Comprehensive Genetic Evaluation of Children With Syndromic Craniosynostosis by a Combination of Cytogenetics, Multiplex Ligation-dependent Probe Amplification and Array-based Comparative Genomic Hybridisation

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Research Article

Keywords: syndromic, craniosynstosis, genetic evaluation, submicroscopic rearrangements

Posted Date: June 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-617776/v1

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 significant incidence makes these cranial deformations an important medical problem. Despite the efforts to clarify the pathogenesis of SC in recent years, its genetic aspects remain largely unknown.

Aiming to elucidate the complex genetic etiology of syndromic craniosynostosis, we conducted an investigation of 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 and gain-of-function variations). 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.

Summary

What is known

• Syndromic craniosynostosis is a genetically determined premature closure of one or more of the cranial sutures, which may result in severe craniofacial dysmorphism and increased intracranial pressure

• Despite the efforts to clarify the pathogenesis of SC in recent years, its genetic and molecular aspects remain largely unclear.

What is new

• Aiming to elucidate the complex ethiology of syndromic craniosynostosis we conducted the largest (so far) genetic screening in Bulgarian paediatric patients.

• The systematic genetic evaluation of children with SC revealed a high prevalence of submicrosopic chromosomal rearrangements – 15.3% (most commonly duplications and gain-of-function variations).

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]. The considerable risk of complications along with significant incidence makes these cranial deformations an important medical problem.

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. Nonsyndromic craniosynostosis (NSC) constitutes about 80% of all cases [4]. Recent studies have shown that genetic variations play a leading role in the pathogenesis of NSC. Considerably rarer are the syndromic forms of craniosynostosis – 20 % of all repoted cases. About 30% of the SC are caused by pathogenic variants in certain genes (*FGFR1, FGFR2, FGFR3, TWIST1, EFNB1, MSX2, RAB23, RUNX2*) [4]. These are called „monogenic" or „mendelian" forms of craniosynostosis. They are inherited in an autosomal dominant pattern (except for *RAB23*) with a variable degree of expression and penetration that yields a large array of clinical manifestations. 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]. Array CGH is however more valuable due to its higher detection rate and whole genome scope.

Despite the serious advancements in the last 2 decades, the genetic basis of craniosynostosis remains rather poorly understood. Trying to clarify the complex factors involved in the pathogenesis of syndromic craniosynostosis we conducted an investigation of 39 children which was carried out by a systematic, combined approach consisting of 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). The presence of clinical or phenotypic resemblance to any well-known monogenic craniosynostosis disorders was considered an exclusion factor.

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 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 oligo array CGH. DNA was isolated from peripheral blood by phenol-chloroform extraction. We used OGT 4x44k format oligonucleotide microarray with targeted CN resolution of 1 probe every 52kb and 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.

Patients' medical reports

The medical history, clinical features and heritage of all patients with pathogenic/probably pathogenic genetic findings are summarized in Table 1. Further patient information can be provided by the authors if requested.

Table 1
Clinical characteristics of our patients with pathological genetic findings

Patient No	Age	Sex	Craniosynostosis	Dysmorphic features	Psychomotor development	IQ	Additional 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

Results

In 27 (about 70%) of our patients, craniosynostosis was simple (a single cranial suture is obliterated). In 10 cases two sutures were simultaneously fused repesenting a complex craniosynostosis. 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) – apparently ballanced reciprocal translocation of chromosomes 2 and 7, inherited from the patient's mother *(*Tables 1 and 2*)*.

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

Patient No	Chromosome analysis	MLPA findings	aCGH findings – ISCN Notation, size	Class
2	46,XY	none	arr[hg19] 1q21.1 (144440748x2, 144510920-146188485x3, 146290655x2) (1.85Mb)	pathogenic
8	46,XX	none	arr[hg19] 14q32.33(105524898x2, 105609511_105787438x0, 105845682x2) (177.93Kb)	probably pathogenic
22	46,XY	del 5q35.3 (*NSD1*)	arr[hg19] 5q35.2 (175,470,501 – 177,136,261x0) (1.66 Mb)	pathogenic
25	46,XY	none	arr[hg19] 1q12q21.2(120322008x2, 142513049_147134234x3, 147203277x2) (4.62 Mb)	probably 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
34	46,XY	del 4q(*TRIML2*)	arr[hg19] 1p22.1 (92258725x2, 92326818_92705290x3, 92767467x2) (378.47 Kb)	probably pathogenic

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 was used to screen for submicroscopic chromosomal rearrangements in all 39 patients. Pathogenic and probably pathogenic submicroscopic defects 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. Five of those defects were duplications and three were deletions.

Table 3
Gene sequences within the chromosome regions of interest in our sample

Patient No	aCGH findings – ISCN Notation, size	Gene sequenses within the chromosome region (according to DGV – Build GRCh37:Feb 2009, hg19)
2	arr[hg19] 1q21.1 (144440748x2, 144510920-146188485x3, 146290655x2) (1.85Mb)	*NBPF20, PDE4DIP, SEC22B, HFE2, RNF115, NBPF25, PFN1P2, NOTCH2NL, POLR3GL, CD160, NBPF, microRNA 6736, NBPF member 9, NBPF member 11, NBPF member 10, NBPF member 8, LOC653513, HFEA2, TXNIP, ANKRD34A, LIX1L, RBM8A, GNRHR2, PEX11B, ITGA10, ANKRD35, PIAS3, NUDT17, POLR3C, PDZK1P1 and GPR89A.*
8	arr[hg19] 14q32.33(105524898x2, 105609511_105787438x0, 105845682x2) (177.93Kb)	*JAG2, MIR6765, NUDT14, BRF1, BTBD6* and *PACS2*
22	arr[hg19] 5q35.2 (175,470,501 – 177,136,261x0) (1.66 Mb)	40 HGNC and 24 OMIM genes, including *NSD1* and FGFR4
25	arr[hg19] 1q12q21.2(120322008x2, 142513049_147134234x3, 147203277x2) (4.62 Mb)	*LINC00624, NBPF13P, NBPF12, PRKAB2, BCL9, CHD1L, FMO5, PDIA3P1, PEX11B, PDZK1, GNRHR2, RBM8A, LIX1L, ANKRD34A, POLR3GL, CD160, SEC22B, HJV, TXNIP, NBPF10, NBPF25P, NOTCH2NLA, LOC653513, NBPF9, NBPF8, PFN1P2, PPIAL4A, LINC00623, NBPF20, FAM72D, PPIAL4G, LINC01138, MIR6077, ANKRD20A12P*
29	arr[hg19] 2p22.3p16.1(36033514x2, 36095582_61287377x3, 61369298x2) (25.19 Mb)	*SLC8A1, THUMPD2, TMEM178A, MAP4K3-DT, MAP4K3, CDKL4, SOS1, LOC375196, DHX57, GEMIN6, SRSF7, GALM, ATL2, CYP1B1-AS1, CYP1B1, RMDN2-AS1, RMDN2, CDC42EP3, QPCT, EIF2AK2, NDUFAF7, CEBPZOS, PRKD3, SULT6B1, HEATR5B, GPATCH11, STRN, VIT1, FEZ2, CRIM1, CRIM1-DT, SRBD1, LINC01121*, SIX2, *SIX3-AS1, MIR548AD, CAMKMT, PREPL, SLC3A1, PPM1B, PLEKHH2, ABCG8, LRPPRC, DYNC2LI1, C1GALT1C1L, THADA, LINC01126, ZFP36L2, HAAO, OXER1, MTA3, KCNG3, EML4, PKDCC, LINC01913, FSHR, LHCGR, GTF2A1L, STON1, STON1-GTF2A1L, PPP1R21, FOXN2, FBXO11, MSH6, KCNK12, MSH2, MIR559, EPCAM, CALM2, STPG4, TTC7A, MCFD2, LINC01119, LINC01118, SOCS5, CRIPT, PIGF, RHOQ, TMEM247, LINC02583, RHOQ-AS1, EPAS1, PRKCE, NRXN1, CCDC85A, EFEMP1, MIR216B, MIR216A, MIR217, PPP4R3B, CFAP36, PNPT1, MTIF2, MIR4426, RPS27A, CLHC1, CCDC88A, PRORSD1P, RTN4, EML6, SPTBN1, RPL23AP32, C2orf73, ACYP2, MIR3682, ERLEC1, CHAC2, GPR75-ASB3, PSME4, GPR75, ASB3, LINC01122, FANCL, VRK2, PUS10, REL, PEX13, LINC01185, PAPOLG, BCL11A*
34	arr[hg19] 1p22.1 (92258725x2, 92326818_92705290x3, 92767467x2) (378.47 Kb)	*C1orf146, BTBD8, EPHX4, BRDT*, TGFBR3

Discussion

Craniosynostosis is frequently classified as [5]: simple - in cases with a single fused cranial suture (sagittal, coronal, metopic or lambdoid - respectively) and complex *or* compound when two or more sutures are prematurely and simultaneously closed.

The distribution of suture involvement in syndromic craniosynostosis in 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 results, which may indicate that the size of our sample is insufficient to be statistically representative in this regard.

*Patient 2 (*Table 1*)* had normal results from conventional chromosome analysis and MLPA. Subsequently we performed aCGH. A pathogenic duplication of the long arm of chromosome 1 (1q21.1) was found (Tables 2 and 3). 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]. Microduplications and deletions in the same locus are associated with an increased risk of psychiatric complications (schizophrenia) in adults [8]. Our patient was diagnosed with ASD, which is congruent with the duplication found in 1q21.1. No direct associations with syndromic craniosynostosis were found. This finding reaffirms the complex genetic etiology of SC and requires further investigation.

In *patient 8* (Table 1), conventional cytogenetics established a normal female karyotype, MLPA also showed a normal result. The patient was screened for submicroscopic rearrangements using aCGH, yielding one probably pathogenic deletion of 14q32.33 (177.93Kb). Several gene sequences have been mapped on this region (Tables 2 and 3). None of these have been connected to premature cranial suture ossification in literature. A submicroscopic deletion of the long arm of chromosome 14 is associated with two conditions – Dubowitz syndome [9] and 14q32.3 deletion syndrome [10]. Due to the patient 8 facial dysmorphism and the presence of gastrointestinal symptoms as well as brachydactyly we are inclined towards Dubowitz symdrome. However 14q32.3 deletion syndrome can not be disproved conclusively at this point since these are extremely rare entities with highly variable phenotypic manifestations. As far as we know neither Dubowitz syndrome nor 14q32.3 deletion syndrome have been associated with craniosynostosis. The association of submicroscopic deletion 14q.32 and SC needs further investigation.

In *patient 22* (Table 1) aCGH revealed a deletion of the 5q35 region (5q35.2-5q35.3). The deletion was 1.665 Mb in size (Tables 2 and 3), 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 [11]. The size and mechanism of formation of 5q35 microdeletions differ depending upon the ethnic origin of the patients [12]. The presented features of our patient (Table 1) - developmental delay, characteristic facial appearance, and macro-dolichocephaly were typical for SoS, although the overgrowth was absent. Childhood overgrowth is a cardinal symptom of SoS, and is present in over 90% of the cases. Our patient's microdeletion includes the *NSD1* and *FGFR4* genes. Overall, the individuals with microdeletions have less prominent overgrowth than patients with *NSD1* variants [13]. Douglas et al. also described a patient with 5q35 microdeletion involving *NSD1* and *FGFR4* genes and craniosynostosis [14]. 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 differenent bone diseases, including craniosynostosis syndromes [15]. 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 [16].

The genetic evalutaion of *patient 25* (Table 1) began with chromosome analysis and MLPA which both showed normal results. Array CGH however, revealed a probably pathogenic microduplication of chromosme 1 (1q12q21.2) spanning across 4.62 Mb (Tables 2 and 3). None of the genes within this chromosome region have been associated with craniosynostosis. 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 (including plagiocephaly), psychomotor delay, seizures and overweight [17]. Brisset's finding clearly differs from our patient 25, most likely due to the additional deletion of 16p. To our knowledge, the evidence of association between microduplication 1q12q21.2 and syndromic craniosynostosis is insufficient to be basis for concrete genotype-phenotype correlations.

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 had mild facial dysmorphism and no other complications). The father had a normal male karyotype (46,XY). 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 [18]. 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 chromosomal region is fairly large, containing a number of genes (Table 3). We found no variations of those sequences that are reported as causative of craniosynostosis with one exception – the *SIX2* gene. It encodes a transcription factor associated with cell differentiation and migration, crucial for the development of several organs (stomach, kidneys and skull). The increased dosage of *SIX2* could lead to early and pronounced ossification of cranial sutures which links with the craniofacial dysmorphism in our patient, making this finding possibly causative. Hufnagel et al. however, report a case with frontonasal dysplasia with sagittal craniosynostosis due to microdeletion of the *SIX2* gene [19]. These findings reaffirm the complex genetic etiology of SC.

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. Array CGH, however showed a submicroscopic duplication of the short arm of 1st chromosome - dupl 1p22.1 (378.47 Kb) which was classified as a probably pathogenic variant. This chromosme region contains 5 gene sequences (Table 3) including the *TGFBR3* gene. 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 bone matrix and play an important role in premature pathological suture closure [21–25]. 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 regarding syndromic craniosynostosis. Additionally our patient presented with hypoplasia of corpus callosum which is characteristic of 1p22 duplications. The disparity between MLPA and aCGH findings found in patient 34 is a result of method limitations.

Conclusion

In an effort to elucidate various genetic factors involved in the pathogenesis of syndromic craniosynostosis we conducted an investigation of 39 children using a combination of cytogenetics, MLPA and array CGH. In total we found 6 patients with pathological genetic variations. This constitutes 15.3% of the children in our sample which corresponds to the data we observed in 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 then the deletions, underlining the importance of increased dosage of certain genes in syndromic craniosynostosis.

Coronal synostosis was the most common anatomical substrate we found, which differs from the established suture involvement distribution in literature, probably due to certain limitations in our sample.

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.

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

DNA - Deoxyribonucleic acid

GRCh – Genome Reference Consortium (human build)

HGNC – HUGO gene nomenclature committee

hg – human genome

ISCN – The International Sustainable Campus Network

kb/kbp – kilobase/kilobase pair

Mb/Mbp – Megabase/Megabase pair

MLPA - Multiplex ligation-dependent probe amplification

NSC - Nonsyndromic craniosynostosis

OGT – Oxford gene technology

OMIM – Online mendelian inheritance in man

PCR - Polymerase chain reaction

SC - Syndromic craniosynostosis

SoS – Sotos syndrome

Declarations

Funding:

No funding was received to assist with the preparation of this manuscript

Conflicts of interest/Competing interests:

The authors have no conflicts of interest to declare that are relevant to the content of this article

Availability of data and material:

Additional data and source material will be provided by the authors if requested

Code availability:

N/A

Author's contributions:

main author, corresponding author - **T. Delchev**; DNA extraction, array CGH screening, array analysis - **S. Hadjidekova**; preliminary clinical selection and diagnostics - **H. Kathom**; DNA extraction, MLPA screening - **S. Bichev**; imaging studies documentation - **Ts. Veleva**; conventional cytogenetic analysis - **I. Boneva**; coordinator, main consultant, academic science advisor - **D. Avdjieva-Tzavella**

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:

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

Consent for publication:

Additional informed consent for publication was obtained from all participants, including their respective family members (and/or legal guardians).

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