Short report

High resolution microarray CGH and MLPA analysis for improved genotype/phenotype evaluation of two childhood genetic disorder cases: ring chromosome 19 and partial duplication 2q

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Abstract

A detailed analysis of the constitutional chromosomal changes in two pediatric patients was performed using high resolution genetic analysis techniques, microarray comparative genomic hybridization (array CGH) and multiplex ligation-dependent probe amplification (MLPA) as well as FISH. The aim was to come to a more precise characterization of the genotype/phenotype relationship. Case 1 was a girl of 25 months, showing areas of hypopigmentation along the lines of Blaschko and no other developmental abnormality. She carried a ring chromosome 19 which we found not to have resulted in loss of subtelomeric sequences, ruling out the possibility that a small subtelomeric loss was causally related to this patient's phenotype. Case 2 was a 9-year-old girl with facial anomalies and mild growth and mental retardation carrying an unidentified addition on chromosome 2p. We found that the addition was duplicated 2q35-q37.3 and that the addition was not accompanied by loss of 2pter or any other chromosomal region. Together with literature data, we hypothesize that pediatric patients with 'pure' trisomy 2q including bands 2q35-q37.1 may have a moderate clinical phenotype as opposed to patients with duplications proximal to 2q33 or patients with duplications 2q3 with

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accompanying distal deletion. These two examples illustrate the additonal value of new, high resolution genetic analysis techniques for a better characterization of the genotype/phenotype relationship in childhood chromosomal disorders.

1. Introduction

Childhood chromosomal disorders are generally described in terms of changes at the chromosome level. However, conventional karyotyping is often unable to detect subtle gains or losses and in many instances the genotype/phenotype relationship remains unclear. This is especially true for carriers of ring chromosomes. Cases have been described with and without loss of chromosome material but a clear correlation with the severity of developmental abnormalities is lacking. Also, when numerical aberrations are concerned, a precise classification of the genetic alterations often remains cumbersome, and consequently correlation with the phenotype can be difficult.

Recently developed genetic analysis techniques, as microarray comparative genomic hybridization (array CGH) and multiplex ligation-dependent probe amplification (MLPA), can be of great additional value in this field. Array CGH was first published by Pinkel et al. [16] greatly improving the resolution and sensitivity of chromosome CGH. In array CGH the target chromosomes are replaced by cloned genomic fragments of which the exact chromosomal location is known. This allows a more detailed detection of aberrations. Moreover, it enables to map the changes directly onto the genomic sequence. MLPA, first described by Schouten et al. [20], is a PCR-based technique capable to semi-quantitatively measure the copy number of up to 50 genes in one experiment. MLPA probe-mixtures have been developed for detection of gains and losses of oncogenes and tumor suppressor genes and new mixtures are still being developed, especially for clinical diagnostic purposes [18].

Until now, these techniques have primarily been used in cancer research, however, they are also suitable for the analysis of DNA copy number aberrations in human genetic disorders. In this paper we show how they allow a more precise characterization of the genotype/phenotype relationship in two pediatric cases that we took as examples. The first concerned a girl of 25 months showing areas of hypopigmentation along the lines of Blaschko but otherwise no anomalies. She was found to carry a ring chromosome 19. The second case was a 9-year-old girl with facial anomalies and mild growth and mental retardation. Chromosome banding analysis showed a duplication attached to the short arm of chromosome 2.

2. Materials and methods

2.1. Subjects

Case 1. The proband was the first daughter born of a 20-year-old mother and a 52-year-old father. There was no family history of mental retardation, abortions or consanguinity.
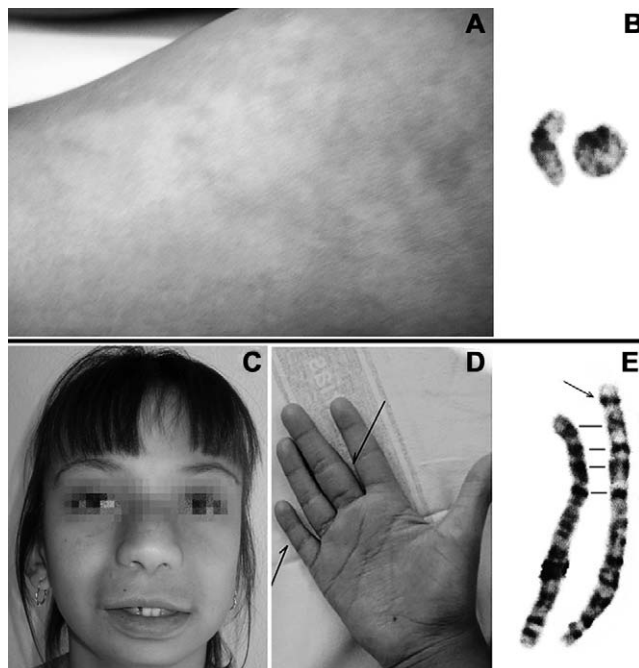
Fig. 1. Photographs of clinical manifestations of the two patients and their corresponding chromosomal abnormality as identified by G-banding. **Case 1**: Skin of the left leg with Hypomelanosis of Ito along the lines of Blaschko (A) and partial G-banded karyotype showing a normal 19 and a ring chromosome 19 (B). **Case 2**: Facial anomalies including high forehead, low-set ears, broad nasal bridge and long philtrum (C), hands with short fifth fingers and increased secondary palmar folds (D) and partial G-banded karyotype showing a normal and a derivative chromosome 2 with extra chromosomal material of unknown origin attached to the terminal part of the short arm (E).

She was born after a pregnancy of 40 weeks without any complications and with a normal weight, length and occipitotemporal circumference. APGAR scores after 1, 5 and 10 min were 9, 9 and 10, respectively. No perinatal complications were reported and psychomotor development was normal. At the age of 18 months she showed areas of hypopigmentation on the left leg distributed along the lines of Blaschko, which was diagnosed as Hypomelanosis of Ito (Fig. 1A). No other pathology or organ defects were noted, and the infant showed normal growth and development.

Case 2. The proband, a 9-year-old girl, was born of a couple already having two sons. There was no family history of mental retardation, abortions or consanguinity. After a full term pregnancy and uncomplicated delivery she was born with poor APGAR scores and she needed resuscitation. Developmental milestones were delayed (e.g. walking at the age of 4 years) and was unable to attend regular school. At the age of 9 years she was 131.6 cm tall (35th percentile) with a weight of 24.6 kg (10th percentile) and an occipitofrontal circumference of 50 cm (8th percentile). Upon physical examination a high forehead, low-set ears, hypertelorism, palpebral ptosis, broad nasal bridge, long philtrum, carious teeth and macrostomie were noted (Fig. 1C. Other manifestations were short fifth fingers and increased secondary palmar folds (Fig. 1D). Further examinations revealed a nystagmus and slow signal conduction of the optical nerve. IQ examination showed moderate mental handicap.

For both subjects informed consent was obtained and the study was approved by the Hospital Central de Asturias.

2.2. Methods

Metaphase spreads were prepared from peripheral blood lymphocytes according to standard procedures. Conventional karyotyping was performed by standard trypsin–Giemsa banding. For array CGH and MLPA, DNA was extracted from peripheral blood lymphocytes according to standard procedures.

Array CGH was performed as described in [4]. Briefly, sample and reference DNA were differently labeled by random priming. Six hundred ng test and reference DNA were hybridized to 5000 cloned DNA fragments (BAC clones) that have been spotted in triplicate on a glass slide (the array). The BAC clones were evenly distributed across the whole genome, with an average resolution of 1.0 Mb and included a set of clones especially for subtelomeric regions [9,13]. Image acquisition, analysis and data extraction were quantified by Imagene 5.5.4 software (Biodiscovery Ltd., Marina del Rey, California, USA), using the default settings.

For MLPA analysis of DNA copy number changes, a specific probe mixture with 48 subtelomeric probe-sets for all chromosomes was used (Salsa P036, MRC-Holland b.v., Amsterdam, The Netherlands). The analysis was performed according to the manufacturer's recommendations. In short, approximately 100 ng of DNA in 5 µl was denatured at 98 °C for 5 min and subsequently hybridized overnight with a mix of sub-telomeric probe-pairs, each consisting of two oligonucleotides (hemiprobes) that recognize adjacent DNA sequences. On day 2, the adjacently hybridized hemiprobes were ligated. After denaturation, PCR was performed with two universal PCR primers, amplifying all probe-pairs in one reaction. Experiments for both test and reference samples were carried out in triplicate. Analysis of the MLPA PCR products was performed on an ABI model 3100 16-capillary sequencer (Applied Biosystems, Warrington, UK).

FISH analysis was carried out using a FITC labeled all-telomere-specific PNA probe (DAKO, Glostrup, Denmark). One hundred µl of probe/hybridization buffer mixture was applied to metaphase slides and denatured by heating 10 min at 80 °C on a hot plate. After hybridization for 24 h at 40 °C, the slides were washed for 5 min in 2 × SSC at RT, three times 5 min in 0.1 × SSC at 45 °C, and then dried in an ethanol series. Finally, the slides were mounted with 20 ul antifade solution (Vectashield, Vector laboratories, Burlingame, USA) containing 0.35 µg/µl 4,6-diamidino-2-phenylindole (DAPI). The slides were analyzed on a Leica DM-RA microscope equipped with a CCD camera (Cohu Inc. San Diego CA, USA), and images were captured with Q-FISH software (Leica, Cambridge, UK).

3. Results

3.1. Case 1

Initial chromosomal analysis of peripheral lymphocytes showed a karyotype 46,XX,r(19) in all metaphases (Fig. 1B). Parental karyotypes were studied and revealed a ring(19) mosa-
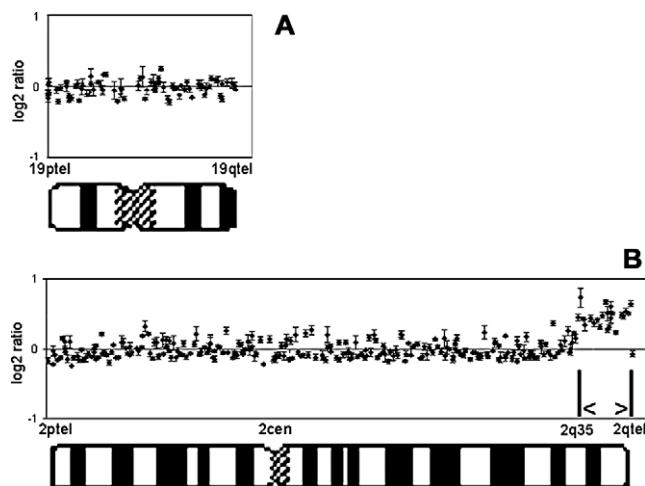
Fig. 2. Part of the results of array CGH analysis. Panel A shows all clones representing chromosome 19, displayed from 19p to 19q (left to right) of **case 1**. All clones displayed log2-ratios of around 0.0, meaning no loss or gain of material. Panel B shows all clones representing chromosome 2, displayed from 2p to 2q (left to right), of **case 2**. On the distal part of the q-arm, a stretch of clones have log2-ratios of around 0.5, signifying a gain of material from 2q35 to 2q37.3.

icism in 20% of peripheral lymphocytes of the mother. The father's karyotype appeared normal. Array CGH analysis showed normal copy numbers for all BAC clones on all chromosomes on the array. Fig. 2A shows the result from the clones on chromosome 19. The results on the mother were identical (data not shown). The center of the BAC clones nearest to the telomeric ends were located at 0.25 Mbp from the 19p-telomere (CTD-3113P16), and 0.14 Mbp from the 19q-telomere (GS1-1129C9), according to the Ensembl Genome Browser, version 26.35.1 (1 November 2004). The results from the MLPA analysis were the same; no losses could be detected on the sub-telomeres of chromosome 19 (Fig. 3A) or any other chromosome. In this assay, the location of the probes was 0.47 Mbp from the 19p-telomere (CDC34), and 0.20 Mbp from the 19q-telomere (LOC125905), according to the Ensembl Genome Browser, version 26.35.1 (1 November 2004). The FISH analysis using a pan-telomeric probe showed a signal that seemed to come from one telomere only (Fig. 4A). However, this probe does not allow to distinguish between the p and the q telomere, and therefore, we can not conclude a possible loss of one of the telomeres.

3.2. Case 2

Chromosomal analysis of peripheral lymphocytes showed a karyotype 46,XX,add(2)(p25) in all metaphases (Fig. 1E). Parental karyotypes were normal. Array CGH showed gain of chromosome bands 2q35-q37.3, i.e. from BAC clone RP11-512o7 at 220.5 Mbp to RP11-556 h17 at 241.8 Mbp, which lies at 1.2 Mbp from the q-telomere (Ensembl Genome Browser, version 26.35.1, 1 November 2004). The translocation of this part of 2q to distal 2p did not appear to have resulted in loss of material on 2p (Fig. 2B). No other copy number abnormality was detected (data not shown). MLPA analysis confirmed the gain of the 2q subtelomere, and showed no copy number changes at the sub-telomeres of other chromo-
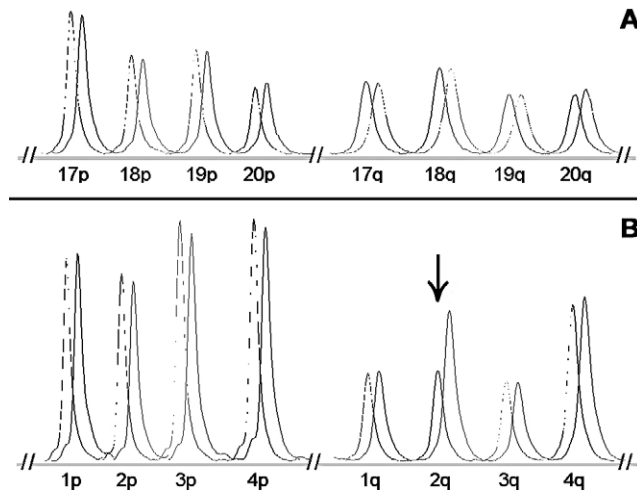
Fig. 3. Part of the results of MLPA analysis. Panel A shows eight pairs of peaks representing probes from the subtelomeric p and q regions of chromosomes 17, 18, 19 and 20 of **case 1**; the left peak of each pair is the result from normal DNA and the right peak of each pair is the result from the sample DNA from blood lymphocytes of the patient. Neither of the peaks displayed a difference in peak surface, meaning no loss or gain of material. Panel B shows eight pairs of peaks representing probes from the subtelomeric p and q regions of chromosomes 1, 2, 3 and 4 of **case 2**; again the left peak of each pair is the result from normal DNA and the right peak of each pair is the result from the sample DNA from blood lymphocytes of the patient. The probe representing 2q (arrow) showed an elevated peak of the sample DNA compared to the normal DNA, meaning a gain of material on the subtelomere of 2q.

somes (data not shown), including 2pter (Fig. 3B). FISH analysis using a pan-telomeric probe showed signals on the two chromosome ends of the derivative chromosome 2. It remains unclear whether the signal at the 2p end represents telomere 2p or 2q (Fig. 4B).
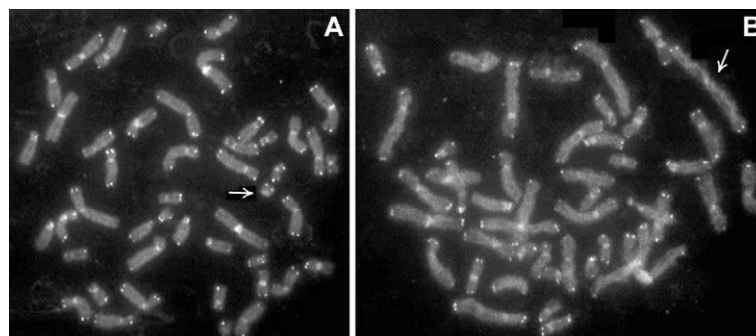

Fig. 4. FISH using a pan-telomeric probe. A: a metaphase of **case 1** with a ring (19) chromosome (arrow) carrying a telomeric signal. B: a metaphase of **case 2** with the derivative chromosome 2 showing a telomeric signal on both chromosome ends.

4. Discussion

4.1. Case 1

Ring chromosomes have been observed for all human chromosomes. They are associated with growth failure (especially when larger ring chromosomes), with malformations and with no or moderate mental retardation [25]. The phenotype is generally thought to be caused by small terminal chromosomal deletions. In addition, rings are unstable because of mitotic crossing-over events, leading to mosaicism. Ring chromosome 19 carriers exhibit exactly this spectrum of phenotypic characteristics [10,12,19,24,28]. However, in neither of these cases loss of subtelomeric material was reported. Flejter et al. [10] demonstrated in one case the deletion of one of the telomeres by pan-telomeric FISH analysis.

By high resolution genetic analysis, we showed with greater certainty that the ring chromosome 19 presented in this paper had not resulted in loss of subtelomeric sequences (at least not up to 0.25 Mbp distance from the p-telomere and 0.14 Mbp from the q-telomere by array CGH, and up to 0.47 Mbp from the p-telomere and 0.2 Mbp from the q-telomere by MLPA). This finding virtually rules out the possibility that a small loss of euchromatin material on chromosome 19 was causally related to the patient's phenotype. The pan-telomeric FISH experiment did not provide useful information.

Hypomelanosis of Ito has been suggested to be the result of genetic mosaicism [14], irrespective of the precise chromosomal abnormality. This would explain the wide variety of chromosomal abnormalities found in patients with hypomelanosis, among others trisomy chromosome 13, trisomy chromosome 14, partial trisomy chromosome 9 and a number of non-reciprocal translocations [7,8,15,17,23]. Kuster and Konig [14] further suggested to designate hypomelanosis of Ito more properly as 'pigmentary mosaicism'. Since ring chromosomes with intrachromosomal telomeric sequences (as in our patient) are unstable and associated with mosaicism [25], it is possible that mosaicism in our patient's neurocutaneous tissues is the cause of her skin abnormalities. Unfortunately, due to the lack of skin tissue samples, we were unable to ascertain this possibility.

4.2. Case 2

Duplications 2q3 usually occur in combination with loss of another chromosome segment [2]. To our knowledge, eight cases of 'pure' (i.e. without acccompanying loss), de novo duplications involving 2q35-q37.3 have been reported previously [1,5,6,11,21,22,27]. The majority of these cases concerned inverted duplications located within the 2q-arm. It has been suggested that inverted duplications are always associated with distal deletion, possibly too small to be detected by cytogenetic analysis [3], a problem that may be overcome by new high resolution genetic techniques, such as array CGH and MLPA. Distal 2q37 deletion is associated with Albright hereditary osteodystrophy-like syndrome, which is characterized by brachydactyly and mental retardation [26]). Therefore, in order to better understand the phenotype-genotype relationship, it is important to know the precise nature of chromosome 2q3 duplications.

Our study concerns a case of a 2q3 duplication translocated to 2pter (no inversion), without any accompanying chromosomal loss. The size of the duplicated region spanned

21.3 Mbp, from BAC clone RP11-512o7 at 220.5 Mbp to RP11-556h17 at 241.8 Mbp, corresponding to chromosomal bands 2q35-q37.3. The fact that we saw no distal 2q loss is in accordance with the lack of arachnodactyly and only mild mental handicap in our patient. In addition, malformations of heart, kidney or brain, associated with duplications more proximal to 2q33, were not observed in our patient. To our knowledge, one case of 'pure' 2q3 duplication similar to our case has been described having trisomy 2q35-q37.1 as certified by FISH [11]. This patient presented with comparable phenotypic anomalies, such as high forehead, broad nasal bridge and low-set ears, clinodactyly and mild growth and psychomotor delay. We suggest that pediatric patients with partial trisomy 2q including at least bands 2q35-2q37.1 as the sole chromosomal abnormality have a moderate clinical phenotype as opposed to duplications proximal to 2q33 or duplications 2q3 with accompanying distal deletion.

In conclusion, in this short report we have shown the additional value of new, high resolution genetic analysis techniques for a more precise characterization of the genotype/phenotype relationship in childhood chromosomal disorders.

Acknowledgements

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