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ORIGINAL ARTICLE

COMPREHENSIVE GENETIC EVALUATION OF BULGARIAN CHILDREN WITH SYNDROMIC CRANIOSYNOSTOSIS

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ABSTRACT

Syndromic craniosynostosis (SC) is a genetically determined premature closure of one or more of the cranial sutures, which may result in severe dysmorphism, increased intracranial pressure along with many other clinical manifestations. The considerable risk of complications along with their significant incidence makes these cranial deformations an important medical problem.

Aiming to elucidate the complex genetic etiology of syndromic craniosynostosis, we investigated 39 children, screened systematically with a combination of conventional cytogenetic analysis, multiplex ligation-dependent probe amplification (MLPA) and array-based comparative genomic hybridisation (aCGH).

Pathological findings were established in 15.3% (6/39) of the cases using aCGH, in 7.7% (3/39) using MLPA and 2.5% (1/39) using conventional karyotyping. About 12.8% (5/39) of the patients with normal karyotype carried submicroscopic chromosomal rearrangements. Duplications were found to be more common than deletions.

Conclusion: The systematic genetic evaluation of children with SC revealed a high prevalence of submicrosopic chromosomal rearrangements (most commonly duplications). This suggests the leading role of those defects in the pathogenesis of syndromic craniosynostosis. The genetic complexity of SC was reaffirmed by the discovery of pathological findings in various chromosomal regions. Certain genes were discussed in conjunction with craniosynostosis.

Key words: craniosynostosis, genetic evaluation, submicroscopic rearrangements, syndromic

List of Abbreviations:

aCGH/array CGH - Array-based comparative genomic hybridisation / **ASD** - Autism spectrum disorder / **CNVs/CN** – Copy number variations / **CRS** – Craniosynostosis / **DGV** – Database of genomic variants / **GRCh** – Genome Reference Consortium (human build) / **HGNC** – HUGO gene nomenclature committee / **ISCN –** The International Sustainable Campus Network / **kb/kbp** – kilobase/kilobase pair / **Mb/Mbp** – Megabase/Megabase pair / **MLPA** - Multiplex ligation-dependent probe amplification / **OGT** – Oxford gene technology / **OMIM** – Online mendelian inheritance in man / **PCR** - Polymerase chain reaction / **TAR** – Thrombocytopenia Absent Radius syndrome / **SC** - Syndromic craniosynostosis / **SoS** – Sotos syndrome

INTRODUCTION

Craniosynostosis (CRS) is the process of premature fusion and ossification of one or more cranial sutures [1]. It has a cumulative incidence of about 1 in 2500 newborn children [2]. When untreated, craniosynostosis can lead to serious medical complications – increased intracranial pressure, mental retardation, hearing or vision defects, behavioural anomalies, craniofacial asymmetry and dysmorphism, seizures [3].

CRS can be classified as ***syndromic*** – when the cranial synostosis is a part of a malformative syndrome or ***nonsyndromic*** – when it presents as an isolated feature.

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Nonsyndromic craniosynostosis constitutes about 80% of all known cases [4]. The syndromic craniosynostosis (SC) is considerably rarer – 20 % of all repoted cases. About 30% of the SC are mainly attributed to pathogenic variants in certain genes (*FGFR1, FGFR2, FGFR3, TWIST1, EFNB1, MSX2, RAB23, RUNX2*) [4]. They are inherited in an autosomal dominant pattern (except for *RAB23*) with variable penetrance and expressivity. Chromosomal anomalies account for about 16% of syndromic craniosynostosis cases [4]. MLPA and array CGH offer similar diagnostic value in literature and can be used in tandem to confirm a certain finding [4].

Despite the scientific achievements in the last two decades, the genetic basis of craniosynostosis remains rather poorly understood. Trying to clarify the complex genetic factors involved in the pathogenesis of syndromic craniosynostosis, we investigated 39 children by carrying out a systematic, combined approach consisting of conventional cytogenetics, MLPA and aCGH.

MATERIALS AND METHODS

Subjects

We investigated 39 children with syndromic craniosynostosis referred to our department in the 2016-2020 year period. 29 of them were male and 10 were female resulting in a sex ratio of 2.9:1. Clinical selection was based on the presence of craniosynostosis along with additional dysmorphic features (SC) - documented by imaging studies (cranial radiography and/or computed tomography).

Methods

Conventional chromosomal analyses at 550 G-band resolution were performed on peripheral blood lymphocytes on all 39 of our patients.

Multiplex Ligation-dependent Probe Amplification (MLPA) is a method used to determine the copy number of up to 45 genomic DNA sequences in a single multiplex PCR based reaction. For this study we used MLPA P245 Microdeletion Syndromes for screening of the most common microdeletion syndromes and MLPA P036 Subtelomeres Mix 1 for screening of subtelomeric deletions/duplications. To confirm alternations discovered with MLPA P036 Subtelomeres Mix 1 we used MLPA P070 Subtelomeres Mix 2B.

Array CGH - the whole genome CNVs screening was carried out by the oligo array CGH. DNA was isolated from peripheral blood by phenol-chloroform extraction. We used the OGT 4x44k format oligonucleotide microarray with a targeted CN resolution of 1 probe every 52kb and a backbone CN resolution of 1 probe every 81kb. The slides were scanned on a GenePix 4100A, two-colour fluorescent scanner (Axon Instruments, Union City, CA, U.S.A.). The arrays were analyzed by CytoSure Interpret Software.

RESULTS

In 27 of our patients, craniosynostosis was simple (a single cranial suture is obliterated). In 10 cases, two sutures were simultaneously fused, repesenting a complex craniosynostosis (two or more sutures are prematurely and simultaneously closed). In the other 2 patients, three cranial sutures were prematurely ossified.

The suture involvement distribution in our sample presented as: coronal in 44.4%, sagittal in 22.2%, metopic - 25.9% and lambdoid in 7.4%.

The analysis of G-banded chromosomes yielded only one pathological finding in patient 29 - 46,XX,t(2;7)(q14;q35) – an apparently balanced reciprocal translocation of chromosomes 2 and 7, inherited from the patient‘s mother *(Tables 1 and 2)*.

MLPA revealed three pathological results - del 5q35.3 (in patient 22), dupl 2p16.1 (in patient 29) and del 4q (in patient 34) representing 7.7% of all participants in our sample *(Table 2)*.

Array CGH - pathogenic and likely pathogenic submicroscopic aberrations were found in 6 patients, representing 15.3% of all tested children *(Tables 2 and 3)*. About 12.8% (5/39) of the patients with normal karyotype carried submicroscopic chromosomal rearrangements. Four of those defects were duplications and two were deletions.

DISCUSSION

The distribution of suture involvement in syndromic craniosynostosis in the literature [4-6] is sagittal in about 50-60%, followed by coronal in 20-25%, metopic in 15% and lambdoid in approximately 5% of all cases. This differs from our findings (see ***Results***). This is probably due to the limited size of our sample.

Patient 2 (Table 1) had normal results from conventional chromosome analysis and MLPA while aCGH revealed a pathogenic duplication of the long arm of chromosome 1 (1q21.1) (*Table 2*). The parents were not available for segregation analysis. None of the genes within this region have been associated with CRS so far. Rare, recurrent chromosome 1q21.1 duplications and deletions have been linked with developmental delay, autism, congenital heart anomalies and macrocephaly in children [7]. Our patient was diagnosed with ASD, which is consistent with the literature we found on duplication 1q21.1. The aforementioned duplication also includes the 1q21.1 recurrent (TAR – Thrombocytopenia Absent Radius syndrome)

Table 1. Clinical characteristics of our patients with pathological genetic findings

Patient No	Age	Sex	Craniosynostosis	Dysmorphic features	Psychomotor development	IQ	Additional personal and familial findings
2	3 years	male	metopic	craniofacial	delayed	72	ASD; mother with pes planus
8	4 years	female	coronal	craniofacial, short neck, thoracic hyperkyphosis, brachydactyly	Normal then regress	74	psychomotor regress, gastrointestinal symptoms
22	2 years	male	sagittal	craniofacial; macro-dolichocephaly; single transverse palmar crease on both hands; umbilical hernia	delayed	21	unilateral hydronephrosis; seizures; cortical atrophy, right fronto-parietal porencephaly and postischemic defects
25	5 months	male	coronal and sagittal	mild craniofacial; upper limb rhizomelia	delayed	60	anaemia, recurrent respiratory infections
29	5 years	female	lambdoid	craniofacial	delayed	58	Dandy-Walker occipital cyst; maternal karyotype 46,XX,t(2;7)(q14;q35)
34	4 years	male	metopic	craniofacial	delayed	64	mitral valve insufficiency; corpus callosum hypoplasia

Table 2. A summary of the results from all genetic assays in our sample

Patient No	Chromosome analysis	MLPA findings	aCGH findings – ISCN Notation and size	Class	Candidate genes
2	46,XY	normal	arr[hg19] 1q21.1 (144440748x2, 144510920-146188485x3, 146290655x2) (1.85Mb)	pathogenic	-
8	46,XX	normal	arr[hg19] 14q32.33(105524898x2, 105609511-105787438x0, 105845682x2) (177.93Kb)	likely pathogenic	-
22	46,XY	del 5q35.3 (*NSD1*)	arr[hg19] 5q35.2 (175,470,501-177,136,261x1) (1.66 Mb)	pathogenic	*FGFR4*
25	46,XY	normal	arr[hg19] 1q12q21.2(120322008x2, 142513049-147134234x3, 147203277x2) (4.62 Mb)	pathogenic	-
29	46,XX,t(2;7)(q14;q35)	dupl 2p16.1	arr[hg19] 2p22.3p16.1(36033514x2, 36095582-61287377x3, 61369298x2) (25.19 Mb)	pathogenic	*SIX2*
34	46,XY	del 4q(*TRIML2*)	arr[hg19] 1p22.1 (92258725x2, 92326818-92705290x3, 92767467x2) (378.47 Kb)	likely pathogenic	*TGFBR3*

region (proximal, BP2-BP3). However, there is insufficient evidence for triplosensitivity, explaining why we found no phenotypic features of TAR syndrome in our patient. Intriguingly, patients 2 and 25 (*see Discussion, Patient 25*) were found to have partially overlapping duplications of 1q21.1. This warrants a further and more detailed investigation of this chromosomal region.

In *patient 8* (*Table 1*), conventional cytogenetics and MLPA showed normal results. The patient was screened for submicroscopic rearrangements using aCGH, yielding one likely pathogenic, homozygous deletion of 14q32.33 (177.93Kb). Several gene sequences have been mapped on this region, none of which have been connected to craniosynostosis. Submicroscopic deletions of the long arm of chromosome 14 are associated with two conditions – Dubowitz syndome [8] and 14q32.3 deletion syndrome [9]. Due to patient 8's facial dysmorphism and the presence of gastrointestinal symptoms as well as brachydactyly, we are inclined towards Dubowitz symdrome (*Tables 1 and 2*). As far as we know, neither Dubowitz syndrome nor 14q32.3 deletion syndrome have ever been associated with craniosynostosis. We were not able to obtain information regarding the biologicical parents of this patient.

In *patient 22* (*Table 1*), aCGH revealed a heterozygous deletion of the 5q35 region (5q35.2-5q35.3). The deletion was 1.665 Mb in size (*Table 2*), encompassing 40 HGNC and 24 OMIM genes, including *NSD1* and *FGFR4*. The array CGH results were confirmed by MLPA. The patient‘s parents were unavailable for testing. This result is consistent with Sotos syndrome (SoS)**.** It is a rare but well-known disorder causing overgrowth in childhood. Ten percent of affected individuals have 5q35 microdeletions [10]. The size and mechanism of formation of 5q35 microdeletions differ depending on the ethnic origin of the patients [11]. The presented features of our patient (*Table 1*) are typical for SoS, although the overgrowth was absent. Our patient's microdeletion includes the *NSD1* and *FGFR4* genes. Overall, the individuals with microdeletions

have less prominent overgrowth than patients with *NSD1* variants [12]. Douglas et al. also described a patient with 5q35 microdeletion involving *NSD1* and *FGFR4* genes and craniosynostosis [13]. Fibroblast growth factor (FGF) and fibroblast growth factor receptor (FGFR) signaling pathways play essential roles in the earliest stages of skeletal development, thus mutations in these genes can cause different bone diseases, including craniosynostosis [14]. Nie et al. speculated that *FGFR4* is involved in growth regulation of face and head structures, although the effect of *FGFR4* on bone development remains unknown and needs further elucidation [15].

The genetic evalutaion of *patient 25* (*Table 1*) began with chromosome analysis and MLPA, both showing normal results. Array CGH, however, revealed a pathogenic microduplication of chromosme 1 (1q12q21.2) spanning across 4.62 Mb (*Table 2*). None of the genes within this chromosome region have been associated with craniosynostosis so far. Brisset et al. present a complex finding of paternally inherited duplication 1q12q21.2 (5.8 Mb) in combination with maternally inherited deletion of 16p11.2 of 545 Kb in a child with several malformations, psychomotor delay, seizures and overweight [16]. Brisset's finding clearly differs from our patient 25, most likely due to the additional deletion of 16p. It is interesting to note that this patient's duplication (which overlaps incompletely with the finding in patient 2) also partially includes 1q21.1 recurrent region (BP3-BP4, distal) but without the *GJA5* gene, thus possibly explaining the absence of congenital heart disease in this patient. To our knowledge, the findings in patients 2 and 25 are the first reported associations between microduplications of 1q12q21.2 and 1q21.1 and syndromic craniosynostosis.

Patient 29 (*Table 1*) presented with a pathological female karyotype - 46,XX,t(2;7)(q14;q35). The same translocation was found in her mother (who presented with mild facial dysmorphism). The father had a normal male karyotype. MLPA revealed a microduplication of the short arm of chromosome 2 - dupl 2p16.1. Several cases with de novo interstitial microduplications involving 2p16.1-p15 are reported in literature with facial dysmorphism, intellectual disability, developmental delay, congenital heart defects and various additional nonspecific features [17]. No associations with craniosynostosis were found. Finally, aCGH was performed, which revealed a large pathogenic duplication of 2p - dupl 2p22-3p16.1 (25.19 Mb). This region is fairly large, containing a significant number of genes which are unrelated to craniosynostosis, with one exception – the *SIX2* gene. This gene encodes a transcription factor associated with cell differentiation and migration, crucial for the development of several organs (including the cranium). The increased dosage of *SIX2* could lead to early and pronounced ossification of cranial sutures, linking with the craniofacial dysmorphism in our patient, making this finding possibly causative. Hufnagel et al. report a case with frontonasal dysplasia with sagittal craniosynostosis due to microdeletion of the *SIX2* gene [18]. These findings reaffirm the complex role of the *SIX2* gene in the etiology of SC, making it a potential candidate for further study.

In *patient 34* (*Table 1*) the conventional cytogenetic analysis showed a normal male karyotype – 46,XY. MLPA revealed a terminal deletion of the long arm of chromosome 4 - del 4q (*TRIML2*) which has no associations with SC, as far as we know [19]. Array CGH, however, showed a submicroscopic duplication of the short arm of 1st chromosome - dupl 1p22.1 (378.47 Kb). This was classified as a likely pathogenic variant. This chromosme region contains 5 gene sequences including the *TGFBR3* gene (*Table 2*). It encodes the transforming growth factor (TGF)-beta type III receptor. These receptors, along with the FGF receptor family are widely expressed in bone cells and in the bone matrix and play an important role in premature pathological suture closure [20-21]. Based on this finding, we hypothesize that the duplication of 1p22.1 containing the *TGFBR3* gene links with the metopic craniosynostosis in our patient, making the finding potentially causative. This particular chromosome region is a promising candidate for further investigation into syndromic craniosynostosis. Additionally, our patient presented with hypoplasia of corpus callosum which is characteristic of 1p22 duplications. The disparity between the MLPA and aCGH findings is a result of method limitations. The patient‘s parents were unavailable for further testing.

In conclucion, we tried to elucidate various genetic factors involved in the pathogenesis of syndromic craniosynostosis by screening 39 children with a combination of cytogenetics, MLPA, and array CGH. In total, we found 6 patients with significant genetic variations. This constitutes 15.3% of the children in our sample, corresponding with the data we observed in the literature. In our study, aCGH had the highest detection rate proving that submicroscopic chromosomal rearrangements play an important role in the pathogenesis of syndromic craniosynostosis. MLPA and conventional karyotyping yielded respectively 7.7% and 2.5% pathological findings. Duplications were found to be more common than the deletions, underlining the importance of increased dosage of certain genes in syndromic craniosynostosis. Coronal synostosis was the most common anatomical variant we found, which differs from the established suture involvement distribution in literature, probably due to sample size limitations. Several genetic variations already connected to different pathological conditions were found in children with syndromic craniosynostosis. Those findings reaffirm the complex role of various

genetic factors in cranial suture patency regulation and warrant further investigation.

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Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Ethics approval: All procedures performed in this study involving human participants were in accordance with the ethical standarts of the institutional research comitee and with the 1964 Helsinki Decladarion and its later amendments of comparable ethical standarts. The study was approved by the Ethics Commitee of Medical Unversity of Sofia.

Consent to participate and for publication: Informed consent was obtained from all participants, including their respective family members (and/or legal guardians) before clinical selection was performed.

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