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Clinical Genetics of Craniosynostosis

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Abstract

Purpose of review—When providing accurate clinical diagnosis and genetic counselling in craniosynostosis, the challenge is heightened by knowledge that etiology in any individual case may be entirely genetic, entirely environmental, or anything in between. This review will scope out how recent genetic discoveries from next-generation sequencing have impacted on the clinical genetic evaluation of craniosynostosis.

Recent findings—Survey of a 13-year birth cohort of patients treated at a single craniofacial unit demonstrates that a genetic cause of craniosynostosis can be identified in one quarter of cases. The substantial contributions of mutations in two genes, *TCF12* and *ERF*, is confirmed. Important recent discoveries are mutations of *CDC45* and *SMO* in specific craniosynostosis syndromes, and of *SMAD6* in non-syndromic midline synostosis. The added value of exome or whole genome sequencing in the diagnosis of difficult cases is highlighted.

Summary—Strategies to optimise clinical genetic diagnostic pathways by combining both targeted and next-generation sequencing are discussed. As well as improved genetic counselling, recent discoveries spotlight the important roles of signalling through the bone morphogenetic protein and hedgehog pathways in cranial suture biogenesis, as well as a key requirement for adequate cell division in suture maintenance.

Keywords

Exome sequencing; CDC45; SMAD6; SMO

Introduction

Craniosynostosis, the premature fusion of the cranial sutures, has a prevalence of between 1 in 1,400 and 1 in 2,100 children [1, 2*], putting it at the borderline of what constitutes a rare disease. Moreover the frequency of non-syndromic midline synostosis appears to be

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Conflicts of interest
None

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increasing, for reasons that are not understood [2*]. Clinical management poses multiple challenges, which are best addressed in a specialist multidisciplinary unit.

Etiological assessment of craniosynostosis should start by recognising the substantial heterogeneity in underlying causes. Awareness of both the striking pathological differences between fusion of different cranial sutures, and the complex interplay of potentially causative factors - intrauterine environment, polygenic background, growth and development of the brain, as well as specific monogenic and chromosomal disorders – is essential [3]. Reviews of the clinical approach to diagnosis and associated phenotypic features are covered in several articles [4–6]; here we focus on recent progress in understanding the genetic underpinnings of craniosynostosis.

A current benchmark for diagnosis

An initial clinical evaluation of a child with craniosynostosis will categorise them according to the cranial vault suture, or sutures, shown to be fused (preferably using 3-dimensional computed tomographic reconstruction); whether any risk factors can be identified in the obstetric or perinatal history; and features suggesting an underlying syndrome, based on positive family history, associated malformations or dysmorphic features, and evidence of significant developmental or cognitive delay. To provide context, Figure 1 shows data (updated from a previously published series [7]), collected from a single specialist unit at Oxford, UK for a 13-year birth cohort (n = 666) with minimum 5-year follow-up. The frequency of different types of craniosynostosis varies widely, with sagittal synostosis being most common (41%) and lambdoid synostosis rarest (1%). Equally striking, the proportion of cases in which a cause is delineated varies widely, from 88% for bicoronal synostosis to only 8% for sagittal synostosis. The high genetic load in coronal synostosis likely reflects the distinct biogenesis of the coronal sutures during embryonic development [8–10].

Although an adverse intrauterine environment may contribute substantially to the origin of many cases of craniosynostosis [11–13], this is difficult to prove in the individual patient [14]. Overall, only 2.4% of cases were attributed to a likely secondary cause in the Oxford series. Of the remainder, 31% were classified clinically as syndromic and 69% as non-syndromic (Fig. 1). Not surprisingly, a positive genetic diagnosis was obtained in a much higher proportion of the syndromic cases (69%) than those initially classified as non-syndromic (5%). However, as discussed below, the recognition that a growing minority of non-syndromic craniosynostosis may have an underlying monogenic basis has important implications for the approach to genetic testing and genetic counselling.

Syndromic craniosynostosis

As shown in Fig. 1, there are six genes frequently (each >0.5% overall) mutated in craniosynostosis: *FGFR2*, *FGFR3*, *TWIST1*, *EFNB1*, *TCF12* and *ERF*. Mutations in the first four of these mostly cause recognisable syndromes (*FGFR2*, predominantly Apert, Crouzon and Pfeiffer; *FGFR3*, Muenke and Crouzon with acanthosis nigricans; *TWIST1*, Saethre-Chotzen; and *EFNB1*, craniofrontonasal syndrome), for which the molecular basis was determined a decade or more ago, so their clinical features and genotype-phenotype

correlations are largely well documented [3–6]. Recent clinical updates have been published on prevalence of tracheal cartilaginous sleeve [15] and progressive postnatal pansynostosis [16] in syndromic craniosynostosis, both of which highlight the particularly high burden of complications arising from *FGFR2* mutations. Additional *FGFR2* mutation-focused phenotype studies have been published on foramen magnum size [17], ophthalmic complications [18] and intestinal malrotation [19]. Complications were found to be differentially enriched in different syndromes: tracheal sleeve, proptosis and exposure keratitis were particularly associated with Pfeiffer syndrome, whereas insidious postnatal pansynostosis was most common in Crouzon syndrome. Recently, useful series have been published on the two most severe - fortunately rare - *FGFR2*-associated craniosynostosis syndromes, Beare-Stevenson cutis gyrata syndrome (BSS) and bent-bone dysplasia (BBD). BSS is caused by two specific heterozygous missense mutations in the juxtatransmembrane region of FGFR2, encoding p.Ser372Tyr and p.Tyr375Cys. Wenger *et al.* [20] reviewed 21 previously published cases of BSS and added two new cases, highlighting the substantial mortality in the first year of life (70%) and developmental delay in survivors. BBD was only recognised as a distinct clinical entity in 2012 [21] and Krakow *et al.* provide a useful overview of the clinical features of 11 cases, including 7 previously unpublished [22*]. Although the missense mutations responsible for BBD, encoding p.Tyr381Asp and p.Met391Arg, lie close to those for BBS, the BBD substitutions localise to the transmembrane region and are associated with a distinct pathophysiology involving enhanced nucleolar rRNA transcripton [23].

Muenke syndrome, defined by a specific 749C>G (p.P250R) mutation in *FGFR3* that represents the single most common nucleotide substitution in craniosynostosis, can only be confidently identified by genetic testing. A clinical survey of 106 subjects provides an overview of the natural history of this disorder [24]; 15% of individuals did not have craniosynostosis, and association with isolated hydrocephalus has been separately described [25]. The mechanisms underlying the clinical variability of Muenke syndrome are not understood, indicating that a wide range of possible outcomes should be mentioned when providing preconceptual and prenatal advice. Not surprisingly there is a diversity of attitudes towards prenatal testing, as recently surveyed amongst five adult couples, in each of which one individual was affected with Muenke syndrome [26].

Two other genes mutated in >1% of craniosynostosis, *TCF12* and *ERF*, were first reported in 2013 [27, 28], so description of the associated natural history is less complete. *TCF12* is discussed in the section on non-syndromic craniosynostosis. *ERF* encodes a negative regulator of ERK1/2, the key signal transducer at the base of the pathway from growth factor receptors through RAS-MAP kinase. Clinical presentation of *ERF* mutations varies from a mild Crouzon-like picture to non-syndromic craniosynostosis. Further published information to augment the original clinical descriptions [28] is still scanty; surprisingly, in view of the relatively high frequency in the Oxford cohort, Paumard-Hernandez *et al.* [29] did not identify any *ERF* mutations in a series of 69 undiagnosed craniosynostosis cases. Chaudhry *et al.* [30] described two subjects with *ERF* mutations, who had features overlapping those originally reported. Surprisingly - given that the pathogenic mechanism of most ERF mutations appears to be haploinsufficiency - a specific missense substitution, p.Tyr89Cys, located within the DNA-binding ETS domain of ERF, was identified in 4 unrelated patients

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or families with Chitayat syndrome, which is characterised by a bilateral accessory phalanx resulting in shortening of the index finger, hallux valgus and respiratory compromise [31]. Although facial features were similar to other subjects with *ERF* mutations, craniosynostosis was not documented. The distinct clinical features might result from altered DNA-binding properties associated with the specific missense substitution, but this has not so far been investigated experimentally.

Recently identified disease genes

Next-generation sequencing has substantially accelerated the discovery of new gene/disease associations in syndromic craniosynostosis. Two examples in 2016 were a mosaic heterozygous mutation of *SMO* in Curry-Jones syndrome (CJS) [32*] and biallelic mutations of *CDC45* in Meier-Gorlin syndrome (MGS) associated with craniosynostosis [33*]. CJS is characteristed by patchy skin lesions, polysyndactyly, diverse cerebral malformations, coronal craniosynostosis, iris colobomas, microphthalmia and intestinal malrotation. *SMO* encodes smoothened, a G-protein-coupled receptor that transduces signalling by the hedgehog family of proteins; the recurrent, mosaic activating c.1234C>T substitution encoding p.Leu412Phe was identified in eight of ten CJS cases analyzed [32*]. CJS has unusual abdominal symptomatology associated with smooth muscle hamartomas on the mesentery and surface of the bowel; motility disorders and upper gastrointestinal bleeding are frequent [34]. The identical *SMO* mutation has also been identified in several tumors, particularly involving the skin or brain; these are potentially treatable using hedgehog pathway inhibitors [35]. *CDC45* encodes a key component of the machinery of DNA replication, present in all eukaryotes, so the identification of mutations in craniosynostosis may appear surprising. Clinical presentation varied from unicoronal or bicoronal synostosis and mild short stature, to a severe phenotype of MGS (defined by the triad of short stature, microtia and a/hypoplastic patellae), combined with multi-suture synostosis. Mutations were found to reduce protein levels, either by affecting splicing or through protein instability (missense mutations); variation in the amount of residual protein activity probably explains the variability of the phenotype observed [33*].

Non-syndromic craniosynostosis

The contribution of genetic diagnoses has been substantially lower in non-syndromic craniosynostosis, <1% in sagittal and metopic synostosis (Fig. 1 and [36]). However diagnostic success rates are higher in unicoronal (13%), multisuture (15%) and bicoronal synostosis (60%) cases (Fig. 1). The single largest contributor to these diagnoses is *TCF12*, which encodes a partner protein of TWIST1 particularly critical for coronal suture development [27]. Two follow-up studies have confirmed the importance of *TCF12* mutations in coronal craniosynostosis, both in the context of familial mutations [37], and in a more general screen of craniosynostosis [29]. Given the haploinsufficiency mechanism of *TCF12* mutations, heterozygous deletions are also expected to be pathogenic and this has been confirmed in two reports [38, 39]. At present, diagnostic labs rely on DNA sequencing to test *TCF12*, pointing to the need for a dedicated diagnostic method such as multiplex ligation-dependent probe amplification (MLPA) to detect *TCF12* deletions. Further analysis of the phenotype associated with *TCF12* mutations is awaited: although the clinical outlook

in most affected individuals is good (and non-penetrance occurs in 50% or more of mutation-positive individuals [27]), a minority may present with learning disability or autistic spectrum disorder [40]. The reasons for this clinical variability require further investigation.

A recent discovery that is likely to change the previously negative diagnostic picture for the midline synostoses was reported by Timberlake *et al.* [41**]. In an exome sequencing study of 132 parent-offspring trios and 59 additional probands with either sagittal or metopic synostosis, these authors reported a significant enrichment of mutations in *SMAD6*, which encodes a negative regulator of signaling through the bone morphogenetic protein (BMP) pathway. Thirteen percent of individuals with metopic, and 3% with sagittal synostosis, were heterozygous for loss-of-function or rare missense variants in SMAD6, and the positive diagnostic rate was higher (24%) in familial cases. Although *de novo* mutations occurred in 3 of the 13 families identified, in the others, the variant was inherited from a parent who was usually unaffected. Confirming that non-penetrance for *SMAD6* mutations is frequent, the Exome Aggregation Consortium [42] did not identify any deficiency of *SMAD6* loss-of-function mutations, yielding a pLI (probability of being intolerant to loss-of-function mutations, also termed *constraint*) value of zero. Timberlake *et al.* [41**] proposed an ingenious explanation for this paradox. Observing that in the only reported genome-wide association study of non-syndromic sagittal synostosis, the strongest signal (odds ratio = 4.6 for the risk allele) was with a single nucleotide polymorphism (SNP) rs1884302 located 345 kb away from the *BMP2* gene [43], these authors genotyped the *SMAD6* mutation-positive individuals for the rs1884302 SNP. They found that 14 of 17 affected individuals harbored at least one risk allele (C), whereas all 13 unaffected individuals were homozygous for the non-risk (T) allele, a highly significant difference that appears to support a digenic disease mechanism involving two different components of BMP signalling. This finding could have major implications for molecular diagnostics, as no genetic testing is currently routinely indicated in either non-syndromic sagittal or metopic synostosis, the two most common clinical presentations of craniosynostosis (Fig. 1). However, the paradoxically low pLI score urges caution in interpretating these data; other groups are currently attempting to replicate the findings to reach consensus regarding future diagnostic use. A further *caveat* is emerging evidence that a similar spectrum of *SMAD6* mutations may predispose to cardiac abnormalities, particularly bicuspid aortic valve with ascending aortic dilatation [44, 45]. This raises the question whether echocardiography should be undertaken on all mutation-positive individuals; clinicians need to have a clear, evidence-based care pathway before offering genetic testing.

Molecular diagnostic approach to craniosynostosis

Although mutations in just six genes constitute three-quarters of all genetically diagnosed cases, the etiology of the remaining quarter is very diverse. Fifty-seven genes were classified as validated “craniosynostosis genes” by Twigg and Wilkie [3], based on identification of mutations in two or more independent cases, and some additional potentially causative genes were highlighted by Lattanzi *et al.* [6]. The long tail of rare genetic diagnoses is apparent in Fig. 1, which shows that in the category of syndromic craniosynostosis with an identified mutation, “other monogenic” (comprising mutations in 20 different genes) is the second

leading causal category after *FGFR2* mutations in Apert syndrome. These rare diagnoses include potentially treatable conditions for which early recognition is particularly important, such as hypophosphatasia (*ALPL*) [46], Albright osteodystrophy (*GNAS1*) [47, 48] and rickets (*XLH*) [49]. A diverse variety of chromosomal abnormalities also occurs in association with craniosynostosis, probably often through non-specific mechanisms involving suboptimal brain growth. The question arises how to design an optimal diagnostic algorithm that accommodates both the simple and complex aspects of the overall presentation.

As a guide, Fig. 2 provides a hierarchical summary of the effort required to make each successful diagnosis. Sixty-six percent of all diagnoses were made using just five diagnostic tests – targeted DNA sequencing of *FGFR2* exons IIIa and IIIc, *FGFR3* exon 7 and *TWIST1* exon 1, plus array CGH, so in many clinical situations it will make sense to start with a combination of these investigations. Moving further down the hierarchy, some tests are more complex (for example, *TCF12* contains 19 coding exons) and the economic argument for using next-generation sequencing is increasingly strong, although orthogonal technology is currently still required to detect specific copy number variations.

Recently the first use of exome and whole genome sequencing for difficult-to-diagnose craniosynostosis was presented [50*]. Of 40 probands studied (previously negative for a wide range of targeted testing), a molecular genetic diagnosis was resolved in 15 (37.5%) of cases. *IL11RA* [51–53] was the only recurrently mutated gene, further underlining the very substantial genetic heterogeneity in rare causes of craniosynostosis. Mutations were classified according to four categories: commonly mutated craniosynostosis genes with atypical presentation; other core craniosynostosis genes; more rarely associated genes; and known disease genes not known to be associated with craniosynostosis. The genes in which mutations occurred were distributed across all four categories, making an argument for the value of a genome-wide search strategy rather than gene panel. Another important finding from this study [50*] was that in 5 of the 15 positive cases, the novel molecular diagnosis had immediate, actionable consequences for genetic or clinical management, either in terms of reproductive diagnostic options or for the medical management of potential complications revealed by the diagnosis.

Genetic counselling in craniosynsotosis

Aside from the uncertainties that face geneticists when counselling about the reproductive implications for many disorders (such as variable expressivity and gonadal mosaicism), a particular issue in craniosynostosis is that 45% of identified genetic causes pinpoint within the *FGFR2* and *FGFR3* genes [Fig. 1]. These genes show markedly elevated apparent mutation rates owing to selective advantage of these mutations when they arise spontaneously in the adult male testis (a process termed selfish spermatogonial selection [54]). Direct methods to identify the source of the originating mutations within individual seminiferous tubules of testes (removed because of incidental pathology) were recently described [55, 56], providing further support for the proposed pathophysiological mechanism. The clinical significance of this knowledge is that sibling recurrence risk for *de*

novo FGFR2 and *FGFR3* mutations is likely to be exceptionally low, making it justified to reassure parents and mitigate demand for prenatal diagnosis [57].

Conclusion

Several initiatives are under way to undertake wide-scale exome/genome sequencing in craniosynostosis, which are expected to yield further novel gene mutations; however these are likely to be either rare, or associated with substantial non-penetrance (as is the case, for example, with *TCF12* and *SMAD6*). Further genome-wide association studies are also in progress, which might, like the *BMP2* SNP, also have possible diagnostic implications.

Partly fuelled by these human genetic studies, fundamental research into the developmental biology of the cranial sutures is continuing to make progress. Maintenance of sutural patency requires a delicate balance between stem cell maintenance, proliferation and osteogenic differentiation [3]; a key goal is to identify the stem cells required to maintain sutural integrity, and delineate their niche (this is likely to involve integration with molecular stress/strain transduction mechanisms, about which very little is currently known). Importantly, markers are now becoming available to mark murine sutural cells at different stages of differentiation including *Gli1* [58], *Prrx1* [59] and *Axin2* [60*]. A detailed understanding of the complex processes underlying normal sutural homeostasis may eventually lead to medical preventions or therapies for craniosynostosis [61]. For the time being, however, surgery continues to be the mainstay of treatment, although lack of consensus about timing and surgical approaches remains a persisting issue in this field [62].

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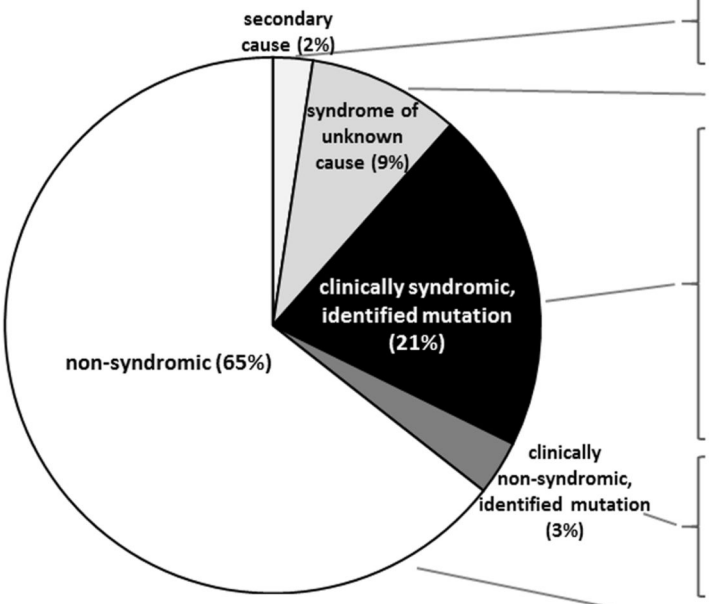
Key points

- The causes of craniosynostosis are very heterogeneous, with monogenic, chromosomal, polygenic and environmental/teratogenic factors all playing an important role
- A specific genetic diagnosis can currently be identified in one quarter of patients with craniosynostosis
- Five percent of patients initially classified as having a non-syndromic diagnosis are subsequently found to harbour a pathogenic mutation; the *TCF12* gene is most frequently implicated (coronal synostosis)
- The recent discovery of *SMAD6* mutations in midline synostosis may have further implications for diagnostic assessment, but both the proposed digenic inheritance mechanism, and potential implications for cardiovascular risk, require further evaluation before clinical implementation
- Craniosynostosis occurs at low frequency in a large number of rare monogenic disorders, many of which have require specific protocols for therapy or screening for additional complications. Accurate and prompt diagnosis requires a combination of careful clinical evaluation and correctly targeted diagnostic testing, proceeding to exome/whole genome sequencing if necessary



suture fused		S	M	UC	BC	L	MS
total		275	129	108	68	9	77
% solved cases		8	13.2	24.1	88.2	11.1	64.9
extreme prematurity	n=7	3	2				2
maternal valproate	n=6		6				
other secondary causes	n=3	1		1			1
syndrome of unknown cause	n=61	22	15	10	4		10
FGFR2-Apert	n=24				19		5
other monogenic	n=23	6	2		2		13
TWIST1-Saethre-Chotzen	n=22			6	12		4
FGFR3-P250R (Muenke)	n=18			3	15		
FGFR2-Crouzon	n=17	5			2		10
chromosomal abnormality	n=16	5	7				4
EFNB1-craniofrontonasal	n=5			3	2		
FGFR2-Pfeiffer	n=4						4
ERF	n=4	1		1			2
TCF12	n=3			1	2		
FGFR3-A391E (Crouzon+AN)	n=2						2
TCF12	n=9			6	2		1
FGFR3-P250R (Muenke)	n=6			2	4		
ERF	n=3	1				1	1
FGFR2	n=2			1			1
TWIST1	n=2			2			
non-syndromic	n=429	231	97	72	4	8	17

Figure 1. Classification and causes of craniosynostosis in a prospectively ascertained 13-year cohort

Data are based on a cohort of patients with craniosynostosis (n = 666) born between 1998 and 2010 inclusive, presenting to a single specialized unit (Oxford, UK) and requiring at least one major craniofacial procedure by the end of 2015. The pie chart on the left shows a broad classification according to presence or absence of syndromic features and identification of a likely secondary or genetic cause. The grid on the right provides a more detailed breakdown according to the pattern of suture involvement and precise diagnosis. Abbreviations for different suture fusions as follows: S, sagittal; M, metopic; UC, unilateral coronal; BC, bilateral coronal; L, unilateral or bilateral lambdoid; MS, multiple suture fusion excluding bilateral coronal or lambdoid. AN, acanthosis nigricans. Data updated from previously published 5-year cohort [7].

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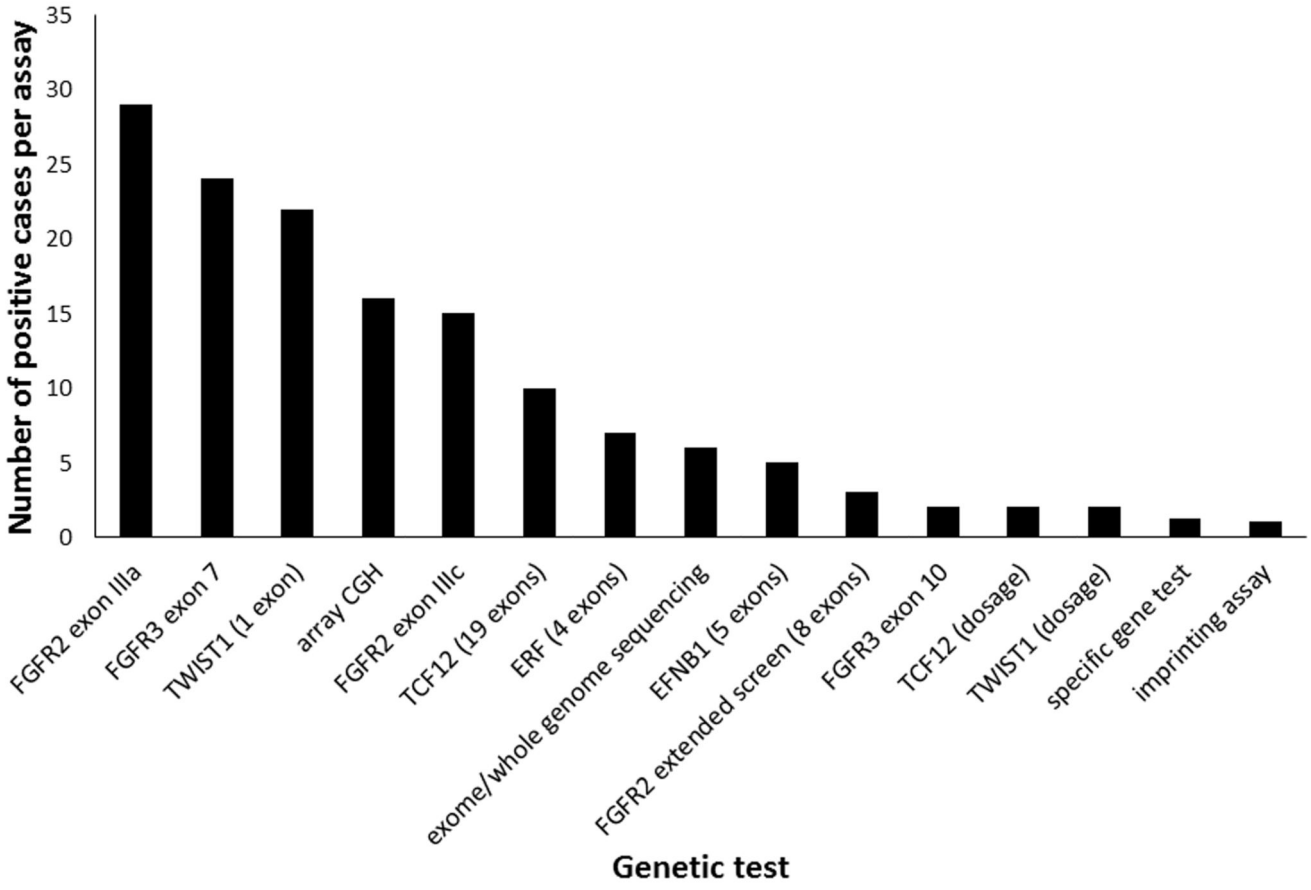

Figure 2. Genetic testing in craniosynostosis

The cohort described in Fig. 1 (n = 666) was analyzed in terms of the number of successes in achieving a positive diagnosis for different genetic tests. The tests are arranged hierarchically with those yielding the highest number of diagnoses at the left.

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