ORIGINAL ARTICLE

Emerging patterns of cryptic chromosomal imbalance in patients with idiopathic mental retardation and multiple congenital anomalies: a new series of 140 patients and review of published reports

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Background: Chromosomal abnormalities are a major cause of mental retardation and multiple congenital anomalies (MCA/MR). Screening for these chromosomal imbalances has mainly been done by standard karyotyping. Previous array CGH studies on selected patients with chromosomal phenotypes and normal karyotypes suggested an incidence of 10–15% of previously unnoticed de novo chromosomal imbalances.
Objective: To report array CGH screening of a series of 140 patients (the largest published so far) with idiopathic MCA/MR but normal karyotype.
Results: Submicroscopic chromosomal imbalances were detected in 28 of the 140 patients (20%) and included 18 deletions, seven duplications, and three unbalanced translocations. Seventeen of 24 imbalances were confirmed de novo and 19 were assumed to be causal. Excluding subtelomeric imbalances, our study identified 11 clinically relevant interstitial submicroscopic imbalances (8%). Taking this and previously reported studies into consideration, array CGH screening with a resolution of at least 1 Mb has been undertaken on 432 patients with MCA/MR. Most imbalances are non-recurrent and spread across the genome. In at least 8.8% of these patients (38 of 432) de novo intrachromosomal alterations have been identified.
Conclusions: Array CGH should be considered an essential aspect of the genetic analysis of patients with MCA/MR. In addition, in the present study three patients were mosaic for a structural chromosome rearrangement. One of these patients had monosomy 7 in as few as 8% of the cells, showing that array CGH allows detection of low grade mosaicisms.

Chromosomal abnormalities are a major cause of mental retardation and congenital malformations. Many chromosomal defects are readily detected by standard or high resolution karyotyping. However, at best, the resolution of cytogenetic analysis is limited to about 5 to 10 Mb. It has long been assumed that a considerable proportion of patients with multiple congenital anomalies and mental retardation (MCA/MR) have submicroscopic chromosomal imbalances, not detectable by routine karyotyping. Such hidden abnormalities have been detected at the subtelomeric regions in around 5% of these patients.[1–4] Following the introduction of the principle of array comparative genomic hybridisation (CGH),[5 6] genome-wide high resolution analysis for DNA copy number alterations became feasible. In analogy with karyotyping, genome-wide array CGH has been termed molecular karyotyping.[7–9] The first papers by Vissers *et al*[10] and Shaw-Smith *et al*[11] reported as much as 15–24% of segmental aneusomies in patients with idiopathic mental retardation and dysmorphism. A few additional studies reported detection rates between 10% and 25%.[12–14] To evaluate the clinical relevance of a chromosomal imbalance, there is a need to collect genotype and phenotype information in a large number of patients. This will allow the determination of the incidence and the genomic distribution of disease causing imbalances and may reveal the underlying mechanisms causing chromosomal imbalances.

In this study we report array CGH data on a new series of 140 patients and review the findings of 292 previously reported patients in order to determine the overall incidence and clinical relevance of each of these chromosomal imbalances. In addition, we provide the first evidence that array CGH screening allows the detection of low grade mosaicism for chromosomal aberrations.

METHODS

Selection of patients

This was a collaborative study between the genetic teams of Leuven and Gent. Patients were selected for the study by clinical geneticists from both teams. The study was approved by the institutional review board and appropriate informed consent was obtained from human subjects. Subjects had mental handicap without known aetiology, but a chromosomal aberration was suspected because of the association with one or more major congenital malformation (such as congenital heart defect, cleft palate, brain malformation, and so on), or dysmorphism (three or more minor anomalies), or both. Ages varied between one and 62 years, with a mean age of 13.1 years. The number of males and females was about equal. All patients had a normal karyotype on G banding analysis at ISCN +550. The presence of a

Abbreviations: BAC, bacterial artificial chromosome; CGH, comparative genomic hybridisation; CNV, copy number variation; MCA/MR, mental retardation and multiple congenital anomalies; PAC, P1 derived artificial chromosome; RTQ-PCR, real time quantitative polymerase chain reaction

subtelomeric abnormality was excluded by fluorescence in situ hybridisation (FISH) or multiplex ligation-dependent probe amplification (MLPA) in 31 of 140 patients. Genomic DNA from each patient was isolated either from blood lymphocytes or from cultured fibroblasts. When consent could be obtained, full phenotypic descriptions of patients with anomalies were submitted to DECIPHER (database of chromosomal imbalance and phenotype in humans using ensembl resources: http://www.sanger.ac.uk/PostGenomics/decipher/).

Array CGH

Bacterial artificial chromosome (BAC) arrays were developed from the 1 Mb clone set of the Sanger Institute which contains 3431 BAC and PAC clones, as previously described.[8 15 16] In short, BAC and PAC (P1 derived artificial chromosome) DNA was isolated from 1 ml bacterial cultures and amplified by two rounds of degenerate oligonucleotide primer polymerase chain reaction (DOP-PCR) using an amino linked primer in the second PCR,[15] and purified on Multiscreen purification plates (Millipore Inc, Bedford, Massachusetts, USA). Purified aminolinked PCR products were spotted in duplicate or triplicate at a concentration of 250 ng/μl on three dimensional CodeLink Bioarray System slides (Amersham Biosciences, Piscataway, New Jersey, USA) with a Lucidea spotter (Amersham Biosciences) or a QArrayMini spotter (Genetix). DNA (300 ng) was labelled by a random prime labelling system (BioPrime Array CGH genomic labelling system, Invitrogen, San Diego, California, USA) using Cy3 and Cy5 labelled dCTPs (Amersham Biosciences). Probe concentration and labelling efficiencies were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, Delaware, USA). Following labelling, hybridisation, and washing of the slides, arrays were scanned at 532 nm and 635 nm using a GenePix 4000B scanner (Molecular Devices) or a GMS 418 scanner (MWG).

Image and data analysis

The scan images were processed with Imagene software (Biodiscovery, El Segundo, California, USA) and further analysed with an in-house developed and freely available software tool, ''arrayCGHbase'' (http://medgen.ugent.be/arrayCGHbase/).[16] In brief, spot intensities were corrected for local background and only spots with signal intensities at least 1.5 times above background were included in the analysis. Where useful, further normalisation of the data was achieved by two dimensional Lowess normalisation using Bioconductor software.[17] Following this normalisation, the values of the duplicates/triplicates on the array and the duplicate experiments were averaged and a $\log_2$ value was calculated. If signal intensity ratios among replicate spots deviated by more than twice the overall standard deviation of all intensity ratios, the spot was not analysed further. At least 95% of the spotted clones fulfilled these quality criteria. The experiment was only scored successful if the standard deviation of the $\log_2$ of the overall spot intensity ratios was less than 0.096. Typically, this SD value for a combined experiment is between 0.035 and 0.06. Clones that have been identified in previous control hybridisations and other studies as being polymorphic were excluded from the analysis.[8 18] Of the 3431 targets on the array, 57 autosomal and eight X chromosomal clones are considered to be polymorphic.

Two or more flanking targets exceeding a value of the mean $\pm$ four times the SD of the $\log_2$ of all intensity ratios for that hybridisation experiment were further investigated to confirm the presence or absence of a genomic imbalance. Single targets showing hybridisation intensity ratios exceeding a value of $\pm$ [$\log_2$(3/2)–2*SD] were also further validated. Validation was undertaken by metaphase FISH for all potential deletions and both metaphase and interphase FISH analysis for all potential duplications larger than 2 Mb in size. Real time quantitative PCR was used to confirm duplications smaller than 2 Mb in size. If in two or more flanking clones the $\log_2$ of the combined intensity ratios exceeded the threshold value of 4×SD, FISH or real time quantitative PCR experiments always confirmed the presence of a chromosomal imbalance. If the intensity ratio exceeded $\pm$ [$\log_2$(3/2)–2*SD] at only one isolated clone in both experiments, a false positive rate of one every seven patients is observed.

FISH

Labelling of the DOP amplified BAC DNA that was used for spotting the arrays was carried out by DOP-PCR on a thermocycler (GeneAmp9700, Applera, Nieuwekerk a/d Ijzer, Netherlands). The reactions were done in a total volume of 50 μl containing 5 μl of 15 μM DOP 1, 2, 3 primermix, 5 μl of 10× PCR buffer w/o $MgCl_2$, which is specially designed for use with Platinum® *Taq* DNA polymerase (Invitrogen), and 5 μl of 50 mM $MgCl_2$. For the dNTPs we used 1 μl of 10 mM dATP, dCTP, dGTP each, 0.7 μl of 10 mM dTTP, 1 μl of 1 mM SpectrumGreen™ or SpectrumOrange™ dUTP (Vysis, Abbott Laboratories, Abbott Park, Illinois, USA) or 5 μl of 10× dNTP mixture containing 1 mM biotin-14-dCTP, 1 mM dCTP, 2 mM dATP, 2 mM dGTP, 2 mM dTTP in 10 mM Tris-HCl (pH 7.5), 1 mM Na_2 EDTA (Bioprime DNA labelling system, Invitrogen). Platinum® *Taq* DNA polymerase (Invitrogen) (0.5 μl), 2 μl of the DOP amplified BAC DNA and H_2O to 50 μl were added. After initial denaturation at 95℃ for 10 minutes, the reaction was as follows: 35 cycles of 95℃ for one minute, 60℃ for one minute, 72℃ for one minute, and a final extension step of 72℃ for 10 minutes.

Purification of the PCR product was carried out with the Qiaquick 8 PCR purification kit (Qiagen NV, Venlo, Netherlands) using QIAvac 6S vacuum according to the suppliers' instructions.

In addition to the region-specific BAC clones used for validation of array CGH results in patients with suspected imbalance, a chromosome 7 centromere specific probe was used for analysis of patient 19 with suspected monosomy 7 mosaicism (see Results). In all, 200 cells were screened for this patient and a control sample by two independent observers.

Before FISH, cells were air dried on slides and pretreated with pepsin followed by fixation with a 1% free formaldehyde solution and subsequent dehydration with ethanol. After hybridisation O/N at 37℃, the slides were washed for one minute in 0.4× SSC/0.3% NP40 solution at 72℃, one minute at 2× SSC/0.1% NP40 solution at RT, and one minute at 2× SSC. The cells were counterstained with DAPI and the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, California, USA). The signal was visualised by digital imaging microscopy with Cytovision capturing software (Applied Imaging, Santa Clara, California, USA). FISH was done as described.[19]

Real time quantitative PCR (RTQ-PCR)

The oligonucleotides were selected by using PrimerExpress 2.0.0 ABI Prism oligo design software (Applied Biosystems, Lennik, Belgium). A penalty score less than 150 was used to analyse the selected oligonucleotides further. The primers and amplicon were separately checked to exclude any repetitive sequences by using the BLAST program from the NCBI browser (http://www.ncbi.nlm.nih.gov/BLAST/) and

the repeatmasker program (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker).

RTQ-PCR was carried out using the qPCR mastermix Plus for SYBR Green I without UNG (Eurogentec, Liege, Belgium) according to the manufacturer's instructions. The final volume of 25 µl contained 0.5 mM of both forward and reverse primers, 12.5 µl of 2× reaction buffer and 5 µl of DNA solution in the range of 2 to 50 ng per reaction. Total genomic DNA from human blood was purified by using an automated version of the purification protocol using Chemagic Magnetic Separation (Chemagen Biopolymer Technologie AG, Baesweiler, Germany).

PCR was carried out in triplicate from each fraction using 50℃ for two minutes and 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds. An 81 base pair DNA fragment within the p53 gene (forward: 5′-CCC AAG CAA TGG ATG ATT TGA-3′ and reverse: 5′-GAG CTT CAT CTG GAC CTG GGT-3′) was used as a control amplicon (Eurogentec). Serial fivefold dilutions of this target ranging from 100 ng to 0.16 ng per experiment served as a standard quantitation curve.

RTQ-PCR was done with the locus-specific oligonucleotides of interest on an ABI PRISM 7000 Sequence Detection System (SDS) according to the manufacturer's instruction manual (Applied Biosystems, Lennik, Belgium). The amplification results and the melting curve were analysed with the ABI Prism 7000 SDS software version 1.1 (Applied Biosystems). The DNA levels were normalised to the gene p53 and relative differences were calculated according to the relative quantitation method.[20]

RESULTS

Array CGH findings in 140 patients with unexplained MCA/MR

One hundred and forty patients with unexplained mental retardation and features suggestive of a chromosomal anomaly (for example, a major malformation or multiple minor anomalies) were analysed on a 1 Mb BAC array. The DNA from each patient was labelled and hybridised with label swap versus the DNA of two other MCA/MR patients, rather than using a ''normal'' reference sample. Dye swap hybridisations for three patients in three hybridisations reduces by half the number of experiments and the cost per patient sample. This approach may be counterintuitive and seem inappropriate in a diagnostic setting. However, *the* ideal reference genome is non-existent owing to large scale copy number variations between the genomes of different ''normal'' individuals.[21 22] To mask benign copy number variation (CNVs), other groups have used pooled DNA of from seven to 10 different male or female subjects as reference material.[11–14] For frequently occurring CNVs, intensity ratios will be reduced. If a CNV were present in 50% of the population, the intensity ratio difference at this locus would be reduced by half. Rather than improving the outcome, this result complicates data interpretation. One disadvantage of using patients as reference in three hybridisations could be that similar imbalances in two or three of the patients would result in equal intensity ratios for the affected region and potentially mask imbalances. However, the finding that the recurrence of a similar chromosomal imbalance in two patients with idiopathic MCA/MR is less than 1% (see below) makes the risk that a similar imbalance would occur in two and three independent patients smaller than, respectively, $1/10^4$ and $1/10^6$.

A chromosomal imbalance was detected in 28 patients (20.1%). An overview of all imbalances is shown in fig 1, and array CGH profiles for aberrant chromosomes are presented as supplementary information. Table 1 summarises the genotype and phenotype of these 28 patients. For eight patients the imbalance spanned more than five clones (>5 Mb in size), for 10 patients between two and five clones (1–8 Mb in size), and for 10 patients the imbalance was only a single clone (<3 Mb). In two patients there was evidence of mosaicism for a structural chromosomal aberration and in one patient a low grade mosaicism for chromosome 7 monosomy was detected (see below). In 17 of 24 patients in whom the parents could be investigated the chromosomal imbalance was de novo by either FISH (deletions or duplications larger than 3 Mb) or quantitative PCR (qPCR) (small duplications). While none of the imbalances smaller than 5 Mb could be detected by high resolution karyotyping, three large deletions (in patients 7, 12, and 15) and two mosaics (in patients 14 and 18) became apparent after retrospective analysis of the karyotype. Eight imbalances (5.7%) involved a subtelomeric region.

All de novo alterations can be considered causal for the MCA/MR phenotype observed in the patients. For four of the 28 patients with a chromosomal imbalance, the parents were not available for genotyping. One of these (patient 1) had a large deletion on 1p36.2 spanning multiple clones. As the observed phenotype in this patient resembles that of patients with known 1p terminal deletions, this imbalance was considered causal. For patients 3, 19, and 20 only one or two clones were abnormal making the causal relation between genotype and phenotype difficult to determine.

For seven of the 28 patients the imbalance (three duplications and four deletions) was inherited from one of the parents. These parents were phenotypically normal with the exception of the father of patient 27, who had mild learning disabilities, and the mother of patient 7, who was similarly affected as the daughter. Patient 27 presented with cleft lip and palate, mild learning difficulties, and a truncus arteriosus. A duplication on chromosome 22q11.2 was detected in this girl and her father. In view of previous reports describing 22q11.2 duplications (including those inherited from normal parents), we assume a direct relation between the 22q11.2 duplication and the observed phenotype in this patient. Patient 10 and one of two imbalances in patient 7 have been listed as polymorphic in the Toronto polymorphism database.[21] In patient 7, the larger deletion on chromosome 5 spanning between 6.8 and 11.8 Mb was also present in the similarly affected mother. Hence this deletion is likely to be causal for the phenotype. In patient 5, the duplicated region in the healthy father and son contains only a single gene, the glycogen branching enzyme (GBE1); dosage effect for this gene seems a rather unlikely cause. In patients 6, 17, and 28, single clone imbalances are inherited and the causal relation between genotype and phenotype remain to be determined.

In summary, we consider that at least 19 of the 28 observed imbalances are causal for the MCA/MR in the patients.

Cytogenetic features of (low grade) mosaic chromosomal imbalances

A further interesting observation in this study was the finding of three mosaics. In patient 16, array CGH revealed increased average intensity ratios for a 12 Mb region compatible with a duplication spanning the long arm of chromosome 13 from band 13q31.3 to 13q33.1 (fig 2A). The average $\log_2$ of the intensity ratio values of the abnormal clones was 0.38. As the theoretical intensity ratio of a duplication is $\log_2$ (3/2) or 0.58, the estimated mosaicism level is 0.38/0.58 or 65%. FISH analysis confirmed the duplication to be present in 60% of cultured lymphocytes (fig 2B).

In patient 14, standard array CGH revealed a 5 Mb deletion at 11q22.1–23.1. FISH with clone RP11-87N22 confirmed the deletion at the 11q22.1 locus in all cells. Forty clones flanking

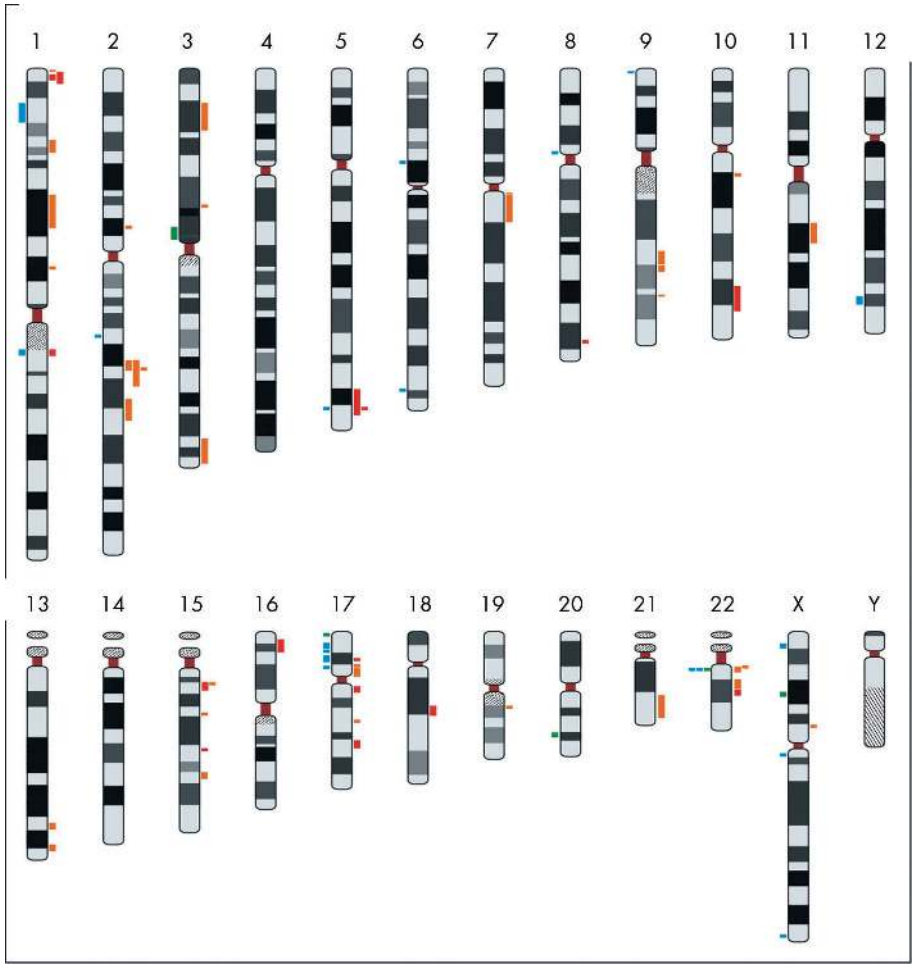

Figure 1 Overview of all published interstitial submicroscopic imbalances detected by array-CGH in patients with mental retardation and multiple congenital anomalies (MCA/MR). Microdeletions and duplications identified in this study are represented by, respectively, red and green bars. Microdeletions and duplications identified by previous array CGH studies[10–14] are indicated by, respectively, the orange and the blue bars. Polymorphic variants from de Vries *et al*[12] are not shown.

this deletion (14 proximal and 26 distal to the deleted segment) showed a mean intensity ratio of 0.21, suggesting a duplication of the adjacent region at 11q21-qter in approximately 35% of the cells (fig 2C). FISH with clone RP11-744N12 located within this presumed duplicated region showed a translocation of 11q21-qter onto chromosome 9 in 6% of the cells, in contrast to the estimated 35% (fig 2D). As this FISH analysis was performed on lymphocytes following stimulation with phytohaemagglutinin, and DNA used for array CGH was extracted from uncultured lymphocytes, we assumed that culturing resulted in clonal selection of the normal cells. FISH on uncultured lymphocytes confirmed this hypothesis and showed three signals of RP11-744N12 in as many as 25% of the nuclei of uncultured lymphocytes.

Array CGH analysis on patient 9 revealed an average intensity ratio of −0.0496 for the clones from chromosome 7 (fig 2E). The level of mosaicism is calculated to be 5%. Interphase FISH analysis by two independent observers using a centromere 7 specific probe revealed a single signal in 10.5% of the nuclei of peripheral white blood cells of the patient while in a control sample a single signal was observed in only 3.5% of the nuclei. The difference between these two proportions was significant ($p<0.01$), thus confirming the presence of the monosomy in approximately 8% of the patient's white blood cells. This finding can probably be explained by the presence of a (pre)malignant clone in this patient.

Review of published reports on MCA/MR patients with submicroscopic imbalances

To obtain insight into the incidence, characteristics, and genomic distribution of imbalances detected by array CGH in MCA/MR patients, all published genomic imbalances were reviewed (fig 1 and table 2).[10–14] From a total of 192 patients screened by arrays at ~1 Mb resolution, 41 imbalances were detected (21%), of which at least 20 (10%) were de novo. Of the 192 patients, 113 were screened for subtelomeric imbalances before array CGH. The number of interstitial imbalances was 35 (18%), of which at least 17 were de novo (8.8%). In addition, de Vries *et al* analysed 100 patients previously shown not to carry subtelomeric imbalances using an array covering the full genome and detected de novo alterations in 10 patients.[12] Five imbalances were likely to be causal, but parents were not available for analysis. Of these 15 imbalances, five were smaller than 1 Mb.

Figure 1 shows that the imbalances were more or less scattered across the genome and appeared mostly randomly distributed over all chromosomes. Some chromosomal regions appeared non-randomly involved. Interstitial aberrations at chromosome 1p36 were detected in two patients in the present study and in three published array CGH cases. Hence, in addition to the 1p36 terminal deletion syndrome—considered to be the most common subtelomeric microdeletion syndrome[23]—interstitial subtelomeric deletions also appear to be common. At two loci (1q21.1 and 5q35.1), both

Table 1 Summary of copy number changes detected by array CGH, short clinical description and parental analysis

Case	Clinical details*	Molecular karyotype	Parents	No of clones	Size (MB)†	Flanking clones
1	Retinal dystrophy, growth retardation, short fingers, low set ears, epicanthic folds	46,XY.arr cgh del(1)(p36.23p36.32)	Not determined	5	4.6–8.1	RP4-785P20, RP11-338N10
2	Microcephaly, ventricular septal defect, large corneae, midface hypoplasia, presacral groove	46,XY.arr cgh del(1)(p36.31p36.32)	De novo	3	2.8–6.0	RP4-785P20, RP11-49J3
3	Short stature, microcephaly, strabismus, unilateral renal agenesis, simple ears	46,XX.arr cgh del(1)(q21.1q21.1)	Not determined	2	0.9–4.0	RP11-533N14, RP11-301M17
4	Epilepsy, brachydactyly type E, scoliosis, absence of some toenails, synophrys	46,XX.arr cgh der(2)t(2;22)(q37;q13)	De novo	chr 2: 4 chr 22: 3	4.0–4.7 1.6–2.9	chr2: RP11-556H17, RP11-15L18 chr22: cN75H12, RP5-925J7
5	Seizures, spasticity, hypotonia, hypoplastic cerebellum and brain stem, Dandy-Walker malformation	46,XY.arr cgh dup(3)(p12.2p12.2)	Inherited (pat)	3	0.5–2.1	RP11-425D6, RP11-359D24
6	Coarse facial features, Dandy-Walker malformation, wide pontine cisterns, right cerebellar lobe atrophy, hirsutism, pigmented nevi	46,XX.arr cgh del(3)(p12.1)	Inherited (pat)	1	0.1–2.5	RP11-474M18
7	Pectus excavatum, sacral dimples, recurrent infections, sparse eyebrows, small high nasal bridge	46,XY.arr cgh del(5)(q34q35.1)del (15)(q13.1)	Inherited (mat)¶ inherited (pat)‡	chr 5: 8 chr 15: 2	6.9–11.8 0.8–3.5	chr5:RP11-505G12, RP11-420L4, chr15:RP11-408F10, RP11-38E12
8	Tetralogy of Fallot, double outlet right ventricle, hypertelorism, high and broad forehead, brachycephaly	46,XX.arr cgh del(5)(q35.1q35.1)	De novo	1	0.2–2.8	RP11-20O22
9	VSD, absent thumbs, growth retardation, hydronephrosis, preductal coarctation of aorta	46,XX.arr cgh del(7)(pterqter) .ish 46,XX[92]/45,XX.−7[8]	De novo	212	158	CTB-164D18,RP4-764O12
10	Axial hypotonia, short stature, stereotypic movements, hypertelorism, strabismus	46,XY.arr cgh del(8)(q24.23q24.23)	Inherited (pat)‡	1	0.2–1.5	RP11-17M8
11	Chondrodysplasia punctata brachytelephalangic type, obesity, short stature, small deeply set nose, hypotonia	46,Y.arr cgh der(X)t(X;9)(p22.32;p23)	De novo	chr 9: 16 chr X: 5	chr 9:13.0–13.9 chr X: 5.4–6.9	chr 9: RP11-187K14, GS1-77L23 chr X: RP11-60N3, CTB-98C4
12	Short stature, microcephaly, VSD, preductal coarctation of aorta, midface hypoplasia	46,XX.arr cgh der(9)t(9;20)(q34.3;q13.33)	De novo	chr 9: 4 chr 20: 7	chr 9: 3.1–4.7 chr 20: 3.5–4.8	del:RP11-399H11, GS1-135I17, dup:RP5-836E13, CTB-81F12
13	Hypotonia, spasticity, abdominal muscle hypoplasia, fine hair, macroglossia	46,XX.arr cgh del(9)(q34q34)	De novo	1	0.1–0.6	GS1-135I17
14	Valvar pulmonary stenosis, cleft uvula, epilepsy, hypoplastic corpus callosum, hypoplastic genitalia	46,XX.arr cgh del(11)(q22.3q23.3) [66]/ der(9)t(9;11)(qter;q21),del(11)(q22.3q23.3)[33]	De novo	chr 9: 6 chr 11: 50	del: 8.5–10.2 dup: 40.4–41.1	del:RP11-531F16, RP11-114K7, dup:RP11-685N10, RP11-469N6
15	Carpal synostosis, macrocephaly, strabismus, oral frenulae, autistic behaviour	46,XX.arr cgh del(10)(q25.1q26.11)	De novo	10	8.2–10.3	RP11-271I13, RP11-355F22
16	Broad thumbs, nasal speech, strabismus, deep hoarse voice, trigonocephaly	46,XX.arr cgh dup(13)(q31.3q33.1) .ish 46,XX[40]/46,XX dup(13)(q31.3q33.1)[60]	De novo	14	12.2–13.9	RP11-388D4, RP11-564N10
17	Microbrachycephaly, almond shaped eyes, wide nasal bridge, large mouth, synophrys	46,XX.arr cgh del(15)(q22.2q22.2)	Inherited (mat)	1	0.2–3.2	RP11-231A23
18	Dysplastic ears, median cleft palate, small penis, brachycephaly, unilateral preauricular fistula	46,XY.arr cgh dup(16)(p13.2p13.3) .ish der(22)t(16;22)(p13.2p13.3;p21)	De novo	9	7.4–8.3	RP11-433P17, RP11-148F10
19	Generalised hypotonia, scoliosis, congenital heart disease, short stature, brachycephaly	46,XY.arr cgh dup(17)(p13.3p13.3)	Not determined	1	0.1–1.7	RP11-135N5
20	Camptodactyly, ectropion, hypoplastic cerebellar hemispheres and vermis, hypertelorism, genital hypoplasia	46,XY.arr cgh del(17)(p12p12)	Not determined	1	0.1–2.3	RP1-27J12
21	Microcephaly, long eyelashes, long columella, deep presacral groove, lacrimal duct stenosis	46,XX.arr cgh del(17)(q11.2q11.2)	De novo	1	0.1–1.9	RP11-474K4
22	Psychiatric disorder, macrocephaly	46,XX.arr cgh del(17)(q23.2q24.1)	De novo	3	1.1–4.2	RP11-115N5, RP11-74H8
23	Small stature, narrow thorax, macrocephaly, downslanting palpebral fissures, prominent maxillary incisors	46,XY.arr cgh del(18)(q12.3q12.3)	De novo	2	1.4–4.6	RP11-486C18, RP11-463D17

Table 1 Continued

Case	Clinical details*	Molecular karyotype	Parents	No of clones	Size (MB)†	Flanking clones
24	Joint laxity, scoliosis, hyperelastic skin, webbed neck, beaked nose	46,XX.arr cgh dup(20)(q13.13q13.2)	De novo	2	0.7–2.7	RP5-1071L10, RP5-994O24
25	Myopia, nasal speech, cleft uvula, pulmonary stenosis, strabismus	46,XX.arr cgh del(22)(q12.2q12.2)	De novo	2	0.7–2.2	CTA-57G9, RP1-76B20
26	Hypotonia, adduction of thumbs, claw toes, syndactyly fingers 3/4, dorsiflexion of the wrists	46,XX.arr cgh del(22)(q13.33q13.33)	De novo	3	1.4–1.9	CTA-722E9, CTB-99K24
27	Cleft lip and palate, truncus arteriosus type I, short neck, ptosis, uteronephrosis,	46,XX.arr cgh dup(22)(q11.21 q11.21)	Inherited (pat)	1	0.1–4.2	XX-91c
28	Epilepsy, microcephaly, abdominal situs inversus, VSD, hypotonia	46,XY.arr cgh dup(X)(p21.3p21.3)	Inherited (mat)	2	0.3–1.2	RP11-37E19, RP6-27C10

*All patients presented with mental retardation. Only the five most relevant dysmorphic features are retained in the table. Full phenotypic descriptions together with the genotype data can be viewed in Ensembl (www.ensembl.org/index.html) through the Decipher DAS server.
†Sizes of the aberrations are shown from a minimal to maximal size in megabases.
‡This imbalance has already proven to be polymorphism.
¶Mother has the same phenotype including mental retardation. Further family could not be investigated.
chr, chromosome; mat, maternal; pat, paternal; VSD, ventricular septal defect.

a duplication and a deletion were observed. Possibly these sites may mark novel microdeletion syndromes caused by recurrent non-homologous recombination in low copy repeats. Of particular interest is the finding of a familial duplication on 22q11.2 in this study, as well as in three previous reported cases (two de novo and one case of unknown origin), further suggesting the recurrent nature of this duplication and the variable phenotypic effect.

DISCUSSION

This study is the largest series of patients reported who have been screened for chromosomal imbalances with a 1 Mb resolution BAC array. In a total of 140 patients, 28 chromosomal imbalances were detected (20%). These included seven duplications, 18 deletions, and three unbalanced translocations. To determine the causal role of these chromosomal aberrations, parents were investigated in 24 of 28 patients. In addition, the Toronto database of normal variants was consulted. About three quarters (17/24) of the observed chromosomal aberrations were de novo and not reported before as a normal variant. In one patient for whom the parents could not be tested, available phenotypic data for similar published cases indicated that the genotype could explain the observed phenotype, and in one patient with inherited deletion the mother was equally affected. This brings the total of clinically relevant imbalances to 19. Taking into account these data and excluding those subtelomeric imbalances that could have been detected by FISH or MLPA/MAPH analysis, our study has identified 11 clinically relevant imbalances (8%) undetectable by karyotyping and subtelomeric screening. This is in accordance with previous findings of 10–15% causal interstitial submicroscopic imbalances in patients with MCA/MR.[10–14] Imbalances identified thus far in MCA/MR patients have been positioned on the human genome map in order to assess their genomic distribution and to detect overlapping regions. This map further confirms that most imbalances are scattered across the genome.

From our data and data from other published reports it has become clear that the clinical application of array CGH poses new challenges. While it is assumed that de novo alterations result in the observed phenotype, only the recurrent association of imbalances with specific phenotypic features will reinforce this causal relation. Hence, it will be essential to collect genotypic and phenotypic information on a large number of MCA/MR patients. In contrast to de novo alterations, many chromosomal imbalances are inherited. Although it is likely that frequently occurring genomic CNVs may not have major disease causing phenotypic effects, rare variants, such as the six familial inherited imbalances detected in this study, should be evaluated with care. In particular, imbalances of regions which are recurrently involved in familial transmission from a normal parent to affected children will pose specific problems for genetic counselling, as illustrated by the 22q11.2 duplication. This is in line with previous observations that 22q11 duplications result in diverse phenotypes from normal to mild to severe, and sharing a tendency for velopharyngeal insufficiency with DiGeorge/VCFS (velo-cardio-facial syndrome) but with other distinctive characteristics as well.[24 25] The 22q11 duplication syndrome may hallmark a novel paradox encountered by molecular karyotyping, as the causal relation between a chromosomal anomaly and an associated phenotype becomes blurred. Hence, imbalances inherited from phenotypical normal parents may contribute to the phenotype through variable penetrance or expressivity, or both, through epigenetic effects, or by uncovering a recessive mutation on the non-deleted allele. To understand the involvement of these variations in the observed phenotypes, it will be necessary not

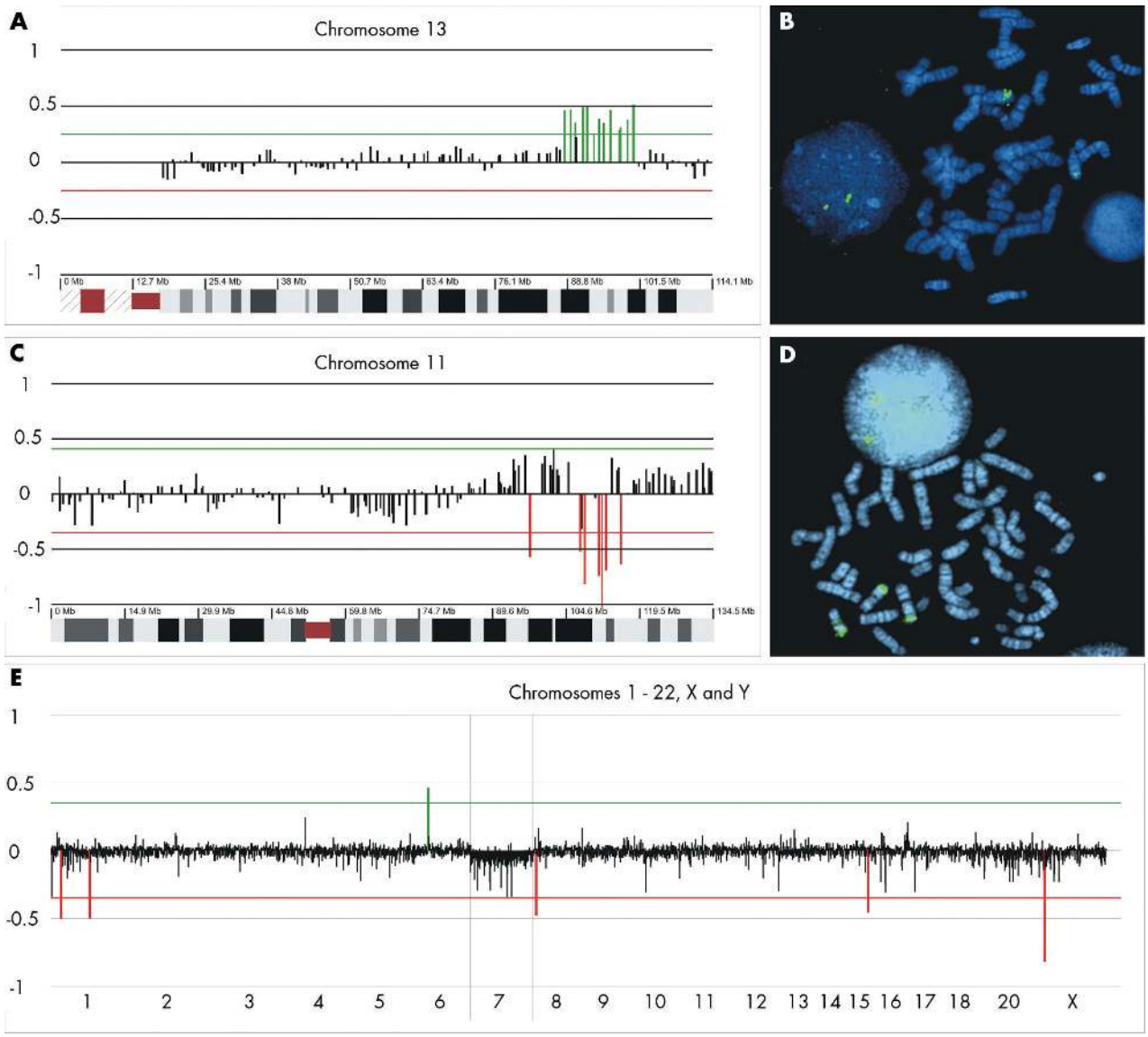

Figure 2 Cytogenetic analysis of patient 16 (panels A and B), patient 14 (panels C and D) with segmental chromosomal mosaicisms, and patient 9 (panel E) with a mosiacism monosomy of chromosome 7. (A) Partial molecular karyotype enlarging the ratio profiles for chromosome 13; in the x axis clones are ordered from the centromere to the q-arm telomere, and the y axis shows the $\log_2$ transformed intensity ratios at each locus. Red lines indicate the threshold for clone deletion or duplication ($\pm$4*SD). (B) Fluorescence in situ hybridisation (FISH) with PAC 1091O16 confirmed that the duplication at 13q32The duplication was present in 60% of the cultured lymphocytes. (C) Partial molecular karyotype enlarging the ratio profiles for chromosome 11. On the x axis clones are ordered from the p-arm telomere to the q-arm telomere and the y axis shows the $\log_2$ transformed intensity ratios at each locus. Red lines indicate the threshold for clone deletion or duplication ($\pm$4*SD). (D) The duplication at 11q24.3 was confirmed with clone BAC 744N12 and was the result of a translocation between 11q and 9q. FISH on cultured and uncultured lymphocytes showed the duplication to be present in, respectively, 6% and 25% of the cells. (E) Molecular karyotype showing the ratio profiles for the chromosomes 1 to 22, X, and Y. Chromosome 7 is positioned between the two vertical lines, and shows $\log_2$ transformed intensity ratios with an average of -0.05.

only to collect benign variation in the genome and information on de novo imbalances associated with disease phenotypes, but also to collect both genotype and phenotype information from patients with familial inherited imbalances and phenotypically normal parents. To start this data collection, both genotype and phenotype data from all patients who consented was submitted at the DECIPHER database (http://www.sanger.ac.uk/Postgenomics/decipher/).

Segmental chromosomal imbalances in mosaic state are causal in several MCA/MR syndromes.[26] The present study illustrates that array CGH may detect segmental chromosomal imbalances which may be overlooked in standard karyotyping when a small number of cells is analysed or when the abnormality is too small to arouse suspicion. A remarkable observation in one of the mosaics was that phytohaemagglutinin stimulation of lymphocytes and subsequent short culture apparently induced a selective growth advantage for the normal cells. Clearly, such culture effects can bias the final cytogenetic observations, as was observed in patient 14. Presently a theoretical model is being developed which should enhance the sensitivity for the detection of low grade mosaicism. Clearly, the presence of a large deletion present in as few as 5% of cells can easily be detected. The ability to detect low grade mosaics will allow the detection of chromosomal aneuploidies in highly contaminated specimens such as aborted fetuses[27] and in the analysis of tumours and leukaemias.[28]

In all reports, including this study, the number of deletions (57) was greater than the number of duplications (24). This may have both a technical and a biological component.

Table 2 Published reports: summary of intrachromosomal copy number changes detected by array CGH

Paper	No of patients*	Intrachromosomal			No of targets on array
		De novo	Familial	Unknown	
Vissers L *et al*[10]	20 (0)	2	2	1	3569
Shaw-Smith *et al*[11]	50 (41)	7	5	0	~3500
Rosenberg *et al*[13]	81(0)	4	7	3	~3500
Schoumans *et al*[14]	41(41)	4	0	0	2600
This study	140 (31)	11	7	3	~3500
Total	**332**	**28**	**21**	**7**	
De Vries *et al*[12]	100	10	0	5	32447

*Number on which subtelomeric imbalances have been excluded before array CGH was carried out.
CGH, comparative genomic hybridisation.

Technically, most threshold algorithms may favour more false negatives for duplication events as compared with deletion events. Most threshold algorithms determine cut offs for both deletions and duplications at equal distance from the mean of all intensity ratios. As the intensity ratios for chromosomal deletions are more distant from the mean (ratio of 1/2) as compared with the intensity ratios observed for duplications (ratio of 3/2), inevitably there is a greater chance that some duplications may be missed. Second, there may be a biological bias. Duplications generally result in a milder phenotype; therefore there may be a selection bias in this patient population. In addition, the frequency of random duplication events in the human genome may be lower than the frequency of deletion events. Van Ommen[29] estimated the frequency of deletion events to be one in every eight births, and the duplication frequency one in every 50 births. This suggests that the number of deletion events is about sixfold greater than the number of duplication events. In patients with MCA/MR, deletions outnumber duplications by approximately twofold.

In conclusion, we confirm that a high percentage of MCA/MR cases hitherto considered idiopathic is caused by submicroscopic chromosomal imbalances. Consequently, screening of selected patients with normal karyotypes seems desirable and feasible. The availability of commercial platforms and improved hybridisation schemes resulting in reduction of costs for these analyses opens the way for implementing array CGH in routine diagnostic analysis. At present it remains unclear what resolution of the array will be optimal for screening MCA/MR patients. Higher resolution arrays may reveal larger numbers of small chromosomal imbalances. However, the finding of only 10% of de novo imbalances in a cohort of 100 patients by a full coverage array may indicate that higher resolution does not necessarily increase the diagnostic yield. More studies using high resolution arrays are needed to compare the incidence of small imbalances in different patient populations. Nevertheless, using a 1 Mb resolution array, some imbalances smaller than 1 Mb are being missed. In addition, the false positive rate may be lowered, especially if the identification of imbalances is based on intensity alterations of three or more aberrant flanking clones.[12] Considering the large percentage of inherited chromosomal imbalances, establishing both benign copy number variations in the human genome as well as developing a comprehensive morbid map of the human genome will be of major importance for understanding which imbalances are causative.

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