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Running title : Genotype-phenotype relevance in 20q13.2 gain

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Abstract :

Interstitial duplication within the long arm of chromosome 20 is an uncommon chromosome structural abnormality. We report here the clinical and molecular characterization associated with pure 20q13.2 duplication in three unrelated patients. The most frequent clinical features were developmental delay, facial dysmorphism, cardiac malformation and skeletal anomalies. All DNA gains occurred *de novo*, ranging from 1.1 Mb to 11.5 Mb. Compared with previously reported conventional cytogenetic analyses, oligonucleotides array CGH allowed us to refine breakpoints and determine the genes of interest in the region. Involvement of *SALL4* in cardiac malformations and *NFATC2* gene disruption in both cardiac and skeletal anomalies are discussed.

Keywords:

20q13.2 microduplication, developmental delay, dysmorphism, cardiac malformation, *SALL4*, *NFATC2*

Introduction :

Interstitial duplication within the long arm of chromosome 20 is an uncommon chromosome structural abnormality that has been studied essentially by conventional cytogenetic techniques. Most of the patients' translocations involving this region hitherto observed, combined 20q trisomies with monosomies for another chromosome [1-11]. Thus, attempt of phenotype-genotype correlation seemed speculative since whole phenotype resulted from trisomies 20q of variable sizes associated with singular monosomies or, occasionally, trisomies. So far only four cases of pure partial trisomy 20q have been cytogenetically and clinically described.

We report here three unrelated patients with overlapping *de novo* gain of 20q13.2 : a new one and two previously published that were more precisely characterized in our study using oligonucleotides array CGH [12,13]. The third published case with pure partial trisomy 20q is defined by an overlapping *de novo* duplication of 20q13.12q13.31 (Iglesias A. *et al*, Clin Dysmorphol, 2006 and personal communication) [14]. The last one described does not overlap latter DNA gains [15]. In this study, oligonucleotides array CGH assayed on two previously reported patients and a new fetal case allowed us to further refine the breakpoints of 20q13.2 gains and specify genes whose expression possibly correlates with the clinical features.

Patient data:

Patient 1:

A 28-year-old gravida 3 para 1 was referred to our maternal fetal medicine service at approximately 21 weeks gestational age (wg) for abnormal findings on routine ultrasound including congenital heart malformation with left heart hypoplasia associated with ventricular septal defect. The parents were unrelated and familial histories were unremarkable. Chromosomal analysis on amniotic fluid showed a normal male karyotype 46,XY.

After genetic counselling and according to the French law, the parents opted for the termination of pregnancy performed at 23 wg. Weight, body length and head circumference were respectively 595 g (75^{th} percentile), 31 cm (75^{th} percentile) and 19.5 cm (25^{th} percentile). External examination showed facial dysmorphism with large forehead, bilateral epicanthus, upslanting palpebral fissures, anteverted nares, prominent philtrum, thin upper lip and retrognathia (Fig. 1a and 1b). Internal examination confirmed severe left heart hypoplasia with mitral and aortic atresia, associated to interventricular septum defect. In addition, the fetus presented on X-rays a brachymesophalangy of the V^{th} fingers. Neuropathological examination revealed multiple olivary heterotopias.

Patient 2:

The clinical history of patient 2 is described by Blanc P. *et al*, Am J Med Gen, 2008 [12]. In brief, the patient was born at 38 wg and exhibited macrosomia. The neonatal period was complicated by laryngomalacia. An echocardiogram demonstrated an asymmetrical aortic valve and a small ventricular septal defect. In addition, early moderate hypotonia was suspected. Neuromotor milestone delay with sitting position at 11 months and autonomous walking at 24 months was an additional feature. At the age of 30 months he had delayed speech as well, and was finally referred for genetic evaluation. Musculoskeletal abnormalities

such as pectus excavatum/carinatum, occipital plagiocephaly and asymmetric bisegmental vertebrae were then observed. Facial dysmorphy included bilateral epicanthal fold, large and high forehead, small bulbous nose with slightly anteverted nares, thin upper lip, dimpled chin and low-set ears with slanted crease to the lobes (Fig. 1c and 1d).

Patient 3:

The girl presented with intellectual deficiency associated with physical signs of joint laxity, hyperelastic skin and scoliosis. Facial dysmorphy included broad nasal tip, beaked nose, hypertelorism, up-slanting palpebral fissures, short philtrum, macrostomia, everted lower lip, thick upper lip, widely spaced teeth, large ear lobe, long and webbed neck (photograph was not available). It is worth-noting that no cardiac anomaly was reported (Menten B. *et al*, J Med Genet, 2006 [13] and DECIPHER N°763 (http://decipher.sanger.ac.uk/)).

Methods:

DNA extraction:

Informed consent for genetic analyses was obtained from the patients' parents according to the local ethical guidelines. Genomic DNA was isolated from fetal pulmonary tissue (for patient 1 referred as P1), and peripheral blood for the other patients (P2 and P3) and their parents. P1 and his parents' DNAs were extracted using Flexigen-DNA (Qiagen, Courtaboeuf, France). For P2 and P3, DNA was isolated from blood leucocytes using the NucleoSpin® Blood L extraction kit (Macherey-Nagel, Hoerdt, France) according to the supplier's protocol. The extracted DNA concentrations were estimated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Conventional Cytogenetic Analysis

Chromosomes were obtained using conventional techniques from P1 pulmonary tissue slides and from synchronized peripheral blood lymphocytes for P2, P3 and all the probands' parents (as previously published for P2 by Blanc P. *et al*, Am J Med Gen, 2008 [12] and for P3 by Menten B. *et al*, J Med Genet 2006 [13]). Chromosomes were stained by RHG banding and GTG banding.

Array Comparative Genomic Hybridisation (aCGH)

Agilent oligonucleotide Human arrays (Agilent, Santa Clara, California, USA) were used to detect chromosomal abnormalities in patients P1 and P3 (180K), and P2 (400K). Microarray was performed in accordance with the manufacturer's instructions.

Briefly, 1µg of genomic DNA was digested with *AluI* (5 units) and *RsaI* (5 units) for 2 hours at 37°C and fluorescently labelled with the Agilent Genomic DNA labelling kit (Agilent Technologies, Massy, France). A human blood donor genomic DNA was used as reference. Cy5-dUTP patient DNA and its gender-matched reference labelled with Cy3-dUTP were denatured and pre-annealed with Cot-1 DNA and Agilent blocking reagent prior to hybridisation (for 24 hours with 180K and 40 hours with 400K) at 20 rpm in a 65°C rotating hybridisation oven (Agilent Technologies). After washing, the slides were scanned on an Agilent Microarray Scanner. Captured images were processed with Feature Extraction 10.7.3.1 software and data analysis was performed with Agilent Genomic Workbench 5.0 (Agilent Technologies). Copy number variations were considered significant if they could be defined by 4 or more oligonucleotides spanning at least 70 Kb and contained at least one gene and were not identified in the Database of Genomic Variants (http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg19). The Genome Browser used to analyse genes content was hg19, Build37 (http://genome.ucsc.edu/).

Fluorescence *In Situ* Hybridisation (FISH):

FISH analyses were performed on P1 pulmonary tissue slides and metaphase spreads from parental lymphocytes and P3 from metaphase spreads and interphasic nuclei from lymphocytes. BAC clones specific for the 20q13.2 region (RP11-244L4, RP11-195N11, RP5 1071L10 and RP5 994024) were used. BAC DNAs were labelled by nick-translation using a FITC-dUTP nucleotide or Rhodamine-dUTP nucleotide (Roche Diagnostics, Rungis, France).

Real-time quantitative PCR

Confirmation of the 20q13.2 duplications in the P1, P3 probands and analysis in the parents were carried out by using real-time qPCR probes specially designed to the region of interest, as previously published for P3 (Menten B. *et al*, J Med Genet 2006 [13]).

For P1, primers were designed using Primer Express v3.0 (Applied BiosystemsTM, Foster City, CA) and primer blast (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Albumin gene exon 12 was used as autosomal reference.

Amplification was performed using a 7900HT real-time thermal cycler (Applied BiosystemsTM, Foster City, CA) and as recommended by Applied Biosystem. Standard curves were generated for each amplicon using increasing amounts of genomic control DNA (from 0.8 ng to 80 ng per well). Reactions were performed with 10 µL Power SYBR® Green PCR Master Mix (Applied BiosystemsTM, Foster City, CA), 1 µL of 500 nM forward primer and 1 µL of 500 nM reverse primer and 8 µL of DNA at 1 ng/µL (8 ng of tested DNA per well).The PCR programme started with 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Dissociations curves, 95°C for 15 sec, 60°C for 15 sec and 95°C 15 sec, were performed at the end of each run to control the specificity of the amplification with the presence of a unique and reproducible melting temperature.

Results

Conventional cytogenetic analysis

Cytogenetic analyses in all parents and probands showed normal karyotypes : 46,XY for P1 and P2 and 46,XX for P3 (as previously published for P2 by Blanc P. *et al*, Am J Med Gen, 2008 [12] and for P3 by Menten B. *et al*, J Med Genet 2006 [13]).

Array Comparative Genomic Hybridisation (aCGH)

In all probands, oligoarray CGH revealed an interstitial gain within the long arm of chromosome 20. In P1, array CGH showed a duplication of 20q13.13q13.2 encompassing a 4.7 Mb region with breakpoints within 49,160,298–53,869,127 bp for minimum size (and 49,152,674–53,911,068 bp for maximum size) (Fig. 2a). P2 analysis showed a duplication of 20q13.2q13.33 encompassing a 11.5 Mb region with breakpoints within 49,954,748–61,454,406 bp for minimum size (and 49,925,528–61,458,333 bp for maximum size) (Fig. 2b). For P3, a gain, comprising of duplications and multiple gain, was observed within 20q13.13q13.2 encompassing 1.1 Mb. First a duplication was observed with breakpoints within 49,216,751–49,494,319 bp, followed by multiple gains between 49,527,585–50,254,914 bp and duplication between 50,286,006–50,333,944 bp (Fig. 2c).

The other changes observed elsewhere on the genome corresponded to CNV previously reported in the database of genomic variants (http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg19).

Secondary technical procedures for array CGH confirmation

FISH experiments on pulmonary tissue slides in P1 and lymphocytes for P3 showed asymmetric signals confirming the DNA gains and excluding an insertion into any other

chromosome. qPCR also confirmed the DNA gains. In parents of P1, qPCR and FISH analyses on lymphocytes showed normal signals indicating a *de novo* origin for the rearrangement seen in the proband.

In P2, GenoSensor Array 300 and metaphase CGH had shown the duplication. As previously published, the appearance of the long arm was suggestive of interstitial duplication involving the 20q13.2 region. Parental karyotype analyses indicated a *de novo* origin for the rearrangement seen in the proband (Blanc P. *et al*, Am J Med Gen, 2008 [12]).

In P3, qPCR was performed to confirm the DNA gain which was found to be *de novo* as previously published (Menten B. *et al*, J Med Genet 2006 [13]).

Discussion

We report interstitial *de novo* gains of 20q13.2 ranging from 1,1 Mb to 11,5 Mb that were characterized in three patients: a 23 wg fetus (P1 with duplication ranging from 49,160,298 bp to 53,869,127 bp) presenting severe congenital heart disease, facial dysmorphy, and brachymesophalangy; a 3-year boy (P2 with duplication ranging from 49,954,748 bp to 61,454,406 bp) presenting developmental milestone delay, macrosomia, cranio-facial dysmorphy, skeletal anomalies, and congenital heart disease; and a girl (P3 with 20q13.13q13.2 multiple DNA gains ranging from 49,216,751 bp to 50,333,944 bp) with developmental delay, facial dysmorphy and scoliosis. Partial trisomy for 20q is rare and most cases reported in the literature resulted from unbalanced segregation products of a balanced reciprocal translocation in a carrier parent, involving chromosome 20 and another chromosome. Moreover, supernumerary marker chromosomes including extra ring 20 chromosome are rare events that involved short arms and pericentric regions but are not extended until 20q13.2 cytogenetic band of long arm [16]. So far, fifteen partial 20q trisomies have been described. Eleven of them resulted from malsegregation of a parental translocation or pericentric inversion of chromosome 20. As the sizes of these trisomies varied and as some of them were associated with monosomies, genotype-phenotype correlation for trisomy 20q has not been clearly established [1-11]. Only four cases of pure partial trisomy 20q have been cytogenetically and clinically described, and among them two have been more precisely characterized and reported here (patients 2 and 3 initially described in Blanc P. *et al*, Am J Med Gen, 2008 [12] and Menten B. *et al*, J Med Genet, 2006 [13], respectively). The third one is defined by an overlapping *de novo* duplication of 20q13.12q13.31, with RP11-75C17 first duplicated clone and RMC20B4087 last duplicated clone, and is thus included in the discussion (Iglesias A. *et al*, Clin Dysmorphol, 2006 and personal communication) [14].

Another case with 20q trisomy published elsewhere was found not to overlap with the region of interest and is therefore not included in the discussion [15].

Among common clinical features, all children shared the developmental delay and dysmorphic features as bilateral epicanthus, upslanting palpebral fissures, large forehead and thin upper lip (Table 1 and Fig. 1). Skeletal and cardiac malformations were present in ¾ of the patients.

The region of interest concerning the four patients includes the three genes *NFATC2*, *ATP9A* and *SALL4* and the microRNA 3194. We focused our study on genes related to musculoskeletal, cardiac or neurodevelopmental functions, as these clinical features are shared by the four cases (Fig. 3b).

Duplication of *SALL4* (sal-like 4) gene is shared by patients 1, 2, and the patient of Iglesias *et al* (2006). *SALL4* encodes a zinc transcription factor whose deletion or point mutation are associated with Duane-radial ray syndrome (Okihiro syndrome), an autosomal dominant disorder characterised by upper limb, mostly radial anomalies, ocular, renal, and congenital heart malformations, especially ventricular septal defects [17] (MIM*607343). It is noteworthy that patients 1 and 2 presented ventricular septal defects [18-21]. In mice model, a role of *Sall4* in the patterning and morphogenesis of the mouse limb and heart has been suggested, and many features of Okihiro syndrome are observed in *Sall4* targeted disruption model [17,22].

Another gene of interest, *NFATC2* (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2), was encompassed in all DNA gains (Fig. 3b). NFAT factors act as transducers in the calcineurin function in heart [23]. In mice and zebrafish models, Nfatc2 has been shown to be required in initiation of heart valve morphogenesis and perpetuation of embryonic valve formation [24-27] (OMIM *600490). Among the cases reported here, two exhibited atresia of mitral and aortic valves (1 and 2) and the patient of Iglesias *et al* (2006) exhibited aortic coarctation. Thus, cardiac malformations could result from a combined abnormal expression of *SALL4* and/or *NFATC2*. However, no cardiac malformation was

described in P3 while a multiple copies of *NFATC2* was observed without *SALL4* copy gain. This phenotypic variability can be ascribed to incomplete penetrance associated with *NFATC2* expression modification, or to functional redundancy of NFAT factors.

Furthermore, a role of *Nfatc2* has been identified in skeletal development, cartilage cell differentiation and osteoblast function [28,29]. *Nfatc2*-deficient chondrocytes exhibited increased proliferation *in vitro*, compared to wild-type cells. Additionally, *Nfatc2* is specifically regulated during chondrogenic differentiation of mesenchymal cells *in vitro*. Therefore, *Nfatc2* is believed to be a repressor/inhibitor of chondrogenesis [28, 30-33]. Thus, Nfact2 could be related to the skeletal features observed in ¾ patients.

In conclusion, we report three unrelated patients, as well as an overview of the patient reported by Iglesias *et al* (2006), all sharing DNA gain at 20q13.2 and similar clinical features including a developmental delay, cardiac malformation, skeletal anomalies and dysmorphic features. This report further highlights the genotype-phenotype correlations with an involvement of *SALL4*, and likely *NFATC2*, in cardiac malformations, and *NFATC2* in skeletal anomalies. It remains that additional patients are needed to further delineate the phenotypic impact entailed by enhanced expression of these genes.

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Titles and legends to figures

Figure 1

Photograph of fetus (P1) at 23 weeks of gestation. Note anteverted nares, prominent philtrum and retrognathia (a, b). Photograph of patient 2 : 30 months-old (c, d). Note large forehead, bilateral epicanthus, small bulbous nose with slightly anteverted nares, thin upper lip, low-set ears with slanted crease to the lobes, pectus excavatum and small umbilical hernia (photograph from Blanc P. *et al*, Am J Med Genet A 2008 with their permission).

Figure 2

Chromosome 20 profiles from aCGH with whole chromosome on the left showing the interstitial gain and on the right, (a) the 20q13.13q13.2 duplication in P1 (b) the 20q13.2q13.33 duplication in P2, and (c) the 20q13.13q13.2 gain in P3. Evidence by FISH analysis of the gain in P3 lymphocytes (d) attested by the presence of an asymmetric red signal on metaphase spread and by the presence of three or four red spots on interphasic nuclei for the BAC clone RP5-944O24.

Figure 3

Chromosome 20 scheme illustrating the *de novo* DNA gains at 20q13.2 in patients 1–3 and patient reported by Iglesias *et al* (2006) using the Database of Genomic Variants browser (hg19). Copy gains regions for the respective cases are shown as black bars (Fig. 3a). Genes included in region partly shared by the four cases are shown in the bottom (Fig. 3b).

Table 1

Summary of clinical and genetic features in present patients and previously reported case with *de novo* DNA gain at 20q13.2. F, female; M; male; Na, not available; Nd, not determined.

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