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ORIGINAL ARTICLE

Missense mutations in the DNA-binding/dimerization domain of *NFIX* cause Sotos-like features

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Sotos syndrome is characterized by prenatal and postnatal overgrowth, characteristic craniofacial features and mental retardation. Haploinsufficiency of *NSD1* causes Sotos syndrome. Recently, two microdeletions encompassing *Nuclear Factor I-X (NFIX)* and a nonsense mutation in *NFIX* have been found in three individuals with Sotos-like overgrowth features, suggesting possible involvements of *NFIX* abnormalities in Sotos-like features. Interestingly, seven frameshift and two splice site mutations in *NFIX* have also been found in nine individuals with Marshall–Smith syndrome. In this study, 48 individuals who were suspected as Sotos syndrome but showing no *NSD1* abnormalities were examined for *NFIX* mutations by high-resolution melt analysis. We identified two heterozygous missense mutations in the DNA-binding/dimerization domain of the NFIX protein. Both mutations occurred at evolutionally conserved amino acids. The c.179T>C (p.Leu60Pro) mutation occurred *de novo* and the c.362G>C (p.Arg121Pro) mutation was inherited from possibly affected mother. Both mutations were absent in 250 healthy Japanese controls. Our study revealed that missense mutations in *NFIX* were able to cause Sotos-like features. Mutations in DNA-binding/dimerization domain of NFIX protein also suggest that the transcriptional regulation is abnormally fluctuated because of *NFIX* abnormalities. In individuals with Sotos-like features unrelated to *NSD1* changes, genetic testing of *NFIX* should be considered.
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INTRODUCTION

Sotos syndrome (MIM #117550) is an overgrowth syndrome characterized by tall stature and/or macrocephaly, distinctive facial appearance and mental retardation.[1] A *de novo* t(5;8)(q35;q24.1) translocation in a patient with Sotos syndrome revealed disruption of *NSD1* at 5q35. Subsequent identification of nonsense, frameshift and submicroscopic deletion mutations of *NSD1* in patients with Sotos syndrome clearly showed that haploinsufficiency of *NSD1* causes Sotos syndrome.[2] *NSD1* encodes nuclear receptor-binding SET domain protein 1, which functions as a histone methyltransferase that activates and represses transcription through chromatin modification.[3] The diagnosis of Sotos syndrome is established by confirming *NSD1* abnormalities,[4] and abnormalities of *NSD1* causes up to 90% of Sotos syndrome cases. However, a part of patients with suspected Sotos syndrome are known to show no abnormalities in *NSD1*,[5] suggesting involvement of another gene.

Recently it was reported that two patients with Sotos-like overgrowth features possessed microdeletions encompassing *Nuclear Factor I-X (NFIX)* at 19p13.2. In addition, a nonsense mutation in *NFIX* was identified in one patient with Sotos-like features.[6] Interestingly, frameshift and donor-splice site mutations were also identified in Marshall–Smith syndrome (MIM 602535) that is osteochondysplasia syndrome characterized by accelerated skeletal maturation, relative failure to thrive, respiratory difficulties, mental retardation and unusual facial features.[7] Therefore, *NFIX* mutations could cause either Sotos-like features or Marshall–Smith syndrome. Whereas the transcripts possessing the nonsense mutation in a patient with Sotos-like features suffered from the nonsense-mediated mRNA decay, transcripts of mutated alleles (by a donor-spice site and two frameshift mutations) in patients with Marshall–Smith syndrome escaped from the nonsense-mediated mRNA decay surveillance and could be translated, suggesting that haploinsufficiency of *NFIX* leads to

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Sotos-like features and dominant-negative effects of the truncated NFIX proteins cause Marshall–Smith syndrome.[6]

In this study, we screened for *NFIX* mutations in 48 Japanese patients who were suspected as Sotos syndrome, but showed neither deletions nor mutations in *NSD1*. Detailed genetic and clinical data are presented.

MATERIALS AND METHODS

Subjects

A total of 48 patients suspected as Sotos syndrome were analyzed for *NFIX* mutations. *NSD1* investigation by sequencing and fluorescent *in situ* hybridization analysis was negative in these patients. In this study, the patients presenting with cardinal features of Sotos syndrome (specific craniofacial features, intellectual disability and overgrowth to same extent) but showing no *NSD1* abnormalities are referred as those with 'Sotos-like features'. Experimental protocols were approved by the Committee for Ethical issues at Yokohama City University School of Medicine. All individuals were investigated in agreement with the requirements of Japanese regulations.

Mutation analysis

Genomic DNA was isolated from peripheral blood leukocytes according to standard methods. DNA for mutation screening was amplified by illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK). Sequencing of exon 1 and high-resolution melting curve (HRM) analysis of exon 2–9 covering the *NFIX* coding region (GenBank accession number NM_002501.2) were performed. For exon 1, the 12 μl PCR mixture contained 30 ng DNA, 0.3 μM each primer, 0.4 mM each dNTP, 1× PCR buffer for KOD FX and 0.3 U KOD FX polymerase (Toyobo, Osaka, Japan). For exons 2–9, real-time PCR and HRM analysis were serially performed in 12 μl mixture on Rotor-Gene Q (QIAGEN, Hilden, Germany). For exon 7, the PCR mixture contained 30 ng DNA, 0.3 μM each primer, 0.4 mM each dNTP, 0.36 μl SYTO9 (Invitrogen, Carlsbad, CA, USA), 0.4 mM each dNTP, 1× PCR buffer for KOD FX and 0.3 U KOD FX polymerase (Toyobo). For the remaining exons, the PCR mixture contained 30 ng DNA, 0.25 μM each primer, 0.36 μl SYTO9 (Invitrogen), 0.2 mM each dNTP, 1× ExTaq buffer and 0.375 U ExTaq HS (Takara, Otsu, Japan). Primers and conditions of PCR are shown in Supplementary Table 1. The PCR products showing an aberrant melting curve were sequenced. All the novel mutations in DNA amplified by GenomiPhi were verified by sequencing of PCR products using genomic DNA as a template. Mutations were checked in 250 Japanese normal controls (500 alleles) by HRM analysis.

Parentage testing

For the family showing *de novo* mutations, parentage was confirmed by microsatellite analysis as previously described.[8] Biological parentage was judged if more than four informative markers were compatible and other uninformative markers showed no discrepancies.

Prediction of functional effect

The effect of the mutations for protein features was predicted by following web-based prediction tools: SIFT (http://sift.jcvi.org/), PolyPhen (http://genetics.bwh.harvard.edu/pph/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org/) and Align GVGD (http://agvgd.iarc.fr/agvgd_input.php).

RESULTS

NFIX mutations

Two heterozygous missense mutations were identified. The c.179T>C (p.Leu60Pro) mutation in patient 1 were not found in her parents, indicating that the mutation occurred *de novo* (Figure 1a). Biological parentage was confirmed by several microsatellite markers (data not shown). The c.362G>C (p.Arg121Pro) mutation in patient 2 was found in his mother (Figure 1a). These two mutations occurred at evolutionary conserved amino acids (Figure 1b) and were absent in 250 Japanese normal controls. Interestingly, the missense changes were located in DNA-binding/dimerization domain of the NFIX protein (Figure 1c). Evaluation with web-based prediction tools strongly suggested that these substitutions are pathogenic (Supplementary Table 2).

Clinical information of the patients

Patient 1 is a product of unrelated healthy parents. The body weight at birth was 2816 g (−0.6 s.d.), height 48.8 cm (0 s.d.) and OFC 33.5 cm (+0.3 s.d.). Neonatal hypotonia was recognized. At 17 months of age, her weight was 9.24 kg (−0.5 s.d.), height 84.9 cm (+2 s.d.) and OFC 48 cm (+1.2 s.d.). The facial appearance showed long/narrow and triangular face, high forehead, midface hypoplasia, prominent ears, epicanthal folds, strabismus, down-slanting palpebral fissures, short nose with anteverted nares, prominent long philtrum, everted lower lip and narrow palate (Figure 1d). Large hands/feet, prominent fingertips, pectus excavatum were also noted. Her primary dentition started at 7 months of age and was completed by 17 months of age. Bone age was estimated as 3 years at 17 months of age and as 5 years at 3 years of age. Bullet-shaped phalanges, which are typical features of Marshall–Smith syndrome, were not observed. She was initially diagnosed as Sotos syndrome. She showed mental retardation and severe developmental delay with developmental quotients of 19. Scoliosis was noted at 18 months of age and surgically treated for several times. Complex partial seizures were noted at 4 years of age and were controlled with phenytoin and zonisamide. At present (17 years of age), prognathia was observed (Figure 1e). Her weight was 40 kg (−2 s.d.) and height 156.5 cm (−0.2 s.d.).

Patient 2 is a male at age of 20 years. The birth weight was 2938 g (−0.4 s.d.), height 51 cm (+0.8 s.d.) and OFC 35.5 cm (+1.4 s.d.). Respiratory insufficiency was noted, but no visceral malformations were pointed out. Bilateral tubing therapy was performed for recurrent bilateral exudative otitis media at 4 years of age. At 14 years of age, his weight was 58.1 kg (+0.6 s.d.) and height 185.7 cm (+3.5 s.d.). Mental retardation was evident as the IQ score (Tanaka–Binet intelligence test) was 59. Craniofacial features included high forehead, down-slanting palpebral fissures and prognathia. He was suspected as Sotos syndrome. His mother showed tall stature, suggesting that c.362G>C led to overgrowth in the mother. Unfortunately, further details of clinical features in the mother are unavailable. Clinical information of two patients is summarized in Table 1.

DISCUSSION

NFIX is a member of the nuclear factor I (NFI) family proteins, which are implicated as site-specific DNA-binding proteins known to function in viral DNA replication and gene expression regulation.[9] NFI proteins form homo- or heterodimers and bind to the palindromic DNA consensus sequence through its N-terminal DNA-binding/dimerization domain.[10] Point mutations in DNA-binding/dimerization domain of NFI protein have been shown to cause loss of dimerization, DNA-binding and replication activities,[11] highlighting the importance of structural integrity of DNA-binding/dimerization domain. It has been reported that the DNA binding domain of SMADs and NFI transcription factors shared considerable structural similarity, and the secondary structure of the DNA-binding domain of NFI was estimated based on that of SMADs.[12] In this study, we identified two heterozygous missense mutations, the c.179T>C (p.Leu60Pro) and the c.362G>C (p.Arg121Pro), in the DNA-binding/dimerization domain. Of note, two mutations are estimated to be localized within α-helical region of DNA-binding domain and at evolutionally conserved amino acids between SMADs and NFI.[12] In addition, two mutations cause substitutions to a proline residue,

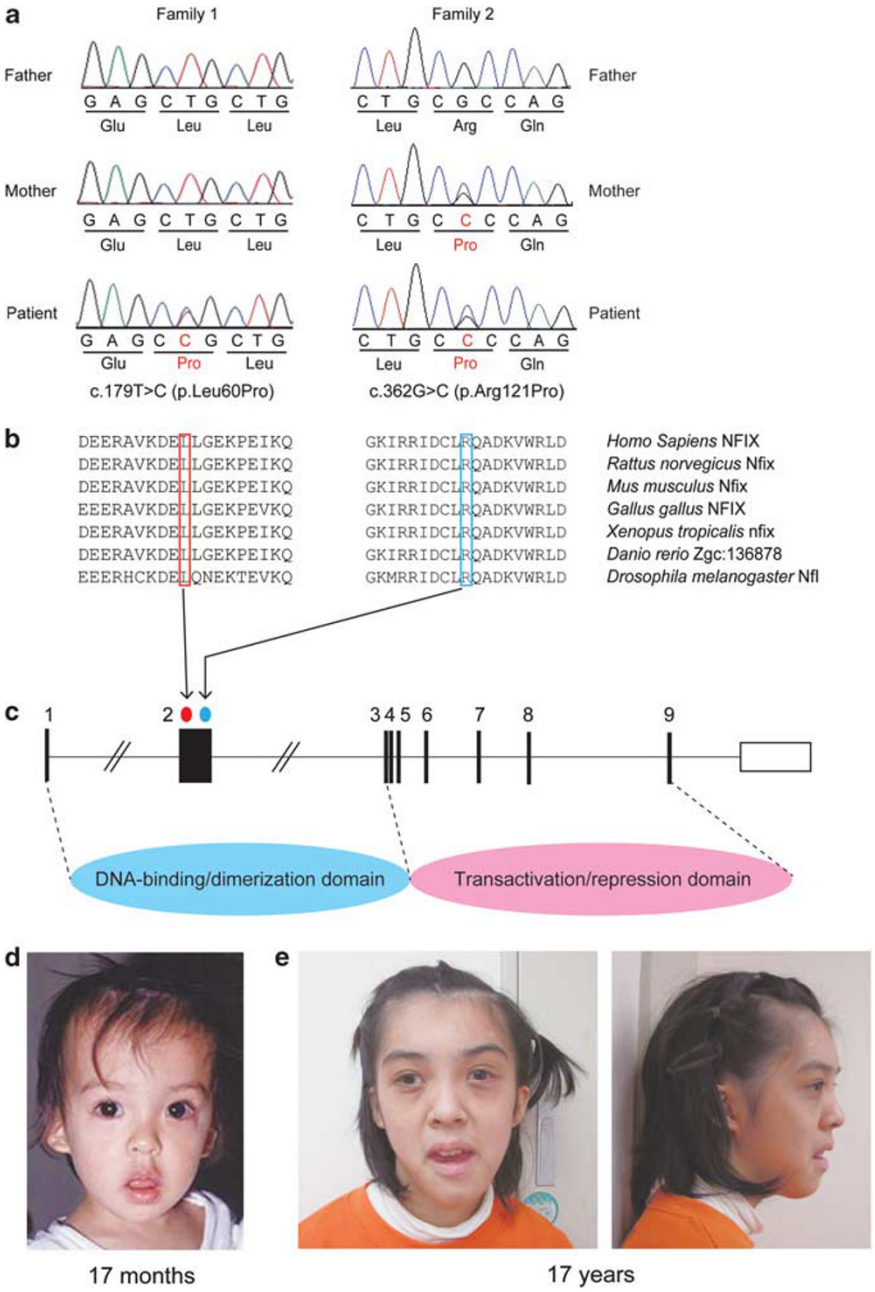

Figure 1 Missense mutations in *NFIX* in individuals with Sotos-like features. (**a**) Electropherogram of family 1 (left) and family 2 (right). The c.179T>C (p.Leu60Pro) mutation occured *de novo*. The c.362G>C (p.Arg121Pro) mutation was inherited from his mother. (**b**) An amino-acid sequence alignments of NFIX protein including amino-acid positions 60 and 121. Protein sequences were obtained through the NCBI protein database and multiple sequence alignment was performed by CLUSTALW web site (http://clustalw.ddbj.nig.ac.jp/). (**c**) Schematic representation of *NFIX* consisting of nine exons. UTR and coding exons are indicated by open and filled rectangles, respectively. The location of mutations is indicated by red (c.179T>C) and blue (c.362G>C) dots. At the bottom, C-terminal DNA-binding/dimerization domain and N-terminal transactivation/repression domain are depicted. Both the c.179T>C and c.362G>C mutations are located in exon 2 encoding a part of DNA-binding/dimerization domain. (**d**) Facial appearance of patient 1 at 17 months of age, showing long/narrow and triangular face, down slanting, short nose with anteverted nares and everted lower lip. (**e**) At 17 years of age, prognathia was noted in patient 1.

which is a unique amino acid characterized by imino radical. Proline has a pyrrolidine ring that restricts the available conformational space; therefore, it has effects on chain conformation and the process of protein folding.[13] Thus, it is very likely that two mutations could affect DNA-binding activity of NFIX protein through conformational changes of the DNA-binding domain.

Because *NFIX* mutations could cause both Marshall–Smith syndrome and Sotos-like features,[6] it is great concern to which of them two patients with missense mutations could be classified. Main clinical features of Sotos syndrome are childhood overgrowth including tall stature and/or macrocephaly, characteristic face and mental retardation. Other minor features are scoliosis, hypotonia in infancy, seizures,

Table 1 Clinical features of two patients with missense mutations in *NFIX*

				Reported by Malan et al.[6]		
		Patient 1	*Patient 2*	*Patient A*	*Patient B*	*Patient C*
Genetics	*NFIX deletion/mutation*	*c.179T>C*	*c.362G>C*	*del 19p13.3*	*del 19p13.3*	*c.568C>T*
Epidemiology	Age at last evaluation (years)	17	14	14	10	27
	Sex	F	M	M	M	F
	Mat/pat age	48/52	?/?	31/33	25/30	31/31
Prenatal growth	Birth weight (g)	2816 (−0.6 s.d.)	2938 (−0.4 s.d.)	4500 (>95)	3110 (10–50)	3600 (50–90)
	Birth height (cm)	48.8 (0 s.d.)	51 (+0.8 s.d.)	53 (95)	49 (50)	52 (95)
	OFC (cm)	33.5 (+0.3 s.d.)	35.5 (+1.4 s.d.)	38 (>95)	33.5 (10)	37.5 (>95)
Postnatal growth	Weight (kg)	9.24 (−0.5 s.d.)[a]	58.1 (+0.6 s.d.)[b]	>P98	>P98	>P98
	Height (cm)	84.9 (+2 s.d.)[a]	185.7 (+3.5 s.d.)[b]	>P98	>P98	>P98
Development						
SS	Autistic traits	−	−	+	+	+
	Behavioral anomalies	NA	−	+	+	+
	Motor retardation	+	+	+	−	−
	Hypotonia	+	+	+	+	−
Overlapped	Mental retardation	+	+	+	+	+
	Degree of delay	DQ19	IQ42	NA	NA	NA
	Speech delay	+	+	+	+	+
	First words (months)	24	18	NA	NA	NA
Craniofacial features						
SS	Long/narrow face	+	−	+	+	+
	Down-slanting palpebral fissures	+	+	+	−	+
	Small mouth	NA	−	+	−	+
	Prognathia	+	+	+	−	−
Overlapped	High forehead	+	+	+	+	+
MSS	Everted lower lip	+	−	+	−	+
	Underdeveloped midface	+	−	NA	NA	NA
	Proptosis	NA	−	NA	NA	NA
	Short nose	+	−	NA	NA	NA
	Prominent premaxilla	NA	−	NA	NA	NA
	Gum hypertrophy	+[c]	−	NA	NA	NA
	Retrognathia	−	−	NA	NA	NA
Eyes						
SS	Hypermotropia	−	−	+	+	−
	Strabismus	+	−	+	−	+
	Nystagmus	−	−	−	−	+
	Astigmatism	NA	NA	−	+	−
MSS	Myopia	NA	−	NA	NA	NA
	Blue sclerae	NA	−	NA	NA	NA
Musculo-skeletal abnormalities						
SS	Abdominal wall hypotonia	−	−	+	−	+
	Pectus excavatum	+	−	+	+	−
	Coxa valga	−	−	+	+	−
Overlapped	Scoliosis	+	−	+	−	+
	Advanced bone age	+	NA	+	+	+
MSS	Abnormal bone maturation	NA	NA	NA	NA	NA
	Bone fractures	−	−	NA	NA	NA
	Kyphosis	−	−	NA	NA	NA
	Umbilical hernia	−	−	NA	NA	NA

Abbreviations: F, female; M, male; Mat/pat, maternal/paternal; MSS, Marshall–Smith syndrome; NA, not ascertained; OFC, Occipitofrontal circumference; SS, Sotot's syndrome.
Growth of patients 1 and 2 is indicated with s.d. and that of patients in the report of Malan *et al.*[6] is indicated with percentile.
[a]At 17 months.
[b]At 14 years.
[c]Suggested the possibility of the adverse drug reaction.

cardiac defect and genitourinary anomalies.[5] On the other hand, main clinical features of Marshall–Smith syndrome are moderate to severe developmental delay with absent or limited speech, unusual behavior, disharmonic bone maturation, respiratory compromise secondary to upper airway obstruction, short stature and kyphoscoliosis.[14] One of remarkable differences between Sotos syndrome and Marshall–Smith syndrome is facial appearances. Although both syndromes has high forehead, Sotos syndrome has a long/narrow face, triangular shaped face with a prominent chin, down-slanting of the palpebral fissures,[1,4–5] whereas Marshall–Smith syndrome has proptosis, underdeveloped midface and prominent premaxilla.[7,14] In patient 1, although some characteristic features of Marshall–Smith syndrome such as everted lower lip, short nose and midface hypoplasia were observed, overall facial appearance, overgrowth features at 17 month of age, scoliosis, hypotonia and seizures were consistent with Sotos syndrome. Similarly, in patient 2, the facial appearance, tall stature and macrocephaly were consistent with Sotos syndrome. In both patients, their body weights were relatively low in comparison with their heights. This is consistent with the fact that, throughout childhood and early adolescence, the height was usually more significantly increased than weight in Sotos patients.[15] In addition, our patients did not show respiratory difficulties, one of specific features in Marshall–Smith syndrome, which cause early death in the neonatal period or early infancy.[7] Thus missense mutations in the DNA-binding/dimerization domain, which may lead to loss of transcriptional regulation by NFIX protein, could cause Sotos-like syndrome in two patients.

Many clinical features including tall statue, mental retardation, speech delay and high forehead are shared between our patients and three patients reported by Malan *et al*.[6] with *NFIX* abnormalities. The recognizable difference is autistic traits. Autistic traits are not observed in our patients but all of Malan *et al*.'s[6] patients. Thus there is a possibility that autistic traits are caused by haploinsufficiency of *NFIX* in Malan *et al*.'s[6] patients, but not by misssense mutations in the DNA-binding/dimerization domain. However, identification of a greater number of cases with *NFIX* mutations is required to confirm this hypothesis.

In conclusion, our report provides further evidences that *NFIX* is a causative gene for Sotos-like features. Abnormalities of *NSD1* are found in majority of Sotos syndrome cases and aberration of other genes including *NFIX* may be found in the minority of Sotos syndrome/Sotos-like features. Genetic testing of *NFIX* should be considered in such patients if no *NSD1* abnormalities were identified.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Leventopoulos, G., Kitsiou-Tzeli, S., Kritikos, K., Psoni, S., Mavrou, A., Kanavakis, E. *et al*. A clinical study of Sotos syndrome patients with review of the literature. *Pediatr. Neurol.* **40,** 357–364 (2009).
2 Kurotaki, N., Imaizumi, K., Harada, N., Masuno, M., Kondoh, T., Nagai, T. *et al*. Haploinsufficiency of *NSD1* causes Sotos syndrome. *Nat. Genet.* **30,** 365–366 (2002).
3 Rayasam, G. V., Wendling, O., Angrand, P. O., Mark, M., Niederreither, K., Song, L. *et al*. *NSD1* is essential for early post-implantation development and has a catalytically active SET domain. *EMBO J.* **22,** 3153–3163 (2003).
4 Visser, R. & Matsumoto, N. in *Inborn Errors of Development* (eds Epstein, C. J., Erickson, R. P., Wynshaw-Boris, A.) 1032–1037 (Oxford University Press, New York, 2008).
5 Tatton-Brown, K. & Rahman, N. Sotos syndrome. *Eur. J. Hum. Genet.* **15,** 264–271 (2007).
6 Malan, V., Rajan, D., Thomas, S., Shaw, A. C., Louis Dit Picard, H., Layet, V. *et al*. Distinct effects of allelic *NFIX* mutations on nonsense-mediated mRNA decay engender either a Sotos-like or a Marshall-Smith syndrome. *Am. J. Hum. Genet.* **87,** 189–198 (2010).
7 Adam, M. P., Hennekam, R. C., Keppen, L. D., Bull, M. J., Clericuzio, C. L., Burke, L. W. *et al*. Marshall-Smith syndrome: natural history and evidence of an osteochondrodysplasia with connective tissue abnormalities. *Am. J. Med. Genet. A.* **137,** 117–124 (2005).
8 Saitsu, H., Kato, M., Mizuguchi, T., Hamada, K., Osaka, H., Tohyama, J. *et al*. *De novo* mutations in the gene encoding STXBP1 (MUNC18-1) cause early infantile epileptic encephalopathy. *Nat. Genet.* **40,** 782–788 (2008).
9 Gronostajski, R. M. Roles of the NFI/CTF gene family in transcription and development. *Gene* **249,** 31–45 (2000).
10 Kruse, U. & Sippel, A. E. Transcription factor nuclear factor I proteins form stable homo- and heterodimers. *FEBS Lett.* **348,** 46–50 (1994).
11 Armentero, M. T., Horwitz, M. & Mermod, N. Targeting of DNA polymerase to the adenovirus origin of DNA replication by interaction with nuclear factor I. *Proc. Natl. Acad. Sci. USA* **91,** 11537–11541 (1994).
12 Stefancsik, R. & Sarkar, S. Relationship between the DNA binding domains of SMAD and NFI/CTF transcription factors defines a new superfamily of genes. *DNA Seq.* **14,** 233–239 (2003).
13 MacArthur, M. W. & Thornton, J. M. Influence of proline residues on protein conformation. *J. Mol. Biol.* **218,** 397–412 (1991).
14 Shaw, A. C., van Balkom, I. D., Bauer, M., Cole, T. R., Delrue, M. A., Van Haeringen, A. *et al*. Phenotype and natural history in Marshall-Smith syndrome. *Am. J. Med. Genet. A* **152A,** 2714–2726 (2010).
15 Cole, T. R. & Hughes, H. E. Sotos syndrome: a study of the diagnostic criteria and natural history. *J. Med. Genet.* **31,** 20–32 (1994).

Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)