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Short Report

Molecular screening for microdeletions at 9p22-p24 and 11q23-q24 in a large cohort of patients with trigonocephaly

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Trigonocephaly is a rare form of craniosynostosis characterized by the premature closure of the metopic suture. To contribute to a better understanding of the genetic basis of metopic synostosis and in an attempt to restrict the candidate regions related to metopic suture fusion, we studied 76 unrelated patients with syndromic and non-syndromic trigonocephaly. We found a larger proportion of syndromic cases in our population and the ratio of affected male to female was 1.8 : 1 and 5 : 1 in the non-syndromic and syndromic groups, respectively. A microdeletion screening at 9p22-p24 and 11q23-q24 was carried out for all patients and deletions in seven of them were detected, corresponding to 19.4% of all syndromic cases. Deletions were not found in non-syndromic patients. We suggest that a molecular screening for microdeletions at 9p22-p24 and 11q23-q24 should be offered to all syndromic cases with an apparently normal karyotype because it can potentially elucidate the cause of trigonocephaly in this subset of patients. We also suggest that genes on the X-chromosome play a major role in syndromic trigonocephaly.

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Trigonocephaly is mostly caused by craniosynostosis of the metopic suture and is characterized by a triangular shape of the head. It can occur as an isolated malformation or as part of a syndrome (1). Lajeunie et al. published a large epidemiological study on trigonocephaly, including 237 French patients (2). In this study, they estimated the prevalence of metopic synostosis to be 1 in 10,000–15,000 births with a sex ratio shifted toward males (3.3 : 1) and

they showed that non-syndromic cases account for 77% of the total sample. Azimi et al. (3) found similar numbers for their series of 25 trigonocephalic cases: 72% were considered to be non-syndromic and the sex ratio (males : females) was 2 : 1. Little is known about the genetic etiology of trigonocephaly and different models of inheritance, such as autosomal dominant, autosomal recessive, and X-linked, have been proposed (4–6). Valproate exposure in uterus, neonatal hyperthyroidism, and intrauterine head constraint are among the known environmental factors that can induce metopic synostosis (7–9). An association of trigonocephaly with chromosomal abnormalities, more specifically with del(9)(p22p24) or monosomy 9p syndrome (OMIM 158170) and del(11)(q23q24) or Jacobsen syndrome (OMIM 147791) is well established (10–14). The critical regions for these phenotypes have been established to be between D9S285 and D9S286 (approximately 8 Mb) and between D11S1316 and D11S912 (approximately 5 Mb), respectively, for the 9p and 11q regions (13, 15). There might be genes at these regions that cause the premature fusion of the metopic suture when in hemizygosity. Once deletion breakpoints seem to vary among patients with these syndromes, it is possible that smaller deletions would lead to milder or even non-syndromic cases. In this context, we have evaluated 76 unrelated patients with syndromic and non-syndromic trigonocephaly to: (a) verify whether non-syndromic trigonocephaly might be caused by microdeletions and (b) further restrict the critical regions for this abnormality in chromosomes 9p and 11q.

Patients and methods

Our total sample consisted of 76 unrelated patients with trigonocephaly. Forty-four families were referred to the Centro de Estudos do Genoma Humano (CEGH), São Paulo, Brazil, 15 were ascertained at the Craniofacial Unit, Oxford, UK, and 20 were referred to Johns Hopkins University, Baltimore, MD. We classified the patients into two groups: group 1 (n = 40) consisted of non-syndromic patients, with trigonocephaly as an isolated feature and group 2 (n = 36) included syndromic patients whose trigonocephaly was associated with other primary abnormalities, such as hand/foot anomalies, malformed ears, cardiac defects, and mental retardation. Patients suspected to have secondary trigonocephaly due to, for example, microcephaly, exposure to teratogens, or hyperthyroidism, were not included in our sample.

Clinical inclusion consisted of two criteria: a triangular head and/or forehead, with or without a metopic ridge and a radiographic documentation on the metopic synostosis. Patients from the UK and the USA with known chromosomal abnormalities were excluded from the research. The inclusion of patients in Brazil was prospective. Karyotype analysis was performed in 22 of the 36 syndromic cases through conventional 450- to 500-band resolution.

DNA preparation

Genomic DNA from either peripheric blood samples or buccal swabs was obtained by established techniques and by QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Parental samples were collected whenever available. Informed consent was obtained for all cases.

Microdeletion screening

Microdeletions were determined through the hemizygosity of microsatellite markers within the critical regions in 9p22-p24 and 11q23-q24 (13, 15). Brazilian and North-American patients were screened with markers D9S286, D9S775, D9S168, D9S269, D9S267, D9S268, D9S1808, D9S1869, D9S274, and D9S285 in chromosome 9 and D11S4094, D11S933, D11S4158, D11S1896, D11S990, D11S4151, D11S4110, and D11S912 in chromosome 11. British patients were additionally screened with markers D9S144, D9S256, D9S1687, D9S1782, D9S156, and D9S1839 in chromosome 9 and D11S1316, D11S934, D11S1351, D11S4091, and D11S4123 in chromosome 11. PCR amplification was carried out using 20–100 ng DNA either with fluorescent-labeled primers (0.3 μM of each; ABI PRISM Linkage Mapping set version 2 or Invitrogen, Carlsbad, CA, USA) or with R6G/R110-dCTP (0.2 mM), in reactions of 10 μl containing 0.25 mM dNTP mix, 1.5 mM $MgCl_2$, 10 mM Tris–HCl buffer (pH 8.5), and 0.7 U of Taq Polimerase Invitrogen. Products were analyzed either in the ABI PRISM 377 or in the MegaBACE Sequencers using the softwares Genotyper System or Genetic Profiler, respectively. Hemizygosity was characterized when the patient inherited a single allele from only one parent. A marker was denominated uninformative when the patient had one single allele and either both parents shared the same allele or

Table 1. Summarized clinical and genetic characterization of the sample

		Non-syndromic		Syndromic		Total	
		n	%	n	%	n	%
Gender	Male	26	65	30	83	56	73.7
	Female	14	35	6	17	20	26.3
Associated sutures fused	Coronal	2	5	2	5.6	4	5.3
	Sagittal	4	10	2	5.6	6	7.9
	Coronal and sagittal	2	5	2	5.6	4	5.3
	Lambdoid	–	–	–	–	0	0.0
	All sutures fused	–	–	1	2.8	1	1.3
Familial cases		4	10	3	8.3	7	9.2
Consanguinity		1	2.5	2	5.6	3	3.9
Test results	Abnormal karyotype					3/22	13.6
	Hemizygosity (9p + 11q)	–	–	3/22	13.6		
		0	0	7	19.4	7	9.2
Total		40	52.6	36	47.4	76	100.0

Unless cited differently, the percentages are related to the total number of individuals in the category.

parental DNA was not available for analysis. This approach has also enabled us to determine the parental origin of deletions.

Results

Characterization of the sample

Our sample consisted of 76 patients with trigonocephaly. Our findings are summarized in Table 1. Different from what is proposed for metopic suture closure, the number of non-syndromic and syndromic patients in our sample was similar: 40 (52.6%) and 36 (47.4%). Most North-American/British patients were non-syndromic (71%), paradoxically; the majority of Brazilian patients were syndromic (63%).

The sex ratio (males : females) for our total sample was 2.8 : 1, with a more significant difference toward affected males in the syndromic group (5 : 1) than in the non-syndromic group (1.8 : 1): (Table 2). Familial recurrence was observed in seven instances: four in non-syndromic cases (10%) and three in syndromic cases (8%). Vertical transmissions were only seen in non-syndromic cases. Pedigrees of familial cases are shown in Fig. 1. Consanguinity was present in one non-syndromic (3%) and two syndromic (6%) families from the Brazilian sample.

Detection and characterization of deletions

No deletions were detected in non-syndromic patients. We found deletions in 9p or 11q in seven of the 36 syndromic patients studied, which represents 19.4% of them. Four of these deletions were only detected through molecular analysis: three of them were missed by conventional karyotype (CR237, CR258, and CR285) and one patient had not been previously karyotyped (CR243). The other three deletions were also detected through karyotype analysis (CR179, CR297, and CR314). Approximate size of deletions and breakpoints are represented in Fig. 2 and Table 3. All deletions were *de novo* with the exception of two cases in which they arose because of an unbalanced segregation of a parental reciprocal translocation. In CR237, a familial case with two brothers affected, the translocation was detected by FISH analysis

Table 2. Comparison between subsamples of patients from Brazil, UK, and USA in respect of the ratios of syndromic to non-syndromic and male to female patients

	Non-syndromic		Syndromic		Total	
	Brazil	UK + USA	Brazil	UK + USA	Brazil	UK + USA
Male	9 (60)	17 (68)	21 (81)	9 (90)	30 (73)	26 (74)
Female	6 (40)	8 (32)	5 (19)	1 (10)	11 (27)	9 (26)
Total	15 (37)	25 (71)	26 (63)	10 (29)	41 (100)	35 (100)

Percentages are in parenthesis.

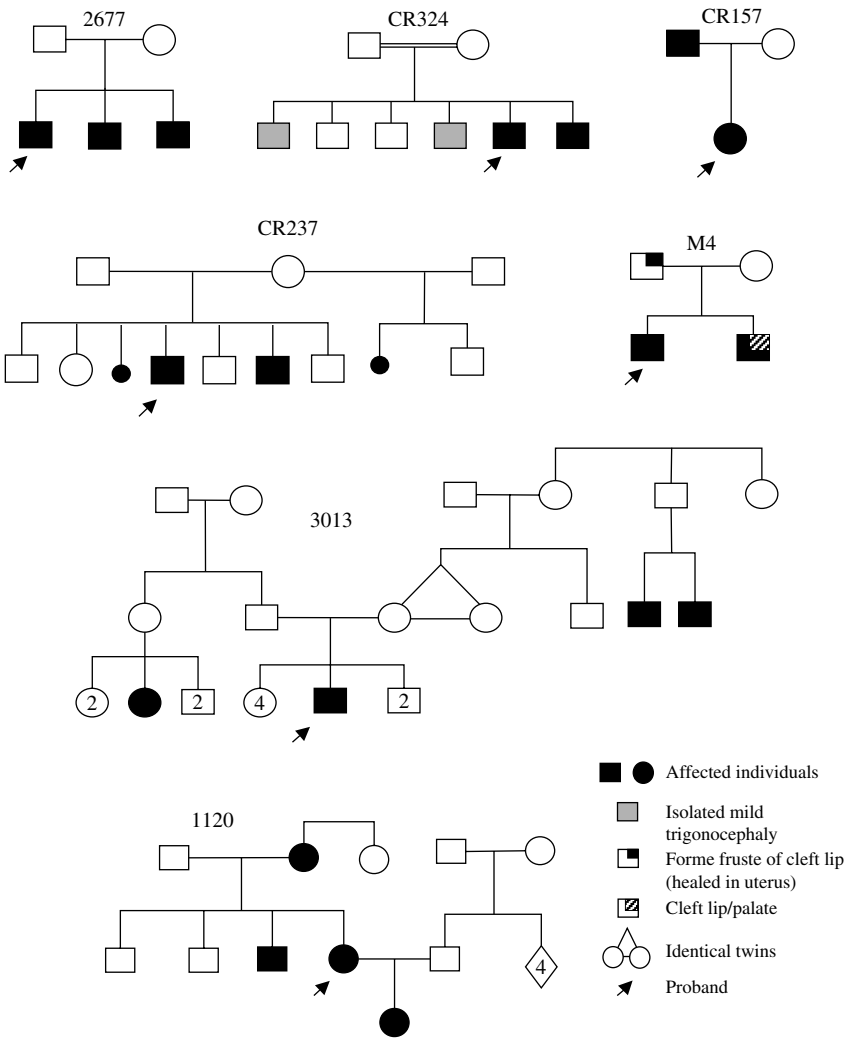

Fig. 1. Pedigrees of the families with trigonocephaly. Families 2677, CR324, and CR237 are syndromic and families CR157, M4, 3013, and 1120 are non-syndromic.

with probes from the short arm of chromosome 9 in maternal metaphases (results not shown) and in CR258, a paternal translocation was suspected after performing high-resolution karyotype in the father. All cases of *de novo* deletions were of paternal origin. The main phenotypic features of deleted patients are summarized in Table 3 and pictures of patients with deletion 9p are shown in Fig. 3.

The proximal breakpoints of our patients with 9p deletions were all within two regions: between markers D9S171 and D9S157 and markers D9S285 and D9S274 (Fig. 2). Only one patient had an interstitial deletion with the distal break between D9S288 and D9S286. The minimal deleted region in monosomy 9p patients lies between D9S286 and D9S285 and our results did not further restrict this region.

CR243 had a terminal deletion with a breakpoint between markers D11S976 and D11S4094 (Fig. 2). Penny et al. (13), defined that the critical region for calvarian suture anomalies in Jacobsen syndrome was distal to D11S1316 (<100 kb from D11S4094) and proximal to D11S912. Therefore, we were not able to restrict this region as well.

Three of our patients (CR289, G2012, and G2600) were homozygous for five consecutive markers at the 11q region, suggesting that they might harbor a deletion in this region. However, FISH analysis with three different probes covering the *CDON* gene (results not shown) and real-time PCR for genomic gene dosage of *CTXL* and *NFRKB* did not confirm our suspicions (Fig. 4–online supplementary material).

Discussion

This work consisted of the genetic evaluation and the molecular screening of 76 patients with

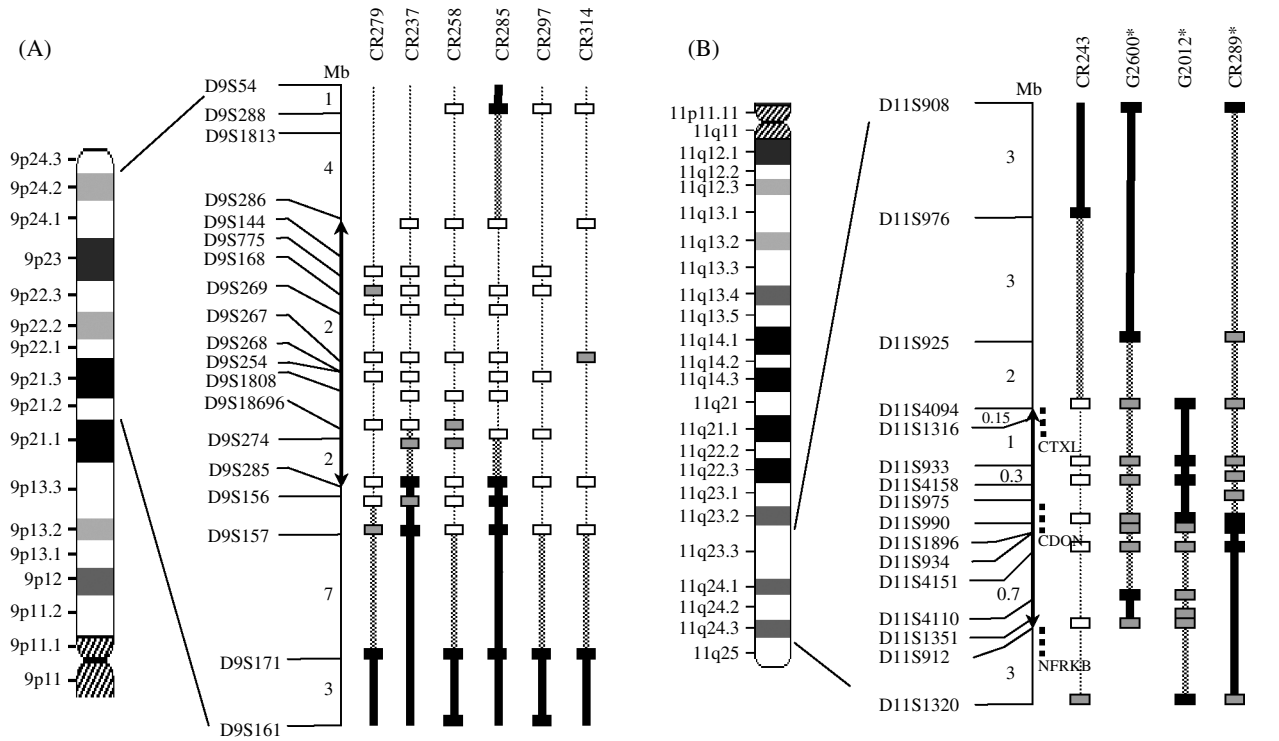

Fig. 2. Schematic figure showing deletions found in 9p (a) and in 11q (b). Positions and relative distances between markers are according to the STS map at NCBI. Black, white, and gray rectangles represent heterozygous, hemizygous, and uninformative loci respectively. Black lines determine non-deleted regions, while fine-dotted lines show deletions found through loss of heterozygosity. Gray lines depict regions where the breakpoints are located in patients with known deletions and uninformative regions in patients with suspected deletions (CR289, G2012, and G2600 in panel b). The line delimited by arrowheads shows the critical regions for monosomy 9p determined by Christ et al. 1999 (a) and for craniofacial abnormalities in Jacobsen syndrome as determined by Penny et al., 1995 (b). The large dots in panel b show the position of genes used in FISH and real-time PCR experiments for patients with five or more consecutive uninformative markers (*). Deletions were excluded in these patients.

trigonocephaly, which is, to our knowledge, the largest cohort screened using molecular techniques till date.

Epidemiological studies propose that the majority of patients with trigonocephaly are non-syndromic (1–3); however, in our series, the number of syndromic and non-syndromic cases was similar. More intriguing was that the prospective Brazilian subsample showed a prevalence of syndromic cases (63%). Two main aspects might have influenced these numbers: the prospective ascertainment criteria in the Brazilian sample, as we did not exclude, a priori, cases with known chromosomal abnormalities and the referral of more severe cases to medical and genetic services in Brazil. It is also possible that the prevalence of syndromic and non-syndromic trigonocephaly is indeed different in the two populations due to their ethnical backgrounds, but further epidemiological studies are needed to investigate this issue.

The sex ratio of our sample confirmed the male prevalence in this type of craniosynostosis (2.8 : 1), being that more significant in the syndromic group (5 : 1) than in the non-syndromic group (1.8 : 1). Despite the different ascertainment methods, the sex ratios in non-syndromic and syndromic groups were the same between the North-American/British and Brazilian subsamples. The higher preponderance of affected males could be explained by a couple of mechanisms, i.e. the presence of important genes in the X-chromosome and/or a lower threshold of this trait in males. The involvement of genes on the X-chromosome may be reinforced by the fact that craniosynostosis also seems to have a higher prevalence in Turner syndrome (16). Escape of inactivation and imprinting of genes on the X-chromosome are other mechanisms that could explain a lower penetrance of a trait or syndrome in females (17, 18).

The rates for familial recurrence, 8 and 10% for syndromic and non-syndromic cases, respectively, were similar to that found by Lajeunie et al. (6%) and Azimi et al. (5.6%). Pedigrees of familial cases suggest a variety of modes of inheritance, such as autosomal dominant, recessive, and X-linked. X-linked inheritance was discarded for family

Table 3. Main phenotypic characteristics of patients with deletion 9p and 11q

Patient	CR179 Del 9p	CR237[a] Del 9p	CR258 Del 9p	CR285 Del 9p	CR297 Del 9p	CR314 Del 9p	CR243 Del 11q
Estimated deletion size (Mb)	24	16	24	13	24	24	17
Origin of deletion	Paternal *de novo*	Maternal 46, XX, t(9;4) (p22.3;q34)	Paternal 46, XY, t(9;?) (9p21.3;?)	Paternal *de novo*	Paternal *de novo*	Paternal *de novo*	Paternal *de novo*
Gender	Male	Male	Male	Male	Female	Male	Male
IUGR	–	–	–	+	–	–	–
Pre-term labor	–	–	–	+	–	–	+
Trigonocephaly	+	+	+	+	+	+	+
Turricephaly	+	+	–	–	–	–	–
Brachycephaly	+	+	+	–	+	–	–
Plagiocephaly	–	+	–	+	–	–	–
Palpebral fissures	ND	Down	Down	ND	Up	Up	Down
Epicanthic folds	+	+	+	+	+	+	+
Strabismus	–	–	–	–	–	ND	+
Flat nose bridge	+	+	+	+	+	+	+
Anteverted nares	+	+	+	+	+	+	+
Long philtrum	+	+	–	ND	+	+	+
Small/malformed ears	+	–	+	+	+	–	–
Cryptorchidism/malformed genitalia	+	+	+	+	[b]	–	–
Inguinal hernia	+	+	–	+	+	–	–
Umbilical hernia	–	+	–	–	+	–	–
Long fingers	+	+	–	+	+	+	–
Small puffy feet	–	–	–	–	–	–	+
Clubfoot	–	–	–	–	–	+	–
Wide space between first and second toes	–	–	+	–	+	+	–
Pancitopeny	–	–	–	–	–	–	+
Cardiopathy	–	–	–	PDA/DXC	–	–	–
Hypotonia	+	+	+	+	+	+	+
Mental retardation	Moderate	Severe	Moderate	Severe	Moderate	Moderate	Moderate

DXC, dextrocardia; IUGR, intrauterine growth retardation; ND, no data; PAD, patent ductus arteriosus; +, present; –, absent.
[a]Characteristics shared by both affected brothers.
[b]Normal external female genitalia with hypoplasic labia.

CR324 through the analysis of polymorphic markers (data not shown). Recurrence in CR237 was actually due to a chromosomal abnormality, as described above. The pattern of segregation of trigonocephaly in these families further confirms the heterogeneity of this condition. Consanguinity in non-syndromic (3%) and syndromic (6%) patients is not increased in our sample, as they are comparable to the values found in normal Brazilian population (1.3–2.9%) (19).

We were able to detect deletions in seven patients, which consist of 19.4% of all syndromic patients. It is also important to observe that three deletions were missed by conventional chromosomal analysis. CR285 had a small interstitial deletion in 9p with a maximum deleted segment of 13 Mb and the other two cases, CR237 and CR258, were not clearly seen even after karyotype revision probably because of the translocated segment. All *de novo* deletions originated in the paternal chromosome, which is not unusual for microdeletions. High incidences of paternally derived mutations have also been reported for other deletion syndromes, such as Wolf–Hirschhorn, deletion 22q11, and Cri-du-chat (20–22).

Although the deletion breakpoints in 9p did not fall into the same position, they were clustered into two distinct regions. Indeed, the short arm of chromosome 9 does not seem to have one main deletion hotspot but rather a series of regions prone to breakages because of their low percentage of CG and high incidence or repetitive sequences such as SINES, LINES, and LTRs (15). These preferential breaks may limit the variation size of the deletions in 9p and make it more difficult to restrict the region responsible for trigonocephaly in this chromosomal segment.

After performing real-time PCR for gene dosage, we were not able to confirm the microdeletions in three patients with five consecutive uninformative markers. Therefore, the presence

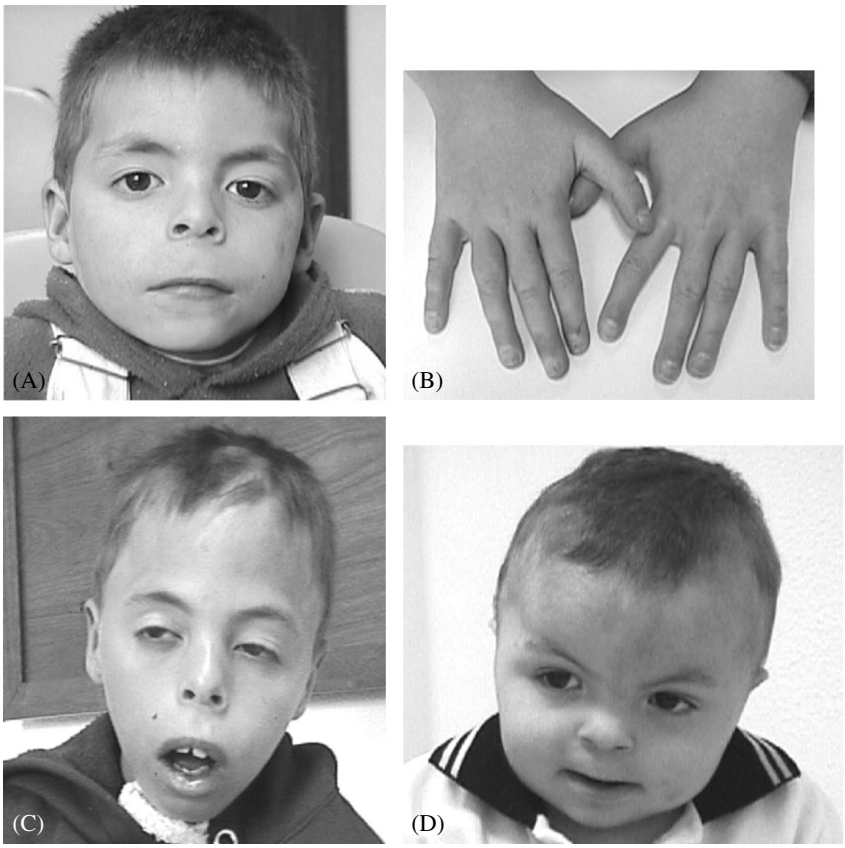

Fig. 3. Facial appearance of three patients with deletion 9p: (a) youngest affected brother from family CR237 at 6 years old, 46,XY, der(9)t(9;4)(p22.3;q34) and his hands (b) showing long second phalanges; (c) proband from family CR237 aged 9 years old and (d) patient CR258 at 1 year of age, 46, XY, der(9)t(9;?)(p21.3;?). For further clinical data see Table 3.

of several consecutive uninformative markers should always be checked by an alternative method.

All patients with deletions were syndromic, suggesting that non-syndromic trigonocephaly is very unlikely caused by microdeletions in these regions. We cannot exclude the possibility that our screening missed some microdeletions because of uninformative markers or because they were smaller than the distance between two markers used. Furthermore, polymorphic marker screening is not a suitable method to

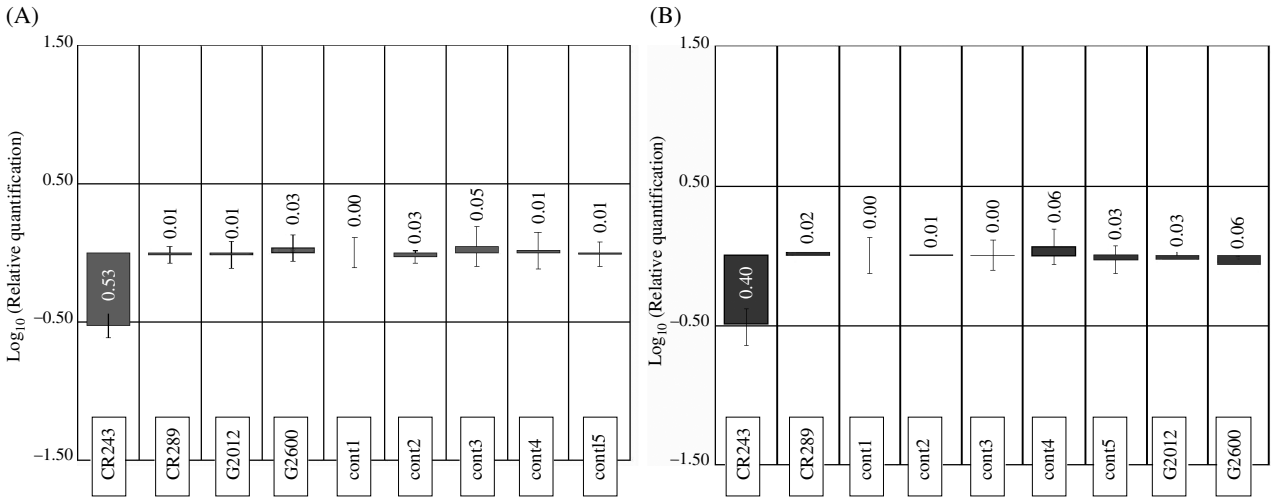

Fig. 4. Real-time PCR graphics showing the relative dosage of genes *NFRKB* (a) and *CTXL* (b) in patients CR289, G2012, and G2600 that were uninformative for five consecutive markers. CR243 was used as the deleted control sample and control 1 served as the normal calibrator sample. For CR243, the relative dosage for *NFRKB* and *CTXL* in relation to the internal calibrator albumin equals −0.5, showing that this individual has only one copy of these genes, while CR289, G2012 and G2600 show a normal dosage for each gene (approximately 1).

detect other abnormalities such as duplications. We believe that a large number of syndromic cases are the result of a variety of chromosomal rearrangements, in opposition to most non-syndromic cases that are probably the result of a more complex inheritance. The development and application of high-resolution methods to detect rearrangements in the whole genome, such as CGH-array, will allow for a better overview of the causes of syndromic trigonocephaly and will help us determine the best screening procedure in cases of syndromic trigonocephaly. However, while these methods are not available for large-scale diagnosis, we propose that karyotype and further molecular microdeletion analysis for the 9p22-p24 and 11q23-q24 regions should be performed for all patients with syndromic trigonocephaly.

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