High-resolution karyotyping by oligonucleotide microarrays : the next revolution in cytogenetics

Gijsbers, A.C.J.

Chapter 3.2

Additional cryptic CNVs in mentally retarded patients with apparently balanced karyotypes

Antoinet CJ Gijsbers, Cathy AJ Bosch, Johannes G Dauwerse, Osdilly Giromus, Kerstin Hansson, Yvonne Hilhorst-Hofstee, Marjolein Kriek, Arie van Haeringen, Emilia K Bijlsma, Egbert Bakker, Martijn H Breuning and Claudia AL Ruivenkamp

Center for Human and Clinical Genetics, Leiden University Medical Center (LUMC), the Netherlands

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Abstract
Apparently balanced chromosome abnormalities are occasionally associated with mental retardation (MR). These balanced rearrangements may disrupt genes. However, the phenotype may also be caused by small abnormalities present at the breakpoints or elsewhere in the genome. Conventional karyotyping is not instrumental for detecting small abnormalities because it only identifies genomic imbalances larger than 5-10 Mb. In contrast, high-resolution whole-genome arrays enable the detection of submicroscopic abnormalities in patients with apparently balanced rearrangements.

Here, we report on the whole-genome analysis of 13 MR patients with previously detected balanced chromosomal abnormalities, five *de novo*, four inherited, and four of unknown inheritance, using Single Nucleotide Polymorphism (SNP) arrays. In all the cases, the patient had an abnormal phenotype. In one familial case and one unknown inheritance case, one of the parents had a phenotype which appeared identical to the patient's phenotype. Additional copy number variants (CNVs) were identified in eight patients. Three patients contained CNVs adjacent to one or either breakpoints. One of these patients showed four and two deletions near the breakpoints of a *de novo* pericentric inversion. In five patients we identified CNVs on chromosomes unrelated to the previously observed genomic imbalance.

These data demonstrate that high-resolution array screening and conventional karyotyping is necessary to tie complex karyotypes to phenotypes of MR patients.

Introduction
Recent advances in molecular cytogenetic technologies provide a resolution that exceeds that of conventional karyotyping and increased the detection of aberrations from 5% to approximately 17% in patients with mental retardation (MR) [13,16,17,19,23,28,31,32,34-36].

A disadvantage of the array technique is the incapability to detect balanced structural abnormalities such as translocations and inversions. Balanced rearrangements have a prevalence of at least 1:500 and in approximately 6% of antenatal patients with a balanced rearrangement an abnormal phenotype is found [20,37]. The abnormal phenotype of these patients can be explained by (1) breakpoint regions directly disrupting genes or transcription regulatory regions [21], (2) indirectly by submicroscopic copy number variants (CNVs) near one or both of the breakpoints [4], (3) the rearrangement hosts 'cryptic' complex chromosomal rearrangements (CCRs) [24], (4) submicroscopic CNVs unrelated to the translocation or inversion [3,6,18], or (5) another unidentified genetic or environmental factor.

Reports of single patients or small series of patients with apparently balanced aberrations have identified unexpected complexity and instability of the human genome [3,6,18,24]. Some studies investigated the difference between additional CNVs in carriers of *de novo* 'balanced' reciprocal translocations and CCRs with normal and abnormal phenotypes [1,11]. In approximately 35% of the phenotypically abnormal patients additional candidate disease-causing CNVs were identified, mostly occurring around the breakpoints of the translocations. In the phenotypically normal cohort no additional genomic CNVs were identified. Sismani and colleagues studied 12 MR patients both with *de novo* and familial apparently balanced translocations for the presence of cryptic CNVs [33]. Two *de novo* and one familial case had additional abnormalities. Recently, Schluth-Bolard and colleagues analysed 47 MR patients with *de novo* and familial apparently balanced chromosomal rearrangements [30]. All familial

rearrangements were inherited from phenotypically normal parents. Imbalances were detected in 16 *de novo* cases (48.5%) and in 4 inherited cases (28.6%).

We report on 13 patients carrying an apparently balanced translocation or inversion detected with conventional karyotyping. High-resolution Single Nucleotide Polymorphism (SNP) array analysis was performed to search for cryptic CNVs. In eight patients additional CNVs were detected. Herein we focus on the interpretation of the detected CNV in relation to the phenotypes of the patients.

Materials and methods

Patients

This study included 13 patients with MR, with or without multiple congenital malformations, and an apparently balanced translocation or inversion observed with conventional karyotyping (five *de novo*, four inherited, and four of unknown inheritance). A summary of the clinical and cytogenetic data of all patients is shown in Table 3.2.1. Karyotyping on GTG-banded chromosomes from cultured lymphocytes of the patient was performed according to standard techniques. The study was approved by the Leiden University Medical Center Clinical Research Ethics Board, conforming to Dutch law and the World Medical association Declaration of Helsinki.

SNP arrays

DNA was extracted from whole blood by a Gentra Puregene DNA purification Kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. The Affymetrix GeneChip Human Mapping 262K *NspI*, 238K *StyI* arrays (Affymetrix, Santa Clara, CA, USA) and Illumina HumanHap300, Human CNV370 BeadChips (Illumina Inc., San Diego, CA, USA) were performed following the manufacturers' instructions and data was analyzed as described previously [17]. Table 3.2.1 shows which SNP array platform was used for each patient.

Evaluation and validation of CNVs

Detected CNVs were evaluated as described previously [17]. The potentially pathogenic CNVs were confirmed with Fluorescence *In Situ* Hybridization (FISH) analysis or another type of SNP array using an independent DNA sample. If parents were available, segregation analysis was performed by FISH or SNP array analysis. FISH analysis was carried out by standard procedures as described previously [9]. BAC clones mapping to the unbalanced chromosome regions were selected based on their physical location within the affected region (http//: www.ensembl.org, Ensembl release 54 - May 2009, Genome build NCBI36).

All potentially pathogenic CNVs were assessed with Ensembl (Ensembl release 54 - May 2009, Genome build NCBI36) and DECIPHER (https://decipher.sanger.ac.uk) for gene content and patients with similar CNVs respectively. Finally, data of all patients with (potentially) pathogenic CNVs was added to the DECIPHER database.

Results

SNP array analysis demonstrated 16 additional submicroscopic CNVs in eight of the 13 patients (61.5%); five out of the five *de novo*, one out of the four familial and two out of the four unknown inheritance cases. In the remaining five patients no additional CNVs were detected. The 16 CNVs consisted of 15 interstitial deletions ranging in size from 59 kb to 10.11 Mb and one interstitial duplication of 2.78 Mb. Results are described in detail in Table 3.2.1.

Table 3.2.1 Cytogenetic, clinical and SNP array data of all patients

ID	Structural aberration	Phenotype	SNP array	Chromosome	Starting SNP	Ending SNP	Size
	CNV near / at breakpoint(s)						
1	inv(5)(q22q31.3) *dn*	Mental retardation *	*NspI* and 317K	4x del 5q14.3, *dn*	rs4920853	rs1862233	59 kb
					rs16902356	rs10051603	83 kb
					rs7715840	rs1062035	320 kb
					rs357509	rs304151	638 kb
				2x del 5q33.3, *dn*	rs6556381	rs9637861	130 kb
					rs6884239	rs3846687	159 kb
2	t(11;12)(q13.3;p12.3), inv(12)(p12.3p13.1) n/a	Mental retardation, psychiatric problems	*NspI*	del 12p12.3p12.3, n/a	rs7306438	rs1865936	2.51 Mb
3	inv(7)(q31.3q34) *dn*	Mental retardation, anxiety disorder, autistic behavior	*NspI*	del 7q21.11q21.3, *dn*	rs2373207	rs17166393	8.29 Mb
	CNV on unrelated chromosome(s)						
4	inv(6)(p21.3q15) n/a	Mental retardation, obesity	*NspI* and 370K	del 18q21.31q21.31, n/a	rs4940582	rs4940754	877 kb
				del 18q21.32q21.33, n/a	rs1877055	rs588677	2.6 Mb
5	t(3;18)(p14.2;q23) mat	Mental retardation *	317K	del 22q11.22q11.23, n/a	rs38114997	rs6003620	630 kb
6	t(2;6)(q37.1;q13) *dn*	Mental retardation *	317K	del 13q12.3q12.3, *dn*	rs1023166	rs7332696	1.24 Mb
7	t(2;6)(q24.1;p24.3) *dn*	Developmental delay *	*NspI*	dup 1p32.3p32.2, *dn*	rs563403	rs6670302	2.78 Mb
	CNV at or near the breakpoint plus additional, unrelated CNV						
8	t(12;14)(q21.3;q32.1) *dn*	Dysmorhpic features*	*NspI*	del 3p12.3p12.3, *dn*	rs7622824	rs11920974	1.15 Mb
				del 4q28.3q31.23, *dn*	rs1519335	rs6838916	10.11 Mb
				del 12q21.31q21.33, *dn*	rs1994104	rs7133204	6.88 Mb
	No additional CNV						
9	t(1:4), pat	Psychomotor retardation	*NspI*	balanced			
10	t(2;9)(q14.2;q32), mat	Mild developmental delay	*NspI*	balanced			
11	t(12;13)(p11.2;q31), n/a	Severe speech delay, mild developmental delay, pectus carinatum, high and thin palatum	317K	balanced			
12	t(9;13)(p22;q14.3), pat	'psuedo'achondroplasia	317K	balanced			
13	t(4;10)(q21.1;q25.2), n/a	Short stature	*StyI*	balanced			

dn, de novo; n/a, (one of the) parents not available; mat, maternally inherited; pat, paternally inherited; *, extended clinical information provided in results

The parental origin of the known *de novo* deletions could be determined in three patients (1, 3, and 8). In all these patients the deletions occurred on the paternal chromosome. In patients 4, 5, and 6 the breakpoints of the deletions were flanked by segmental duplications (according to the Database of Genomic Variants, DGV; http://projects.tcag.ca/variation).

CNVs at or near the breakpoint regions

Eight CNVs in three patients were located at or near one of the breakpoints of the apparently balanced chromosome abnormalities (Table 3.2.1). Two of these patients (1 and 3) had more complex chromosome abnormalities.

Patient 1

The patient was a 1-year old boy with MR, sleeping problems, grand-mal seizures, sensorineural deafness, severe loss of vision, epicanthic folds, strabismus, ventricular septal defect (VSD) and general hypotonia. He was initially diagnosed with a *de novo* paracentric inversion of region 5q22q31.3 (Fig. 3.2.1a). Additional SNP array screening showed four *de novo* interstitial deletions on band 5q14.3 (Fig. 3.2.1b and c) and two *de novo* interstitial deletions on band 5q33.3 (Fig. 3.2.1b and d). The presence of the six deletions was confirmed by a different SNP array platform (NspI, Affymetrix). Based on the SNP array data the inversion breakpoints were revised to 5q14.3 and 5q33.3. These breakpoints were confirmed by high resolution G- banding. The 6 deletions contain eight known coding genes (*COX7C*, *RASA1*, *CCNH*, *TMEM161B*, *MEF2C*, *EBF1*, *RNF145* and *UBLCP1*).

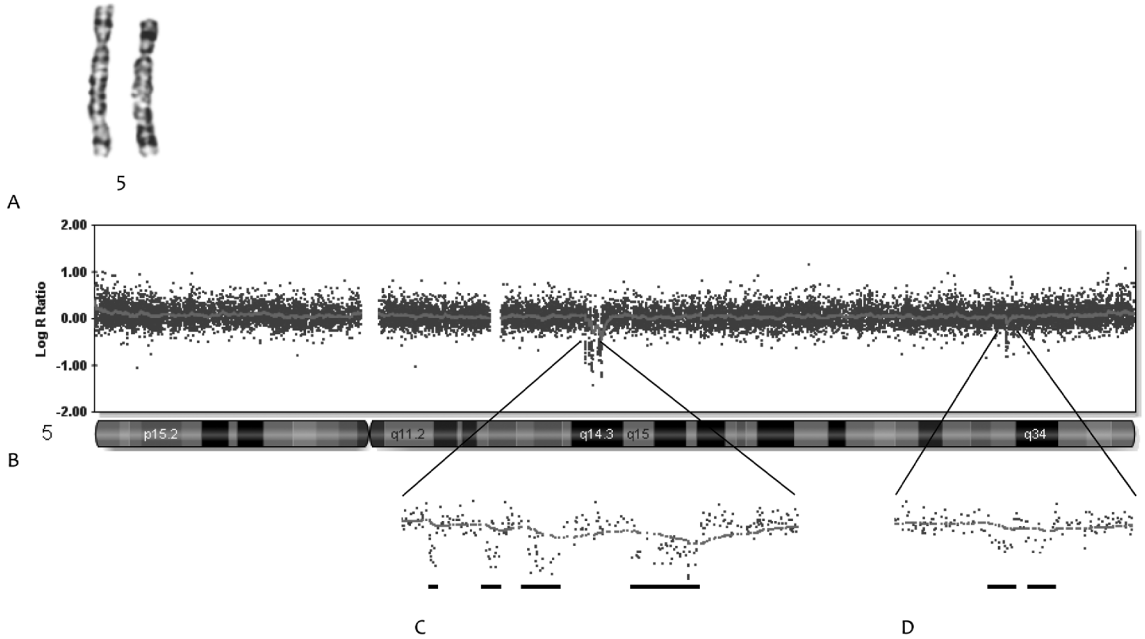

Figure 3.2.1 Cytogenetic and molecular results for patient 1. (a) Partial karyotype showing chromosome 5. Right: abnormal chromosome 5 with inversion 5q14.3q33.3. (b) SNP array copy number plot (Illumina HumanHap 300 BeadChip) for chromosome 5. (c) Four deletions at 5q14.3 and (d) two deletions at 5q33.3

Patient 2

The patient was a 61-year old male with MR and psychiatric problems. Conventional karyotyping showed a translocation between the long arm of chromosome 11 and the short arm of chromosome 12. The breakpoints were determined at 11q13.3 and 12p12.3. In addition, a pericentric inversion of region 12p12.3q13.1 was observed in the derivative chromosome 12. Subsequent SNP array analysis identified an interstitial

deletion on band 12p12.3. The parents were not available for testing. The 2.51 Mb deleted region contains eight known coding genes (*RERG*, *PTPRO*, *EPS8*, *STRAP*, *DERA*, *SLC15A5*, *MGST1* and *LMO3*).

Patient 3

The patient was a 15-year old mentally retarded girl. She had a severe anxiety disorder and autistic features. The patient is described in detail by Dauwerse and colleagues [10]. Conventional karyotyping showed a *de novo* paracentric inversion of region 7q31.3q34. However, FISH analysis in order to map the exact breakpoints identified an insertion of region 7q31.31q35 within band 7q21.3. SNP array screening demonstrated a *de novo* interstitial deletion at the insertion site from chromosome bands 7q21.11 to 7q21.3. The deletion contains approximately 40 known coding genes.

CNVs on unrelated chromosomes

Six CNVs in four patients were detected on chromosomes not related to the chromosomes involved in the balanced inversions or translocation (Table 3.2.1).

Patient 4

The patient was a 42-year old male, diagnosed with MR and obesity. Conventional karyotyping showed a pericentric inversion of chromosome region 6p21.3q15 (Fig. 3.2.2a). Additional SNP array analysis identified two interstitial deletions on the long arm of chromosome 18 in chromosome bands q21.31 and q21.32 (Fig. 3.2.2b and c). The patient's mother and brother showed a normal karyotype and normal SNP array results. His father was not available for testing. The two deletions contain 13 known coding genes (*NEDD4L*, *ALPK2*, *MALT1*, *ZNF532*, *SEC11C*, *GRP*, *RAX*, *CPLX4*, *LMAN1*, *CCBE1*, *PMAIP1*, *MC4R*, *CDH20*).

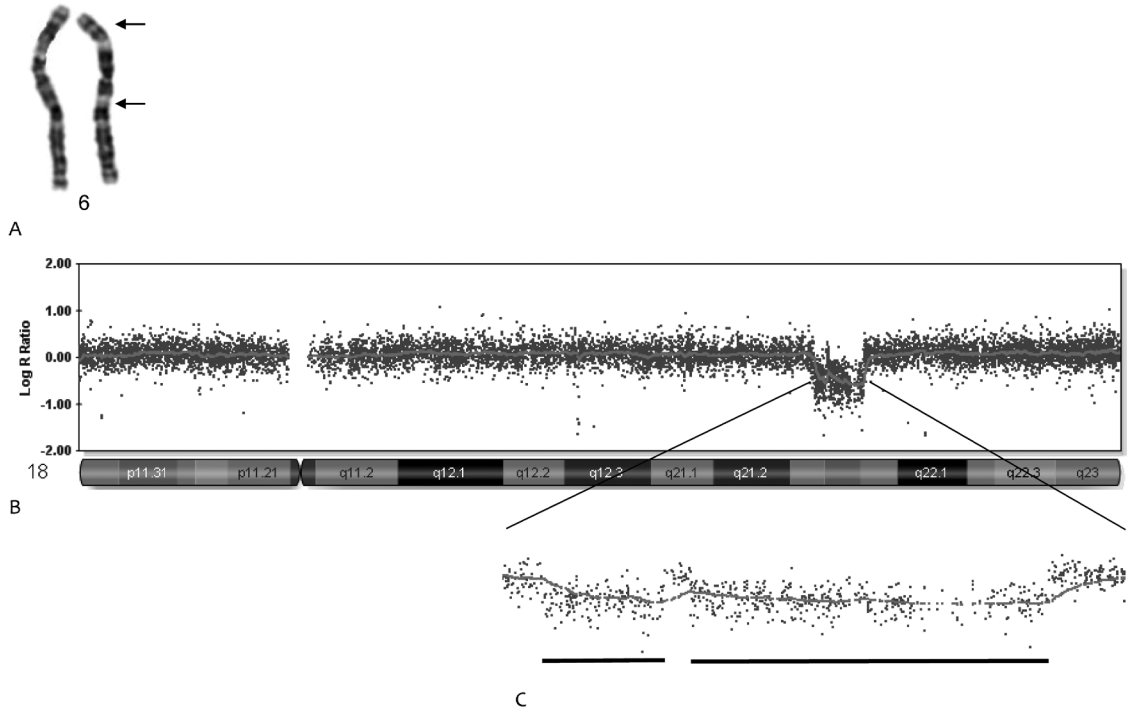

Figure 3.2.2 Cytogenetic and molecular results for patient 4. (a) Partial karyotype showing chromosome 6. Right: abnormal chromosome 6 with inversion 6p21.3q15. (b) SNP array copy number plot (Illumina HumanCNV370 BeadChip) for chromosome 18. (c) Two deletions at 18q21.31q21.31 and 18q21.32q21.33.

Patient 5
The patient was a 46-year old female with MR, deafness, heterochromia of the iris, a depigmented forelock, hypertension and hypothyroidism. Mutations in the coding region of *MITF* (Waardenburg syndrome) were excluded by sequencing. Conventional karyotyping showed a balanced translocation t(3;18)(p14.2;q23). SNP array analysis identified one additional deletion on the long arm of chromosome 22 between bands 22q11.22 and q11.23. Conventional karyotyping and SNP array results of the father were normal. The mother was not available for testing. However a healthy sister of the proband showed the same t(3;18)(p14.2;q23). The 22q deletion contains four known coding genes (*RTDR1*, *GNAZ*, *RAB36* and *BCR*).

Patient 6
The patient was a 5-year old boy with mild MR and hyperactivity. He had several dysmorphic features, including microcephaly, coarse hair, hypotelorism, a narrow nasal bridge, a long columella, large ears, pectus excavatum, syndactyly of 2-3 toes, and patchy depigmentation of the skin. Conventional karyotyping identified a *de novo* balanced translocation t(2;6)(q37.1;q13). SNP array results revealed an interstitial deletion on the long arm of chromosome 13 band q12.3. In the 1.24 Mb deletion five known coding genes (*KIAA0774*, *SLC7A1*, *UBL3*, *KATNAL1*, *HMGB1*) are located. FISH analysis on the parents showed that the deletion occurred *de novo*. Parental DNA for SNP array analysis was not available.

Patient 7
The patient was a 1-year old boy with mild developmental delay, a unilateral cleft palate and dysmorphic features, including protruding ears and a unilateral preauricular earpit, long eyelashes, prominent arched eyebrows and strabismus. On his philtrum he had a dimple. X-ray of the spine showed posterior fusion defects of several thoracic vertebrae. Conventional karyotyping showed a *de novo* balanced translocation t(2;6)(q24.1;p24.3). SNP array results revealed a *de novo* interstitial duplication on chromosome 1p32.3p32.2 that contains 23 known coding genes. The parental origin of this duplication could not be determined with SNP array analysis.

CNV at or near the breakpoint plus additional, unrelated CNV
Patient 8
The patient was a new born girl with low-set ears, a prominent forehead, pulmonary stenosis and a VSD. Conventional karyotyping showed a *de novo* translocation t(12;14)(q21.3;q32.1). Subsequent SNP array analysis detected three *de novo* deletions on chromosome bands 3p12.3, 4q28.3q31.23, and 12q21.31q21.33, the latter at the breakpoint of the translocation. The deletions contain in total 52 known coding genes.

Discussion
The development of high-resolution array platforms allows the detection of CNVs in carriers of apparently balanced chromosome aberrations. In this study we have analyzed 13 MR patients with previously detected apparently balanced chromosomal rearrangements. Three of the patients had a breakpoint-associated imbalance, four had an imbalance on an unrelated chromosome and one patient had both an additional imbalance near the breakpoint of a translocation as well as cryptic deletions on unrelated chromosomes.

CNVs at or near the breakpoint regions

Three of the 13 patients showed additional CNVs at the breakpoints (patients 1-3). The deletions in patients 1 and 3 are highly likely contributing to the patients' phenotypes, since both are *de novo* and other patients have been reported with deletions in the same regions.

The 5q14.3 deletion region of patient 1 is recently described, five patients showed a 5q14.3 microdeletion and phenotypic similarities, including severe MR with absent speech, epilepsy, hypotonia and stereotypic movements [22]. The minimal overlapping region in their study encompassed the *MEF2C* gene. The phenotype of patient 1 is therefore most probably caused by haploinsufficiency of the *MEF2C* gene. None of the 12 breakpoint regions of the 6 *de novo* deletions in patient 1 contained low copy repeats (LCRs). It is therefore not likely that non-allelic homologous recombination (NAHR) underlies this complex single chromosome rearrangement. Poot and colleagues proposed that such a complex single chromosome rearrangement may be the result of mismatched repair of multiple double-strand breaks that co-localize in a chromosome at the time of DNA-damage induction [25].

None of the genes in the 12p12.3 deletion of patient 2 could be directly related to the phenotype. To our knowledge, a deletion of the same region has not been reported yet. Since we were not able to investigate the parents, pathogenicity of the deletion remains uncertain.

The phenotype of patient 3 overlaps with patients reported with *de novo* 7q21.1q21.3 deletions [8]. As explained previously disruption of the C7orf58 gene in band 7q31.31, one of the insertion breakpoints, may explain anxiety disorder and/or autistic features [10].

CNVs on unrelated chromosomes

In four patients the translocation or inversion appeared balanced, but SNP array analysis detected cryptic CNVs on unrelated chromosomes (patients 4-7). Haploinsufficiency of one or more genes in the deletions of patients 4, 5, and 6 may have contributed to the patients' phenotypes.

Partial deletions of the long arm of chromosome 18 lead to variable phenotypes. The region 18q12.1q21.33 could be associated with mild to severe MR [12,14], explaining the phenotype of patient 4. Furthermore, *MC4R* (melanocortin 4 receptor) may have contributed to the obesity in this patient. The *MC4R* gene is a member of the melanocortin receptor family and represents a G-protein coupled seven transmembrane receptor. Genetic studies related melanocortin receptors to genetically determined obesity [5,7].

The deletion detected in patient 5 overlaps partly with a known microdeletion syndrome. The recurrent 22q11.2 distal deletions are either approximately 1.4 Mb or 2.1 Mb in size with a common proximal breakpoint flanked by LCR22-4 [2]. They differ at the distal breakpoints flanked by either LCR22-5 for the smaller deletion or LCR22-6 for the larger deletion [2]. The breakpoints for the deletion of patient 5 are LCR22-5 and LCR22-6. The same deletion has been reported before in one patient and her healthy father [26]. This patient had a congenital heart defect, normal appearance and psychomotor development, and minimal dysmorphic features. The clinical features of patient 5 do not resemble the previously reported patient.

Patients with larger 13q deletions than patient 6 have been reported, but presented no recognizable phenotype [29]. One patient, with a deletion of 1.43 Mb on

13q12.3 (DECIPHER ref. 2154), is partly overlapping with the deletion in our patient. The common clinical features are MR, microcephaly, and large ears. The smallest region of overlap contains two genes, *KATNAL1* and *HMGB1*, which might be responsible for the overlap in phenotype.

The clinical relevance of the *de novo* interstitial 1p duplication in patient 7 is not clear. Interstitial duplications of chromosome 1p are rare and are associated with a variable phenotype [15]. One patient has been described with a similar duplication, however this patient also carried a deletion within 1p36.32 [15].

CNVs at or near the breakpoint plus additional, unrelated CNV

All three deletions in patient 8 occurred *de novo* and it is highly likely that haploinsufficiency of one or more genes have contributed to the patient's phenotype. Each deletion partly overlaps with patients described in the DECIPHER database (refs. 2059, 790 and 1020), although no similar phenotypes have been described.

General discussion and conclusion

Since there is no applicable technique available yet to detect balanced chromosome rearrangements and cryptic imbalances in one experiment, both high-resolution array screening and conventional karyotyping were necessary to unravel the complex karyotypes described in this paper. In 61.5% of our patients with an apparently balanced aberration, we detected an additional cryptic CNV.

In all five *de novo* cases an additional cryptic CNV was identified. Previous published data have shown cryptic imbalances in approximately 30-50% of MR patients with *de novo* apparently balanced chromosome rearrangements [11,33]. The high occurrence in our study is probably due to the small sample size. In only two of the four patients with an inherited translocation or inversion, one of the parents showed a similar phenotype as the proband. No additional CNVs were detected in these two cases. In one of the other familial cases an additional CNV not related to the translocation was detected (patient 4). Since, DNA of the mother was not available the inheritance of this CNV could not be determined.

By SNP haplotype analysis we could determine for three of the patients that the deletions had occurred in the paternal allele. This observation is consistent with previous studies suggesting that male gametogenesis is more susceptible to this type of chromosome abnormalities [11].

In the remaining patients where no additional abnormalities were detected the presence of cryptic imbalances explaining the phenotype obviously cannot be excluded. It is possible that higher resolution arrays may reveal smaller aberrations that could have been missed in our analysis. Alternatively, the breakpoints of the apparently balanced rearrangements might disrupt putative disease genes or cause a position effect giving rise to the abnormal phenotype.

With the advent and application of high-resolution array screening it was demonstrated that man is more genetically variable than previously considered [27]. Each individual (healthy or not) presents multiple CNVs in its genome. Yet, unless reported in healthy individuals or patients with similar phenotypes, the pathogenicity of a substantial number of CNVs remains uncertain. The clinical interpretation of CNVs is even more difficult in patients with multiple chromosomal aberrations like the ones described in this paper, as the phenotypes might be the result of a combination of two or more chromosome aberrations [3,6,18].

The findings described here once more illustrate that genomic CNVs are an important cause of phenotypic abnormalities in carriers of apparently balanced chromosome aberrations [1,3,6,11,18,24,30,33]. We recommend re-evaluating MR patients with an apparently balanced karyotype (translocation, inversion or CCR) by whole-genome high-resolution array analysis. Furthermore, we advise to first investigate MR patients with a SNP array analysis and if more complex abnormalities are suspected a follow-up by conventional karyotyping [17].

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