

ARTICLE

Differential allelic expression of *SOS1* and hyperexpression of the activating *SOS1* c.755C variant in a Noonan syndrome family

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Noonan syndrome (NS) is a genetic condition characterized by congenital heart defects, short stature and characteristic facial features. We here present the case of a girl with moderate learning disabilities, delayed language development, craniofacial features and skin anomalies reminiscent of NS. After a mutation screening of the known NS genes *PTPN11*, *SOS1*, *RAF1*, *KRAS*, *GRB2*, *BRAF* and *SHOC2* we found the heterozygous c.755T>C variant in *SOS1* causing the p.I252T amino-acid substitution, which was considered possibly pathogenetic by bioinformatic predictions. The same variant was present in the proband's mother, displaying some NS features, and maternal grandfather showing no NS traits, but also by a healthy subject in 1000 genomes project database without phenotype informations. The functional analysis revealed that *SOS1* c.755C activated the RAS-ERK intracellular pathway, whereas no effects on RAC-JNK cascade have been detected. After a comparison between the sequence of *SOS1* cDNA from peripheral blood and *SOS1* genomic DNA, we showed for the first time a differential allelic expression of the *SOS1* gene in healthy individuals, thus occurring as a physiologic condition. Interestingly, we found that the mutated allele C was 50% more expressed than the wild-type allele T in all familial carriers. The comparable amount of *SOS1* mRNA between mutated individuals and the controls indicates that the variant does not affect *SOS1* expression. The present study provides a first evidence of allelic imbalance of *SOS1* and pinpoints this condition as a possible mechanism underlying a different penetrance of some *SOS1*-mutated alleles in unrelated carriers.

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INTRODUCTION

Noonan syndrome (NS; OMIM 163950) is one of the most common Mendelian syndromes. The incidence of affected individuals is estimated to be between 1:1000 and 1:2500 with an equal male to female ratio. NS is a clinically heterogeneous disorder predominantly characterized by variable developmental delay, short stature, dysmorphic facial features, skeletal abnormalities, congenital heart defects, neck abnormalities, chest deformities and predisposition to myeloproliferative disease; mild mental retardation, bleeding diathesis, lymphedema, hearing difficulty and cryptorchidism are also occasionally observed in affected individuals.¹ The diagnosis is carried out by a clinical-based scoring system, comprising major and minor criteria.² NS is numbered among the RASopathies, a class of developmental disorders caused by germline mutations in genes encoding components or regulators of the RAS/MAPK pathway. Each RASopathy exhibits a specific clinical phenotype, but, given the involvement of a common mechanisms leading to the RAS/MAPK pathway dysregulation, they share many overlapping traits, including craniofacial dysmorphism, cardiac malformations, cutaneous and musculo-skeletal abnormalities, neurocognitive impairment, hypotonia and an increased cancer risk³ with the exception of Legius syndrome.⁴

Compared with the other RASopathies, NS is characterized by a significant locus heterogeneity caused by the involvement of the mutated version of several RAS/MAPK pathway genes: protein

tyrosine phosphatase, non-receptor type 11 (*PTPN11*), son of sevenless 1 (*SOS1*) and *RAF1* in ~60–70% of NS cases, and more rarely *NRAS*, *KRAS*, *BRAF*, *MEK*, *SHOC2*, *CBL*, *RIT1* and *RRAS* genes.^{1,5–7} Both the genetics and the allelic heterogeneity can explain only in part the variable and complex NS phenotype. Furthermore, despite the identification of several NS genes, for ~30% of patients the pathogenetic mutation remains unknown and additional NS genes remain to be identified.

As far as NS genotype/phenotype correlation, mutations in the *PTPN11* gene are responsible for a wide clinical spectrum, characterized by a very high incidence (of at least 80%) of congenital heart disease, mainly pulmonary valve stenosis, typical facial features, cryptorchidism and bleeding diathesis. Patients with *SOS1* gene mutations display a rather distinctive form of NS with macrocephaly, ptosis, some cutaneous findings (hyperkeratotic skin, sparse eyebrows, sparse slow growing curly hair) similar to those of Cardio-facio-cutaneous syndrome and a low frequency of short stature and intellectual disability. Similarly to what observed in NS subjects with *PTPN11* mutations, among individuals with *SOS1* mutations cardiac defects have been reported to occur in the majority of cases (>85%), with a high prevalence of pulmonary valve stenosis, a relatively high occurrence of atrial and ventricular septal defects and a low prevalence of hypertrophic cardiomyopathy.⁸ *RAF1* gene mutations are strongly associated with hypertrophic cardiomyopathy, intellectual disability,

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short stature and skin features of NS-like with lentiginos syndrome (multiple lentiginos, café-au-lait spots, multiple nevi). Patients with *KRAS* mutations are affected by variable intellectual disability and may manifest features overlapping those of Costello and Cardio-facio-cutaneous syndrome. Familial patients account for ~20% of NS cases and present mostly an autosomal dominant inheritance with a near-complete penetrance and variable expressivity.⁹ Many affected adults are diagnosed only after the birth of a more obviously affected infant, and in fact milder or subclinical phenotypes have been reported in *PTPN11* and *SOS1* mutation carriers.^{10,11} The reported different degrees of severity of NS phenotype in both familial and sporadic carriers, for the same or similar functional mutations, are suggestive of the involvement of *cis/trans* regulatory factors, modulating the effects of specific mutations that should be investigated.

We here demonstrate the hyperactivating function of the *SOS1* c.755T>C variant occurring in a NS family, and for the first time we show a differential allelic expression (DAE) of *SOS1* gene that might have a role in both the complete penetrance and the variable expressivity of the NS clinical phenotype.

MATERIALS AND METHODS

Mutation screening and sequencing of GC-rich stretches

Peripheral lymphocyte DNA was obtained from patient, parents, maternal grandparents and controls using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Informed consent for molecular genetic studies on DNA and RNA was signed by all the analyzed patients. Sequencing was performed on the coding region of *PTPN11* (NG_007459.1), the nine exons of *SOS1* (NG_007530.1), and three exons of *RAF1* (NG_007467.1) (exons 7, 14 and 17), where mutations have been previously identified,^{10,12,13} *KRAS* (NG_007524.1), *GRB2* (NG_029556.1), exons 6–11–12–13–15 of *BRAF* (NG_007873.3) and exon 2 of *SHOC2* (NG_028922.1). Primer sequences are published elsewhere.^{10,11} PCR was carried out in a 50 μ l reaction volume containing 150 ng of genomic DNA, 0.2 μ M primers, 100 μ M dNTPs, 10 \times reaction buffer, 50 mM MgCl₂ and 2.5 U GoTaq DNA Polymerase (Promega, Madison, WI, USA) with the following cycling profile: 4 min initial denaturation at 95 °C, 35 cycles as follows: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

The primer sequences for the amplification of the *SOS1* promoter region and for the *SOS1* 3'-UTR regions are reported in Supplementary Table S1. The GC-rich stretch, located in the *SOS1* promoter region, was divided into four fragments and amplified using AccuPrime GC-Rich DNA Polymerase (Life Technologies, Carlsbad, CA, USA). The PCR was carried out in a 25 μ l reaction volume containing 100 ng of genomic DNA and 10 μ M primers, following the manufacturer's instructions. The fragments were amplified with the following cycling profile: 5 min initial denaturation at 95 °C, 30 cycles as follows: 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min. The PCRs for the *SOS1* 3'-UTR regions were carried out using GoTaq DNA Polymerase (Promega) in a 25 μ l reaction volume containing 100 ng of genomic DNA, 10 μ M primers, 100 μ M dNTPs, 10 \times reaction buffer and 50 mM MgCl₂. The following cycling profile was carried on: 4 min initial denaturation at 95 °C, 30 cycles as follows: 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The specificity of all the amplified PCR products was checked by 1.5% agarose gel electrophoresis. PCR products were bi-directionally sequenced using the Terminator v3.1 Cycle Sequencing Kit (Life Technologies), and resolved on an automated ABI-3130xl DNA genetic analyzer (Life Technologies). Output data were analyzed by SeqScape software v2.5 (Life Technologies).

The data concerning the variant and the phenotype of the proband have been submitted to the public database LOVD (https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=SOS1): the patient ID is 59 and the variant accession number is *SOS1_00027*.

Plasmids and mutagenesis

Plasmid h*SOS1* (human *Sos* WT) was a kind gift of G. Scita (IFOM, Milano); h*SOS1*, h*SOS1*-M269R and h*SOS1*-R497Q were previously described.¹¹

The h*SOS1* plasmid was used as template for site-directed mutagenesis, performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) to create the h*SOS1*-I252T mutated plasmid. Pairs of complementary mutagenic primers (I252T-Fw: 5'-TCGCATAGTAGATACAC ATGAACCTTAGTG-3' and I252T-Rev: 5'-CACTAAGTTCATGTGTATCTAC TATGCCGA-3') were designed. Hemagglutinin (HA)-c-Jun NH₂-terminal kinase 1 (JNK1) and HA-tagged extracellular regulated kinase1 (HA-ERK1) have been previously described.¹⁴

Biochemical analysis

Human embryonic kidney HEK293 cells were grown in DMEM plus 10% FBS and antibiotics. Cells were transiently transfected with equal amounts of the full-length h*SOS1* or the mutated isoforms of h*SOS1* and either HA-tagged JNK1 or ERK1 using the JetPEI (Polyplus, New York, NY, USA) method according to the manufacturer's instructions. Twenty-four hours after transfection the medium was replaced with serum-deprived medium and left for 18 h. HA-ERK1- and HA-JNK1- transfected cells were then stimulated with epidermal growth factor (EGF) (20 ng/ml) for 10 and 15 min, respectively. Cells were then collected in lysis buffer (Tris-HCl 50 mM, pH 7.5, NaCl 150 mM, Triton X-100 1%, glycerol 10%, NaF 50 mM, NaVO₃ 1 mM and cocktail of proteases inhibitors) and equal amounts of proteins were immunoprecipitated using anti-HA antibodies (BABC0, Richmond, CA, USA) and subsequently incubated with protein G slurry. Beads were washed and resuspended in SDS sample buffer. Samples were separated on SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were then incubated overnight at 4 °C with the primary antibodies: anti-HA (1:1000), anti-phospho ERK1/2 (1:1000, Cell Signalling, Danvers, MA, USA), anti-phospho-JNK1 (1:500, Cell Signalling) or anti-*SOS1* (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the incubation with the horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Little Chalfont, UK), the membranes were developed using ECL plus kit (GE Healthcare), according to the manufacturer's instructions. Band intensities were then quantified by densitometry using ImageJ software (National Institute of Health, USA).

RNA extraction and qPCR

RNA extraction from blood of patients and controls was performed using the Tempus Blood RNA tubes and the Tempus Spin RNA Isolation kit (Life Technologies).

Total RNA (1 μ g) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies). PCR was performed using 1 μ g of cDNA and specific primers for *SOS1* transcript (NM_005633.3) *Sos1*-ex6-fw: 5'-GCAGACATTTTAAAGCTGGTTGG-3' and *Sos1*-ex6-*rev*: 5'-CTCTGCTAA GTCTTCAAAGCAG-3' for the amplification of the c.755T>C region or *Sos1*-3utr-fw: 5'-TTCACCATCTAAGATTATGT-3' and *Sos1*-3utr-*rev*: 5'-CATTT TGTTTGGGTGTGTGG-3' for the amplification of the rs1059310:T>C SNP region using GoTaq DNA polymerase (Promega) and direct sequenced as described above. The pick height in electropherograms derived from direct sequencing was measured using ChromasPro software. Statistical analysis was performed using a student's *t*-test.

qPCR was performed using GoTaq qPCR master mix (Promega) and primers qPCR-*Sos1*-fw: 5'-TCCACGAAGACGACCAGAAT-3' and qPCR-*Sos1*-*rev*: 5'-GGGACTGTCCAAATGCTTA-3'. GAPDH was amplified as an internal control.

RESULTS

Case report

A 9 years and 5-month-old female (patient III-2, Figure 1) born to healthy unrelated parents was referred for genetic assessments because of moderate learning disabilities. Family history was noncontributory.

At the time of her birth, her mother was 31 and her father 32-year-old. Pregnancy was not problematic. She was born after 40 weeks of gestation with weight of 3070 g (25th centile), a length of 50 cm (50th centile) and occipitofrontal circumference of 35 cm (75–90th centile). Delivery was carried out after a prolonged labor with cesarean section

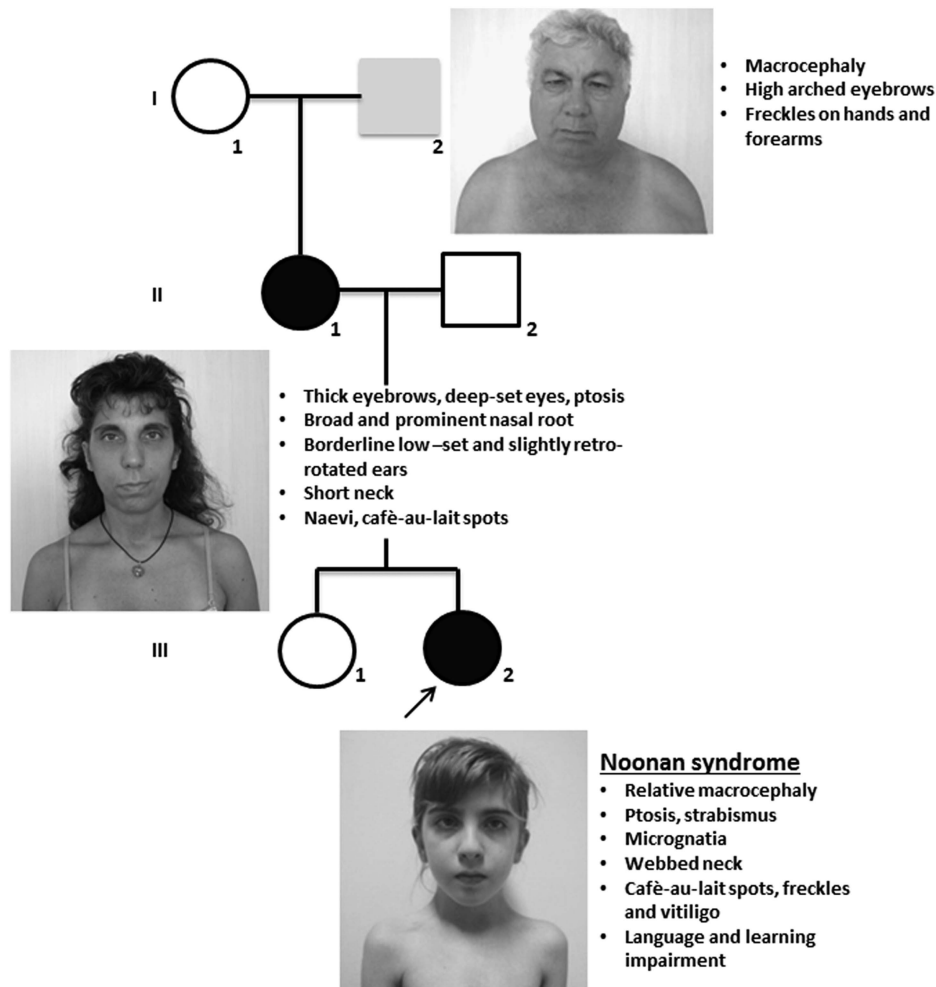


Figure 1 Pedigree with photographs and summary of clinical features of NS family members bearing the c.755T>C variant in *SOS1*. Proband (III-2), shown by the arrow, and her mother are colored in black to indicate that they display the full NS phenotype. Her maternal grandfather is colored in gray to indicate a milder phenotype (see also Table 1).

because of a previous cesarean section. Apgar score was 4 and 10 at 1 and 5 min. She walked unassisted at 12 months and was toilet trained at 30 months. She was able to say her first words at 10 months; however at kindergarten her speech was not easily understandable and her language was still composed of simple sentences at almost 10-year-old. Neuropsychological evaluation showed borderline cognitive skills. At the time of clinical evaluation at 9 years and 5 months, a myopathic appearance of the face with bilateral ptosis was evident, together with strabismus, a high forehead, micrognathia and slight webbing (Figure 1). Face was asymmetric, the left side being smaller. Cutis marmorata was more evident at the lower limbs than at the upper limbs. Abnormalities of skin pigmentation included café-au-lait spots, lentigines and vitiligo areas at the anterior face of both legs, sternum, groin and right scapula. First manifestations of vitiligo were evident on the legs at 7 years. Occipitofrontal circumference was 53 cm (75th centile), weight 25 Kg and height 127 cm (between the 10th and 25th centile). Hypotonia was appreciable at the upper limbs. Brain MRI scan images, carried out when the patient was 9 years and 10-months-old, showed a small arachnoid cyst in the left temporal lobe. EEG was normal; antinuclear antibodies were detected as the result of autoimmune diseases screening carried out for vitiligo. Ptosis, relative macrocephaly, webbing, borderline cognitive skills, café-au-lait spots, lentigines, hypotonia and vitiligo as possible sign of an autoimmune

disorder suggested to investigate the spectrum of the Neuro-cardio-facio-cutaneous syndrome, especially NS. Heart and kidneys ultrasound carried out on the basis of the diagnostic hypothesis were normal. Karyotype was 46,XX; fragile X syndrome was ruled out through DNA evaluation. Clinical genetics examination of the proband's parents and maternal grandparents was carried out subsequently the *SOS1* testing. Proband's mother (II-1) was evaluated at 41 years. Figure 1 shows her thick eyebrows, bilateral ptosis, broad and prominent nasal root, short neck, her ears are mildly low-set and posteriorly rotated. Skin examination revealed four café-au-lait spots in subscapular region and a number of pigmented naevi on chest and arms. Skin creases pattern over proximal interphalangeal joints of both hands was simplified. Hypotonia was appreciable at the upper limbs. Occipitofrontal circumference was 57 cm (>97th centile) and height 164 cm (>50th). Developmental and/or learning disabilities were denied; she appeared indeed a very appropriate person. As for the proband's maternal grandfather (I-2), at the time of clinical evaluation at 71 years (Figure 1), there were no relevant dysmorphic features except high-arched eyebrows. Occipitofrontal circumference was 59.5 cm (>97th centile), height 166.5 cm (10th). There were lentigines at the upper limbs and hands. Phenotype of the proband's father (II-2) and maternal grandmother (I-1) was unremarkable.

Mutation analysis

Patient III-2 was subjected to a mutation screening of the known NS genes *PTPN11*, *RAF1*, *SOS1*, *KRAS*, *SHOC2*, *BRAF* and also *GRB2*, which was considered as a candidate gene in a previous study.¹¹ We found the heterozygous variation c.755T>C in exon 6 of *SOS1* (NG_007530.1), leading to the p.(I252T) amino-acid substitution. The same variant was present in the mother and maternal grandfather, both displaying some clinical NS features (Table 1). *SOS1* encodes for a 150 kDa Ras-GEF (guanine exchange factor) protein characterized by a RAS-GEF (Cdc25) domain, conserved histone-like fold, Dbl homology (DH) and pleckstrin homology (PH) domains, a helical linker, a RAS exchange motif (REM) and a proline-rich region. *SOS1* is also known to activate a distinct pathway mediated by RAC1, a GTPase belonging to the Rho-family.¹⁵ Ile252 is an invariant residue in a hydrophobic core of the *SOS1* DH domain that, competing with RAS for binding to the allosteric site, might act as an intramolecular inhibitor of RAS-GEF activity.¹⁶ Bioinformatic analysis with PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) identified the p.(I252T) variant as 'probably damaging' with a score of 0.674 and the analysis with SIFT predicted the amino-acid change to 'affect protein function', given that the Ile residue is highly conserved during evolution.

The c.755T>C variation has been already reported in a NS familial case, but no validation studies have been carried out.⁸ A more recent NMR-based study, despite detecting a functional effect of p.(I252T) variant in solution, failed to show ERK activation in serum-activated cells.¹⁷ Furthermore, this variant is present in the SNP databases (dbSNP database <http://www.ncbi.nlm.nih.gov/SNP/> rs142094234: T>C, chr2.hg19:g.39051253A>G) with a frequency <0.001, being observed only 1 time out of 1000 genomes (<http://www.1000genomes.org>). For these reasons we wanted to check a possible pathological effect of this variant.

Table 1 NS cardinal features met by family members carrying the *SOS1* c.755T>C variant

	Proband	Mother	Grandfather
<i>I. Craniofacial features</i>			
Typical (A)		+	+
Suggestive (B)			+
<i>II. CHD</i>			
III. Short stature	-	-	-
<i>IV. Chest deformity</i>			
<i>V. Family history</i>			
First-degree relative with definite NS (A)		+	
First-degree relative with suggestive NS (B)		+	+
<i>VI. Other</i>			
Intellectual disability, cryptorchidism and lymphatic dysplasia (A)			
One of intellectual disability, cryptorchidism, lymphatic dysplasia (B)	+	-	-
^a Skin anomalies	+	+	+, less suggestive
Scores	I. A+2B	I. A+1A	I. B+1B

The matched features have been further characterized as 'major (A)' and 'minor (B)' according to the scoring system for NS.²

Abbreviations: CHD, congenital heart defect; NS, Noonan Syndrome. Definite NS is diagnosed if I. A plus one other major sign or two minor signs are matched; or when I. B plus two major signs or three minor signs are present.²

^aSkin anomalies has not been included in the scoring system.

Functional study of *SOS1* c.755T>C (p.(I252T))

To investigate whether the *SOS1* c.755T>C (p.(I252T)) variant could lead to a functional variation of the Ras pathway activity, we verified whether it hyperactivates the ERK1 downstream effector. At this purpose we transfected HEK293 cells with the mutant Sos-p.I252T plasmid, the wild-type human *SOS1* or the mutant isoform *SOS1* c.806T>C (p.(M269R)), previously reported to activate the RAS-ERK pathway¹³ as a positive control and compared the ERK1 phosphorylation levels of each *SOS1* isoform before and after EGF stimulation. We found that the Sos-p.I252T substitution significantly increased ERK1 phosphorylation level, with a level comparable to the c.806T>C (p.(M269R)) mutant-positive control (Figure 2a and b).

As *SOS1* is also involved in RAC1 pathway signal transduction regulation, we sought to determine whether this pathway might be affected by detection of variations in JNK activation. HEK293 cells have been transfected, under EGF stimulation, with the mutant p.I252T, the human wild-type *SOS1* and the mutant c.1490G>A (p.(R497Q)), previously reported to activate the RAC-JNK pathway,¹¹ as a positive control. After HEK293 cells transfection with the Sos-p.I252T plasmid, JNK was not found to be significantly overactivated under EGF stimulation, in comparison with the Sos-wt or the JNK activating *SOS1* c.1490G>A (p.(R497Q)) mutant (Figure 2c and d).

DAE analysis of *SOS1* gene

Because the c.755T>C variant has also been detected in a healthy individual, being reported in the 1000 genomes SNP database, we hypothesized that *SOS1* alleles may be differentially expressed as well-affecting/unaffected variants sporadically occurring in population, as described for other genes,¹⁸ and that this mechanism may have a role in the penetrance of potential pathogenetic variants. As *SOS1* allelic expression was never investigated, we studied the expression of the common SNP rs1059310:A>G, localized within the *SOS1* 3'-UTR, in five heterozygous healthy controls. The *SOS1* rs1059310:A>G SNP was PCR amplified from both genomic DNA and peripheral blood cDNA and subjected to direct sequencing. The heights of the A and G peaks in the resulting electropherograms were evaluated and the A:G allele ratio of both genomic DNA and cDNA was compared. The A:G allele ratio was about 50:50 in genomic DNA as expected but was significantly different (A:G allele ratio mean 62:38) in cDNA samples (Figure 3a).

To verify whether the *SOS1* c.755T>C alleles also showed DAE, a fragment of *SOS1* exon 6 including the c.755T>C variant was PCR amplified from both genomic DNA and peripheral blood cDNA of family carriers and subjected to direct sequencing. Sequence analysis of both genomic DNA and cDNA allowed us to establish that the C:T allele ratio in the cDNA of all familial carriers was 62:38, indicating that the mutated allele C was expressed 1.6-folds more than the wild-type allele T (Figure 3b and c). The comparable ratio value of mRNA level of *SOS1* alleles in both NS family members and healthy subjects indicates that *SOS1* DAE can normally occur.

To test whether the higher expression of the mutated allele could result in an increased amount of the total *SOS1* mRNA in our NS patients, we performed a qPCR on total RNA from the NS family members and the previously described controls. The total amount of *SOS1* mRNA was comparable between mutated individuals and controls, which included also the non-mutated healthy grandmother (I-1) of the proband (Figure 3d).

Search for SNPs' heterozygosity of *SOS1* promoter region and miR-124-binding site in NS patients and controls showing DAE

Starting from the central assumption that in the absence of *cis*-regulatory polymorphisms, both copies of an autosomal gene will contribute equal amounts of messenger RNA, we searched for SNPs' heterozygosity at *cis*-regulatory sites responsible for *SOS1* transcription, mRNA stability and/or translational efficiency in all the three NS family members here described and in the five controls showing DAE. We sequenced the *SOS1* promoter region (chr2.hg19: g.39,347,347_39,348,479 UCSC database) and the 3'UTR validated miR-124-binding site into which the SNP rs115465194:A>G is located.¹⁹ We observed a homozygosity condition of the most frequent allele for each of the eighteen SNPs analyzed (1000 genomes database <http://www.1000genomes>, Supplementary Table S2).

DISCUSSION

We here report on the functional study of *SOS1* c.755T>C (p.(I252T)) variation that we found in a NS family from Northern Italy. The c.755T>C variant is located in exon 6 within the *SOS1* DH domain and it has so far been identified only in another NS patient.⁸ As already observed by Lepri *et al*,⁸ we predicted that the variant might

lead to a structural perturbation of the DH fold, destabilizing the masking of the distal RAS-binding site. The *SOS1* DH domain interacting with the PH domain both inhibits exchange activity for RAC1 and promotes the interaction with the cell membrane inhibiting RAS activation.¹⁵ As the *SOS1* c.755T>C variation has been recently reported also in a healthy subject (<http://www.1000genomes.org>), its role in activating the RAS pathway needed to be demonstrated. Furthermore, a study based on NMR methodology aimed at detecting defects in RAS GTPase cycling showed that p.I252T variant failed to stimulate ERK activation in serum-activated cells, whereas the assays carried out in solution allowed to detect ERK hyperactivation. This apparent contradictory result was explained by the layered interdomain interactions and association with cell membranes that would prevent the hyperactivation effect of this variant.¹⁷ We here show that the *SOS1* c.755T>C (p.(I252T)) variant detected in our NS family hyperactivates the RAS pathway. Accordingly, several other missense mutations have been identified in the DH domain of *SOS1* (Tartaglia *et al*¹) and in particular the c.797C>A (p.(T266K)) and c.806T > C (p.(M269R)) changes in exon 6 have been shown to be pathogenic mutations.¹³

The three affected members of the NS family here described did not show any mutation in the known NS genes other than the c.755T>C *SOS1* variant. Our proband's phenotype resembles that of *SOS1*-mutated individuals, being associated with relative macrocephaly, ptosis and absence of short stature, whereas her cutaneous findings – lentigines, café-au-lait spots – are similar to those of NS-like with lentigines rather than those found in Cardio-facio-cutaneous syndrome and patients with *SOS1* gene mutations,⁸ that is, hyperkeratotic skin, sparse eyebrows, sparse slow growing curly hair. Our proband IQ was borderline to the lower limit of the normal range and displayed

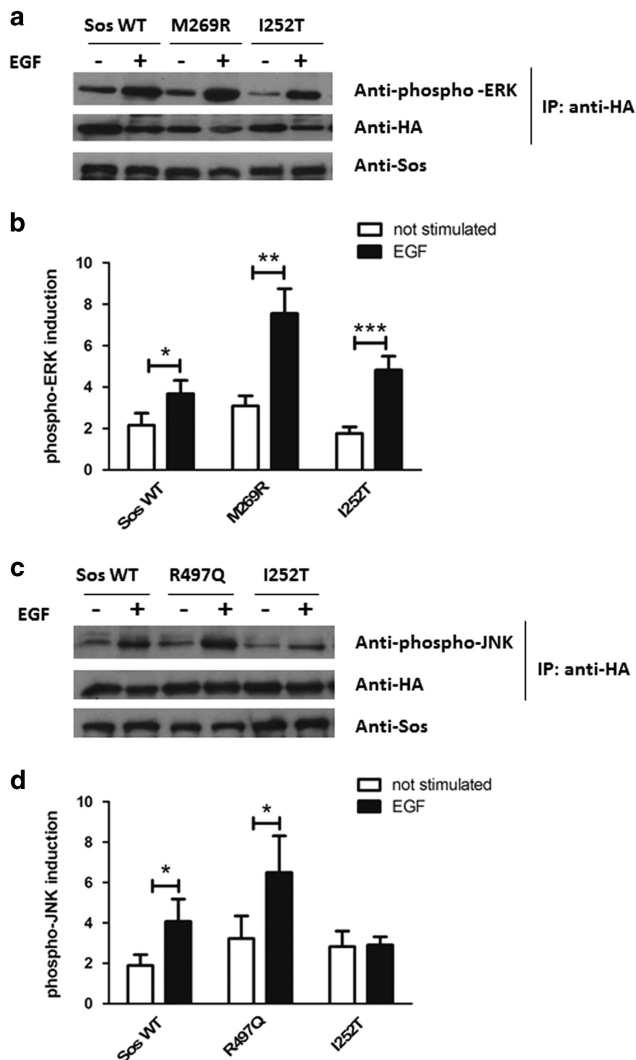


Figure 2 ERK and JNK activation assays. (a) h*SOS1* (Sos WT) or M269R or I252T together with HA-ERK1 were expressed in HEK293 cells. Cells were serum starved for 18 h and then stimulated with EGF (20 ng/ml) for 10 min. HA-ERK1 was immunoprecipitated with anti-HA antibodies and the level of phosphorylated ERK1 (upper row) and HA-ERK1 (middle row) in the immunoprecipitates was detected with anti-phospho-ERK and anti-HA antibodies, respectively. The level of the different *SOS1* constructs was detected in total cell extracts with antibodies against *SOS1* (bottom row). (b) The graph represents the mean \pm SEM of phospho-ERK1 induction normalized to HA-ERK1 intensity in the immunoprecipitates. The quantification was carried out on three independent experiments, and the data are expressed as the ratio normalized to the unstimulated condition. Mann-Whitney test has revealed a significant increase of ERK1 phosphorylation upon EGF stimulation in comparison with the not-stimulated condition for each *SOS1* construct (Sos WT+EGF vs Sos WT not stimulated * P <0.05, M269R+EGF vs M269R not stimulated ** P <0.01, I252T+EGF vs I252T not stimulated *** P <0.001). (c) h*SOS1* (Sos WT) or R497Q or I252T together with HA-ERK1 were expressed in HEK293 cells. Cells were serum starved for 18 h and then stimulated with EGF (20 ng/ml) for 15 min. HA-JNK1 was immunoprecipitated with anti-HA antibodies and the level of phosphorylated JNK1 (upper row) and HA-JNK1 (middle row) in the immunoprecipitates was detected with phospho-JNK and HA antibodies, respectively. The level of the different *SOS1* constructs was detected in total cell extracts with antibodies against *SOS1* (bottom row). (d) The graph represents the mean \pm SEM of phospho-JNK1 induction normalized to HA-JNK1 intensity in the immunoprecipitates. The quantification was carried out on three independent experiments, and the data are expressed as the ratio normalized to the unstimulated condition for each *SOS1* construct. Mann-Whitney test has shown that EGF stimulation significantly increases the level of JNK phosphorylation in *SOS1* WT and in R497Q-transfected cells in comparison with the not-stimulated condition (Sos WT+EGF vs Sos WT not stimulated * P <0.05, R497Q+EGF vs R497Q not stimulated * P <0.05).

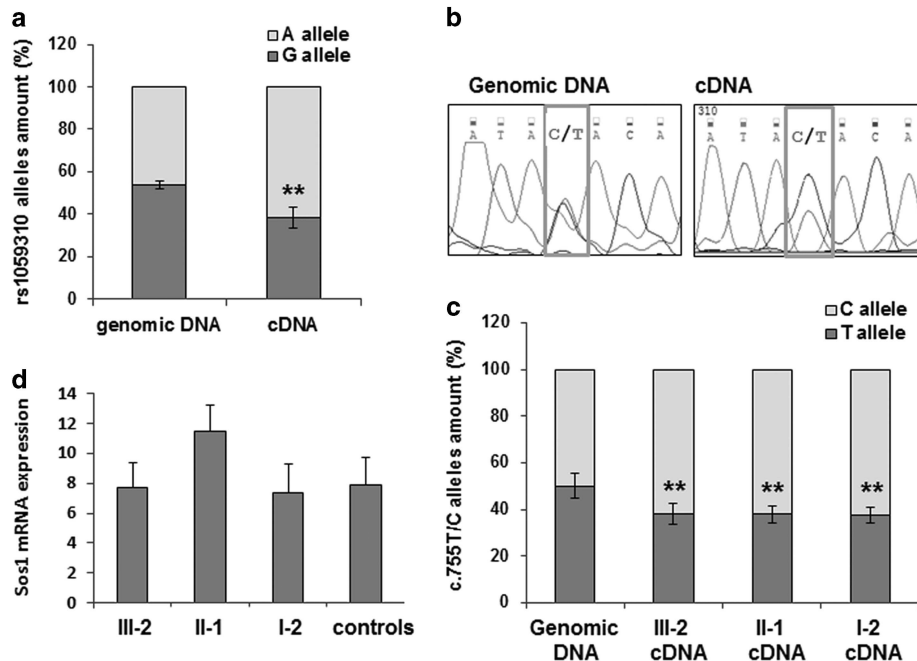


Figure 3 *SOS1* allelic expression analysis. (a) A:G allele ratio of the common rs1059310:A>G SNP in genomic DNA and cDNA of five controls (student's *t*-test $**P < 0.005$). (b) Representative electropherograms of PCR amplified fragments from genomic DNA or cDNA of individuals heterozygous for the *SOS1* c.755T>C variant (boxed). (c) The height of the peaks in the above electropherogram has been measured and the C:T allele ratio calculated for genomic DNA and cDNA. Statistical analysis was performed on five independent PCR amplifications. The genomic bar represent the mean \pm SD of the allele ratio of all the subjects (student's *t*-test $**P < 0.005$ compared with genomic). (d) qPCR of *Sos1* mRNA after retrotranscription of total RNA extracted from blood samples of mutated family members and controls. The control group includes the maternal grandmother (I-1) of patient III-2. GAPDH was used in qPCR as an internal normalizer. The histogram shows the means \pm SD of three independent experiments.

impairment of language and speech development and of learning at school, in agreement with the observation of a low frequency (11%) of intellectual disability in *SOS1*-mutated patients that, if present, usually causes only minor impairment in affected individuals.⁸ The *SOS1* c.755T>C variant was also found in the proband's mother and in her maternal grandfather. As detailed in Table 1, both the proband and her mother fulfill the diagnostic criteria for a definite NS if family history is accounted for. The maternal grandfather, displaying only macrocephaly, short and broad neck, high-arched eyebrows, freckles on hands and forearms, has a phenotype less suggestive for NS, even taking into account that increasing age makes characteristic facies more subtle. The previously reported NS patient bearing the *SOS1* c.755T>C variant⁸ was familial as well and belonged to a cohort of subjects with features fitting NS, or highly suggestive of NS, but, because the phenotype was not further detailed within the cohort, comparison between the two *SOS1* c.755T>C-mutated families cannot be performed. Despite the apparent genetic homogeneity, the proband and her mother show the full NS phenotype, whereas the grandfather has features that seem mixed up with those of the general population. An incomplete expression of the full NS phenotype with subclinical traits has already been reported in some *SOS1* mutation carriers.^{10,11} Interestingly, the inherited c.1490G>A (p.(R497Q)) *SOS1* mutation, activating Rac1 pathway, has been detected together with a validated pathogenetic *de novo* *RAF1* mutation in the same patient, showing the possibility of the co-expression of different mutant effectors of the RAS pathway, leading to the onset of variable NS phenotypes.¹¹ Similarly to what occurs in the family here described, in the *SOS1* c.1490G>A (p.(R497Q)) mutation family there was a fading in the craniofacial phenotype when dating back to the third generation, but they all showed keratosis pilaris, a *SOS1*

distinctive ectodermal abnormality.^{8,10} The functional study here reported, showing a strong activation of RAS-ERK signalling following EGF treatment that, not detected for RAC-JNK, excludes the direct hyperactivation of the Rac1 pathway, consistently with the association between skin involvement and *SOS1* c.1490G>A (p.(R497Q)) mutation. The presence of further NS-specific signs as a mild thorax deformity and CHD (pulmonary valve stenosis, hypertrophic cardiomyopathy) in the proband was interpreted as the result of the occurrence of the *RAF1* mutation.¹¹ Therefore, it is possible that specific isoforms of modifier genes might modulate the onset of some NS traits in affected individuals of the same family. Alternatively, considering the mild expression of the proband's phenotype even if fitting the full NS, the different genetic background and/or the different environmental influences could have contributed to decrease the expression of the c.755T>C *SOS1* variant across the family generations, that is, with the degree of relationship.

Another possible explanation of the clinical heterogeneous expressivity despite homogeneity of DAE in the three family members could come from a tissue-specific allelic imbalance or a tissue differential sensitivity to the genetic variation: for example, differently compared with her family members, the proband's brain may 'behave' like blood tissue.

The detection of the *SOS1* c.755T>C also in a healthy subject (<http://www.1000genomes.org>), even if with a very low frequency (< 0.001), prompted us to hypothesize that epigenetic mechanisms might influence the penetrance and/or variable expression of *SOS1* c.755T>C pathogenetic allele and therefore enhancing or decreasing the activating effect of this variant. We first investigated and verified the occurrence of DAE. The patterns of allelic imbalance represent a common biological phenomenon affecting human gene expression.

It was estimated that in lymphoblastoid cell lines ~20% of genes display population-average ratios of allelic expression larger than 40:60 or an higher than expected variance in allelic expression among heterozygous individuals.¹⁸ This condition might reflect the presence of different, possibly unknown, causative variants interfering with the complex modulation of gene expression.^{20,21} Accordingly, analyzing the common rs1059310:A>G *SOS1* SNP in five healthy heterozygous individuals, we verified the occurrence of *SOS1* DAE. Because the rs1059310 A allele resulted more expressed than the G allele in all analyzed individuals (60:40 ratio), we speculate that it might be in linkage disequilibrium with a putative gene expression regulatory motif. As far as the c.755T>C variant, our family members showed a higher expression of the mutated allele in respect to the wild type, possibly increasing the activating effect of the variant. The fact that the mutated allele did not increase the total *SOS1* mRNA level, but resulted in an allelic expression imbalance, suggests that the c.755T>C variation does not affect *SOS1* expression. We speculated that this variant occurs, in this family, in *cis* with a putative *SOS1* SNP, increasing the level of mutated mRNA, or alternatively, in *trans* with a SNP leading to a decrease of the normal mRNA, resulting in a higher level of the *SOS1* protein able to increase the activation of the Ras downstream Erk1 effector. We searched for the heterozygosity of SNPs located in regulatory regions, mainly in the promoter and in proximity to the validated miR-124 binding site in the 3'UTR,¹⁹ but we did not detect this condition in the NS family members. It has been estimated that in over 40% of all genes individual in human population at functional *cis*-regulatory sites is heterozygous^{22,23} and DAE affects from 20 to 50% of genes.¹⁸ These functional variants remain difficult to identify as regulatory regions can reside hundreds of thousands of bases away from the transcriptional start site.^{24,25}

We propose that DAE can explain the lack of NS phenotype in the control despite carrying the above variant, which probably occurred in *trans* with a putative SNP increasing the allele expression and that variations in the expression of the normal or mutated *SOS1* allele may be involved in the expression intensity of the NS phenotypes, which in some cases differs among unrelated individuals affected with the same mutations, as it has been already proposed for neurofibromatosis type 1.²⁶ However, as no clinical information is available about the control individual carrying the c.755T>C *SOS1* variant, a very mild NS phenotype, similar to our proband's mother, cannot be ruled out.

In conclusion, the obtained results demonstrated the pathogenic role of *SOS1* c.755T>C variant. The present study provides the first evidence of DAE for *SOS1* gene in both NS and healthy individuals, suggesting that DAE is a physiological condition during *SOS1* gene expression. Moreover, it adds for the first time the evidence that DAE, at least analyzed in blood tissue, is not sufficient to explain the reduced penetrance and/or expressivity of the NS phenotype across family generations, which is a well-known phenomenon.

SOS1 mutations account for 10–13% of NS patients¹³ and probably in some cases, when a certain mutated allele is underexpressed, although functionally activating, it is not so penetrant to cause the disease. The coupling of both *SOS1* sequencing and the DAE analyses might be recommended to evaluate the pathogenetic potential of the identified *SOS1* variant suggesting, when the mutated mRNA is underrepresented, sequence analysis of additional NS genes.

The findings here reported pinpoint on the role of possible epigenetic mechanisms leading to DAE, which might emerge as

modifier factors able to modulate the phenotypic effects of a specific mutation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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