responsible of different types. Consequently, there is no real decision tree in the gene analysis order.

The aim is to replace Sanger sequencing by massive parallel sequencing (MPS) in order to get a rapid, confident and costless diagnosis. Instead of studying the genes one by one, they are analysed all at once. But do we get the same results?

METHODS

An AmpliSeq™ custom panel was designed including 17 genes: *AAAS, AIRE, CDKN1C, Cited2, CYP11A1, CYP11B2, GPX1, MC2R, MCM4, MRAP, NNT, NR0B1, NR5A1, PBX1, PRDX3, StAR, TXNRD2*. To evaluate the MPS strategy, 27 patients, previously studied by Sanger sequencing (up to 10 genes), were sequenced on an Ion Torrent PGM™ (n=18) or Proton™(n=9) System. The Bio-informatics pipeline used was the one implemented in the Torrent Suite™ software (Alignement – Variant Calling). Statistical tools (logit models and complementary log-log link function) were used to compare the results in R software, considering Sanger as the "gold standard". Each base theoretically covered by MPS and Sanger is included. 2 covariates were considered: sequencing depth and Mapping quality.

RESULTS

86% of our theoretical targets (exons+/-10bp) and 90 % of the designed targets are well sequenced (>30X). (75%/77% are above 100X). The average read depth is 430X.

Within the 10 genes studied in Sanger, the table below shows the number of patients studied by gene and the number of variations found within the common regions targeted by Sanger sequencing and MPS. The contingency table shows the common variants found by Sanger and MPS (Real Positives RP), the variations found only by Sanger (MPS false negative FN), only by MPS (MPS false positives FP) and the base pair targeted for which no variations was seen, nor by Sanger or MPS (real negatives RN)

Gene	*AAAS*	*CITED2*	*CYP11A1*	*CYP11B2*	*MC2R*	*MRAP*	*NNT*	*NR0B1*	*NR5A1*	*STAR*
Nb Patients	1	3	15	6	11	10	18	17	8	21
Nb Sanger Variations **n=247**	5	3	12	67	31	0	63	8	11	47
Nb MPS Variations **n=237**	5	2	16	53	27	0	73	8	6	47

	Sanger V	**Sanger NV**
MPS V	214 (RP)	23 (FP)
MPS NV	33 (FN)	292 286 (RN)

Contingency table **V: Variations, NV: No Variations**

The Sensitivity of MPS is 86,6% and Specificity 99,9%. The no detected variations were in *NR5A1* (regions nor covered due to GC rich despite the design) or in *CYP11B2* (mapping quality MAPQ was at 0 due to its high homology with *CYP11B1)*. False positives (FP) found by MPS are due to homopolymer as we could attend with Ion Torrent technologies. Only one FP in *NR5A1* remains unexplained.

FALSE NEGATIVES: Sanger Variations / MPS No Variations

Chr	Position hg19	Ref	Alt	Gene	Comments
chr18	13915410	C	A	MC2R	first position of the bed
chr18	13915410	C	A	MC2R	first position of the bed
chr18	13915410	C	A	MC2R	first position of the bed
chr18	13915410	C	A	MC2R	first position of the bed
chr5	43653243	C	T	NNT	Not covered
chr5	43609343	C	T	NNT	Not covered
chr5	43609343	C	T	NNT	Not covered
chr5	43612984	insA	NA	NNT	Different position in MPS
chr8	143996363	A	G	CYP11B2	MAPQ=0
chr8	143995994	C	T	CYP11B2	MAPQ=0
chr8	143994266	A	G	CYP11B2	MAPQ=0
chr8	143996059	A	G	CYP11B2	MAPQ=0
chr8	143995994	C	T	CYP11B2	MAPQ=0
chr8	143994321	T	C	CYP11B2	MAPQ=0
chr8	143994266	A	G	CYP11B2	MAPQ=0
chr8	143996683	T	C	CYP11B2	MAPQ=0
chr8	143996059	A	G	CYP11B2	Not covered
chr8	143996005	T	C	CYP11B2	Not covered
chr8	143995994	C	T	CYP11B2	Not covered
chr8	143995994	C	T	CYP11B2	different position in MPS
chr8	143995981	G	A	CYP11B2	MAPQ=0
chr8	143995994	C	T	CYP11B2	MAPQ=0
chr8	143995981	G	A	CYP11B2	MAPQ=0
chr8	143994349	T	C	CYP11B2	MAPQ=0
chr8	143994321	T	C	CYP11B2	MAPQ=0
chr8	143994266	A	G	CYP11B2	MAPQ=0
chr9	127244955	G	A	NR5A1	Not covered
chr9	127244955	G	A	NR5A1	Not covered
chr9	127244955	G	A	NR5A1	Not covered
chr9	127262802	C	G	NR5A1	Not covered
chr9	127244955	G	A	NR5A1	Not covered
chr9	127262802	C	G	NR5A1	Not covered
chr6	139694572	C	A	CITED2	Not covered

FALSE POSITIVES: MPS Variations / Sanger No Variations

Chr	Position hg19	Ref	Alt	Gene	Comments
chr15	74637346	C	A	CYP11A1	FP homopolymer
chr15	74640101	GA	AG,G	CYP11A1	FP homopolymer
chr15	74631715	GCCCCA	GCCCA,GCCCAC	CYP11A1	FP homopolymer
chr15	74640101	GA	AG,G,GG	CYP11A1	FP homopolymer
chr5	43612985	T	A	NNT	2nd line for insertion in VCF
chr5	43612985	T	A	NNT	2nd line for insertion in VCF
chr5	43655821	G	A	NNT	RP not studied in Sanger initially
chr5	43612985	T	A	NNT	2nd line for insertion in VCF
chr5	43612985	T	A	NNT	2nd line for insertion in VCF
chr5	43628542	A	T	NNT	FP homopol VV à côté
chr5	43655821	G	A	NNT	FP homopolymer
chr5	43702887	A	G	NNT	VP not studied in Sanger initially
chr5	43609201	G	GT	NNT	FP homopolymer
chr5	43612985	T	A	NNT	2nd line for insertion in VCF
chr5	43609170	T	TTA	NNT	FP homopolymer
chr5	43612976	A	AAT	NNT	FP homopolymer
chr5	43612983	ATT	ATA,AT	NNT	RP in a position more 5' in Sanger
chr5	43628542	A	T	NNT	FP homopolymer
chr8	143998426	C	T	CYP11B2	RP not studied in Sanger initially
chr8	143998426	C	T	CYP11B2	RP not studied in Sanger initially
chr8	143995996	G	C	CYP11B2	RP in a position more 5' in Sanger
chr8	143998426	C	T	CYP11B2	RP not studied in Sanger initially
chr9	127253318	C	G	NR5A1	FP ? C:312 83% 0+ 312-/G: 63 17% 0+,63-

CONCLUSIONS

Complete molecular investigation by MPS was faster and cheaper. All regions insufficiently covered(<30X) by MPS still need to be studied by Sanger sequencing. Taking this into account, no variations would have been missed except in *CYP11B2*. This underlines problem of pseudogenes or homologous genes in MPS .

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