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Letter to the Editors-in-Chief

Incidental finding of unreported large duplication in *F8* gene during prenatal analysis: Which management for genetic counselling?

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ABSTRACT

Detection of incidental finding and variant of unknown significance (VUS) during prenatal diagnosis has particularly increased with the emergence of genetic tests such chromosomal microarray analysis (CMA). Many factors and clear guidelines need to be applied in the interpretation of the potential clinical consequences of unreported complex copy number variations (deletions/duplications).

From a clinical case where an unreported and not completely intragenic duplication in *F8* gene has been identified in a 12-week-old fetus without haemophilia A history documented in the family, we will examine and study the difficulties of interpretation and the challenges that the detection of such variant has on genetic counselling.

1. Introduction

Interpreting the clinical relevance of unreported sub-microscopic rearrangements, especially when detected by chromosomal microarray (CMA), is crucial for genetic counselling and requires particular caution before concluding that they are causally associated with a disease. In prenatal diagnosis, one important limitation of CMA is the fact that many small size (< 5 Mb) sub-microscopic deletions and duplications, also called Copy Number Variations (CNVs), cannot be clearly classified as benign or pathogenic as they have not yet been characterized. In cases with sonographic fetal abnormalities, detection rate of pathogenic CNV turns around 27%. Regarding the frequency of CNV of uncertain significance (VUS), estimation of their frequency varied depending on the different platforms ranging from 0 to 4% to 9–12% in case of high-resolution whole genome arrays [1]. By contrast with the clinical significance of deletion CNVs in a loss-of-function gene that can be easily interpreted, it is not the case for duplication CNVs.

In case of incidental finding of unreported CNV with a high suspicion of being possibly pathogenic, a number of criteria and guidelines should be applied before concluding that they are responsible for a hereditary disorder [2,3]. In this case report, identification of a large unreported duplication in the *F8* gene in a 12-week-old fetus provides the opportunity to highlight the difficulties of interpretation of such VUS for an optimal genetic counselling.

2. Materials and methods

2.1. Array CGH and MLPA

Oligonucleoride array-CGH was performed in the DNA of fetuses using SurePrint G3 Human CGH Microarray ISCA 4x180K v2 (AMADID 031748; Agilent Technologies, Santa Clara, CA, USA). The 180 K kit (180,000 probes) has an overall median probe spacing of 13 kb. Analysis was performed according to the protocol provided by the supplier (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, version 6.3). Arrays were scanned using a SureScan High Resolution Microarray Scanner (Agilent) and results were analyzed using CytoGenomics Analysis software v2.5 (Agilent).

MLPA analysis was performed with the SALSA MLPA P178 FVIII kit (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions.

3. Results and discussion

We here present the case of a 35-year old pregnant woman of dichorionic diamniotic male twins. In one of the fetuses, whole genome CMA analysis was carried out at 12 weeks of gestation after the detection of multiple malformations by ultrasound consistent with a diagnosis of trisomy 13. In addition to an abnormal karyotype of trisomy 13, a 75 kb duplication of the *F8* gene (arr[hg19] Xq28(154176025_154250982)x2 mat) was also identified. In the second fetus, molecular analysis revealed normal karyotype with the presence of the same 75 kb partial duplication of *F8* gene exons 1 to 13 detected in the first fetus. As this 75 kb partial *F8* duplication was not already reported in the database or literature, this variant was classified by the geneticists as uncertain clinical significance or VUS (Variant of Unknown Significance). Because this CNV implicates the *F8* gene (OMIM 300841) which encodes the coagulation Factor VIII, the pregnant patient was transferred to our Haemophilia center for medical counselling.

Briefly, haemophilia A (HA) (OMIM 306700) is an X-linked congenital bleeding disorder, caused by a lack or dysfunction of coagulation Factor VIII. HA severity was defined by FVIII:C levels in plasma activity, classified as severe (< 1%), moderate (1–5%), or mild (5–40%) (N: 50–150). No HA history was documented in the family. MLPA analysis showed the presence of the duplication in the DNA of the pregnant patient and her mother with normal FVIII:C activities measured at 225% and 139% respectively by one-stage assay. This variant was not detected in the patient's brother and father (Fig. 1). At this stage of analyzes, major difficulties for the interpretation of this

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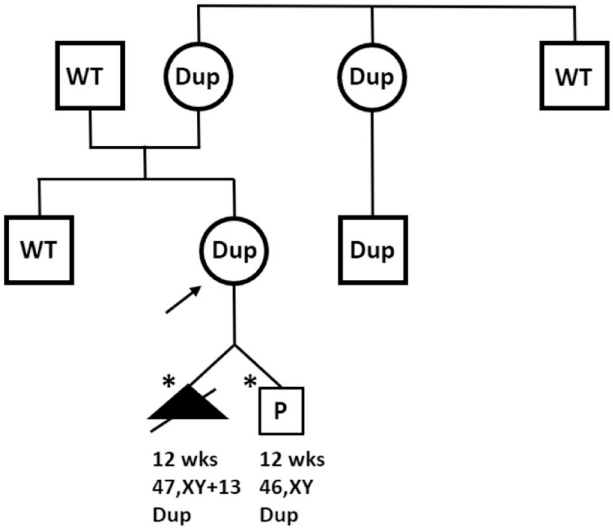

Fig. 1. Transmission of the 75 kb partial duplication of *F8* gene exons 1 to 13.
*Oligonucleotide array-CGH was performed only in the DNA of fetuses.
MLPA analysis was performed in all family members described in this fig.
WT (wild type) and Dup (duplication) indicates the absence or presence of the duplication respectively.
Males are indicated by squares, females by circles. Index care (proband) is shown by arrow.
Pregnancy (P).

kind of variant will be detailed and reviewed below.

3.1. First difficulty: rarity and unreported

In prenatal diagnosis, array-CGH designs requires consensus between a higher resolution that can detect a majority of clinical relevant and a lower resolution more suitable for the identification of chromosome abnormalities with low number of CNV with uncertain significance or incidental findings. In Belgium, national consensus recommends to use at least 60 kb arrays for an average resolution of 400 kb [4].

CNV detected by microarray testing are classified under three categories: clearly pathogenic, clearly neutral or unclassified variants (VUS). This last category corresponds to a deletion/duplication that has not previously been described, has not been detected in controls so far studied and reported (*e.g.*, the Database of Genomic Variants (http://projects.tcag.ca/variation), DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER), European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA)), and for which there is incomplete data on the genes in the region. Consensus approach consists in reporting those with a high suspicion of being possibly pathogenic depending on several factors: genomic size, number of genes content, partial or total involvement of a highly penetrant monogenic disorder, affecting an X-linked gene in a XY fetus, similar CNVs already described [4].

CNVs of one or more exons in *F8* are relatively rare in HA with an estimated occurrence rate of < 5% for deletion and 1% for duplication [5]. If all deletions CNVs are associated with a severe phenotype, *F8* duplications were linked to different haemophilia severities depending on localization, length of exons involved, and whether the resulting proteins were in-frame or out-of-frame (Table 1).

3.2. Second difficulty: nature of the variant

Unlike unreported SNPs (Single Nucleotide Polymorphisms) whose pathogenicity can be studied by *in silico* prediction algorithms (Polyphen-2, SIFT, Align-GVGD, MutationTaster2) or deletions CNVs affecting a well-known loss-of-function gene, large duplication CNVs can have different consequences on gene functions depending on the duplication breakpoints, duplications locations and gene reading frames. Indeed, CMA analyses were unable to provide either genome position, *i.e.* in tandem or translocated to a different genomic position outside the target gene, or orientation information, for example direct or inverted duplication. Without resolving breakpoints by molecular analyses, it is impossible to predict the effect of duplications CNVs on gene structure and to correlate with phenotypes.

In case of incidental finding, parental testing as well as siblings may be necessary to determine the “genotype-phenotype” correlation. At this time of analysis, the normal FVIII:C activities, measured in the pregnant and her mother were not contributory as the majority of haemophilia carriers have factor levels that are considered adequate for hemostasis, attributed to the random X-chromosome inactivation which occurs early in the embryonic life [6]. However, translocated recombination outside the target gene does not seem to be applicable to our family given that the CNV segregates over at least three generations.

3.3. Third difficulty: not completely intragenic

In our case report, breakpoint CMA analysis revealed an unreported 75 Kb duplication delimited from *F8* exon 1 to 13 with a 5′ extragenic *F8* localization. The clinical significance of not completely intragenic duplication CNV is difficult to interpret. In the Newman et al. study, most interstitial duplications are tandem and lie in direct orientation, thus preserving, at breakpoint junctions, one intact gene copy on the duplication allele [7].

What can be observed in the scientific literature or in most famous HA database [http://www.factorviii-db.org/] is that most large *F8* duplications identified in HA are intragenic, disrupting the reading frame. However, very few are not completely intragenic due to one of the two junctions being located outside of *F8*. For those located upstream of the gene, consequences on the FVIII vary greatly from no association with HA to severe or mild phenotype (Table 1). In case of tandem direct orientation, a normal phenotype could be explained if the duplicated sequences would not compromise transcription or translation of an intact *F8* mRNA transcript producing normal FVIII levels [8]. In case of interruption of the open reading frame (ORF) of the *F8* cDNA, a severe phenotype should appear. The phenotype could

Table 1
Inventory of large > 50 bp duplications in the F8 gene.

CNV ID	Localisation	Database	Exons duplicated in F8	Genes implicated	HA phenotype	Reference
Duplication with one extragenic border						
5217	NA	Eahad variant database (http://www.eahad-db.org/)	1 to 5	Partial F8	Severe	Rost et al. J Thromb Haemost. 2008; 6:1996–1999.
esv3577569	chrX:154,201,126 - 154,277,543 [hg19]	Database of Genomic variants (http://projects.tcag.ca/variation)	1 to 6	Partial F8, partial FUNDC2	Benign	Uddin M et al. Genet Med. 2015; 17: 747–752.
289,235	chrX:154,235,454-154,310,067 [hg19]	DECIPHER (https://decipher.sanger.ac.uk/)	1	Partial F8, FUNDC2, partial CMC4 and MTCP1	Benign	Not reported
627	chrX:154,208,417-154,309,447 [hg19]	DECIPHER	1 to 6	Partial F8, FUNDC2, partial CMC4 and MTCP1	Unknown	Not reported
1877	chrX:154,20,8417–154,309,447 [hg19]	DECIPHER	1 to 6	Partial F8, FUNDC2, partial CMC4 and MTCP1	Unknown	Not reported
288,464	chX:154,156,799-154,310,067 [hg19]	DECIPHER	1 to 13	Partial F8, partial FUNDC2	Likely benign	Not reported
NA	chrX:154,117,967-154,609,974 [hg19]	CHAMP (https://www.cdc.gov/ncbddd/hemophilia/champs.html)	1 to 22	Partial F8, FUNDC2,CMC4, MTCP1, BRCC3, VBP1, RAB39B, CLIC2	Likely benign	Lannoy et al. Eur J Hum Genet. 2013; 21:970–976.
	chrX:154,146,529-154,229,174 [hg19]		2 to 14	Partial F8	Severe	
NA	chrX:154,238,748-154,449,116 [hg19]	/	1	Partial F8, FUNDC2,CMC4, MTCP1, BRCC3 and partial VBP1	Mild	Lannoy et al. Haemophilia 2015; 21:516–522
NA	chrX:154,147,182-154,378,045 [hg19]	/	1 to 14	Partial F8, FUNDC2,CMC4, MTCP1, BRCC3	Unknown	Jourdy Y et al. Haemophilia. 2017; 23:e316–e323.
	chrX:154,182,749-154,235,426 [hg19]		2 to 11	Partial F8	Probably associated with severe	
NA	chrX:154,224,460-154,722,370 [hg19]	/	1 to 3	Partial F8, FUNDC2,CMC4, MTCP1, BRCC3, VBP1, RAB39B, CLIC2, TMLHE	Unknown	Jourdy Y et al. Haemophilia. 2017; 23:e316–e323.
NA	chrX:154,176,025-154,250,982 [hg19]	/	1 to 13	partial F8	Benign	This study
Intragenic duplications						
NA	NA	/	11 to 14	Partial F8	Severe	Vinciguerra ch et al. Blood 2007 110:1149
NA	NA	/	2 to 4 6	Partial F8	Unknown* Unknown*	Johnsen JM et al. Blood Adv. 2017; 1:824–834.
NA	NA	CHAMP	2 to 10	Partial F8	Severe	Venceslá Á et al. Haemophilia. 2012; 18:708–713.
NA	NA	CHAMP	2 to 14	Partial F8	Severe	Miller CH et al. Haemophilia. 2012; 18:375–382
NA	NA	CHAMP	2 to 25	Partial F8	Severe	Rost et al. J Thromb Haemost. 2008; 6:1996–1999.
NA	NA	CHAMP	5 to 21	Partial F8	Severe	Johnsen JM et al. Blood Adv. 2017; 1:824–834.
NA	NA	CHAMP	5 to 25	Partial F8	Severe	Rost et al. J Thromb Haemost. 2008; 6:1996–1999.
NA	NA	CHAMP	6	Partial F8	Unknown	Zimmermann MA et al. J Thromb Haemost. 2010; 8:2696–2704.
NA	NA	CHAMP	7 to 11	Partial F8	Severe	Zimmermann MA et al. J Thromb Haemost. 2010; 8:2696–2704.
NA	NA	CHAMP	7 to 22	Partial F8	Severe	Zimmermann MA et al. J Thromb Haemost. 2010; 8:2696–2704.
NA	NA	CHAMP	13	Partial F8	Moderate-mild	Casula L et al. Blood. 1990; 75:662–670
NA	NA	CHAMP	14	Partial F8	Severe	Rost et al. J Thromb Haemost. 2008; 6:1996–1999.
NA	NA	CHAMP	14 to 21	Partial F8	Severe	Rost et al. J Thromb Haemost. 2008; 6:1996–1999.
NA	NA	CHAMP	22	Partial F8	Severe	Zimmermann MA et al. J Thromb Haemost. 2010; 8:2696–2704.
NA	NA	CHAMP	23 to 25	Partial F8	Severe	Zimmermann MA et al. J Thromb Haemost. 2010; 8:2696–2704.
NA	NA	CHAMP	23 to 26	Partial F8	Severe	Zimmermann MA et al. J Thromb Haemost. 2010; 8:2696–2704.
NA	NA	CHAMP	24	Partial F8	Severe	Rafati M et al. Haemophilia. 2011; 17:705–707

NA: not available.
* Severe patient with two reportable variants.

also be less severe if the DNA fragment is duplicated in the opposite direction or in tandem inversion in one intron of the F8 gene generating a new rearranged intron. In this situation, the duplication should not interfere with the ORF of the *F8* but rather with the efficiently of the splicing process. The phenotype of such rearrangement would depend on the number of normal mRNA transcripts produced during splicing [9].

Fortunately, the VUS found in this family was ultimately classified as "benign" after 10 weeks of analyses necessary to collect and study blood samples of other family members (Fig. 1). Indeed, genetic testing extended to other members of the family identified the same VUS in the healthy patient's cousin who was found to have a FVIII:C in the normal range. The mother gave birth to a healthy boy late 2018. In agreement with the European recommendations [10] to promote the use of international variant databases, this unreported duplication CNV was submitted to the EAHAD DATABASES for Haemophilia A (F8) Variants [http://www.factorviii-db.org/].

4. Conclusion

As illustrated by this report, many factors must be taken into account for the interpretation and counselling about the potential clinical consequences of incidental findings/VUS. Difficulties in interpretation of the unreported 75 kb partial duplication of *F8* gene exons 1 to 13 found in both fetuses were shared with the parents before they were referred the Haemophilia treatment Center for specialist counselling.

Contributorship statement

NL designed the review, studied the literature, and wrote the manuscript. CL and AVD recruited the patients, collected data and revised the manuscript. CH reviewed the data analysis and revised the manuscript.

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Declaration of competing interest

The authors state that they have no conflicts of interest to declare.

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