**Sesn1 is a novel gene for left–right asymmetry and mediating nodal signaling**

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Remarkable progress has been made in understanding the molecular mechanisms underlying left–right asymmetry in vertebrate animal models but little is known on left–right axis formation in humans. Previously, we identified **SESN1** (also known as **PA26**) as a candidate gene for heterotaxia by positional cloning of the breakpoint regions of a de novo translocation in a heterotaxia patient. In this study, we show by means of a zebrafish sesn1-knockdown model that Sesn1 is required for normal embryonic left–right determination. In this model, developmental defects and expression data of genes implicated in vertebrate left–right asymmetry indicate a role for Sesn1 in mediating Nodal signaling. In the lateral plate mesoderm, Nodal signaling plays a central role in left–right axis formation in vertebrates and is mediated by FoxH1 transcriptional induction. In line with this, we show that Sesn1 physically interacts with FoxH1 or a FoxH1-containing complex. Mutation analysis in a panel of 234 patients with isolated heterotaxia did not reveal mutations, indicating that these are only exceptional causes of human heterotaxia. In this study, we identify **SESN1** as an indispensable gene for vertebrate left–right asymmetry and a new player in mediating Nodal signaling.

**INTRODUCTION**

Heterotaxia (OMIM606325) is a group of congenital disorders characterized by a misplacement of one or more organs according to the left–right axis. It occurs in ~1/10000 newborns and is often accompanied by severe organ malformations. Although in recent years detailed knowledge on the molecular mechanisms underlying internal left–right asymmetry in vertebrate animal models has been obtained, still very little is known on the conservation of these pathways in humans and on the causes of human heterotaxia. To date, four genes are known to be implicated in non-syndromic heterotaxia: **ZIC3** (1,2), **ACVR2B** (3), **LEFTYA** (4) and **CFC1** (5). However, heterozygous mutations in either of these genes account for less than 3% of sporadic heterotaxia (3–6). Three of these genes, **ACVR2B**, **LEFTYA** and **CFC1**, had initially been identified as functional candidate genes through studies in animal models. All three genes are components of Nodal signaling, a conserved mechanism driving asymmetric expression of genes in the embryonic lateral plate mesoderm (LPM) (Fig. 1). This asymmetric gene expression is the molecular basis of morphological left–right asymmetry in all vertebrate model organisms. Previously, we identified **SESN1** (also known as **PA26**) as a candidate gene through positional cloning of the breakpoint regions in a patient with heterotaxia and a de novo translocation 46,XX,t(6;18)(q21,q21) (7). To validate the function of **SESN1** in left–right axis formation, we started a simultaneous

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approach of both functional analysis of **sesn1** in zebrafish and mutation analysis in patients with isolated heterotaxia. Our results confirmed that Sesn1 is indispensable for left–right axis formation in zebrafish embryogenesis and provided evidence for a role of **sesn1** in mediating Nodal signaling.

**RESULTS**

**Zebrafish sestrin genes**

**SESN1** belongs to a gene family comprising two additional sestrin genes in the human and mouse genome (**SESN2** and **SESN3**) (7). Also in zebrafish, we identified and characterized three homologous sestrin genes (Supplementary Material, Fig. S1): **sesn1**, **sesn2** and **sesn3** (GenBank accession mRNA: DQ858215, DQ858216 and DQ858217). On the basis of the highest nucleotide and amino acid sequence identity, the presumed zebrafish orthologs of the human sestrins could be distinguished (Supplementary Material, Fig. S2 and Table S1). Zebrafish Sesn1 shows 76.6% amino acid identity to the human protein. Three alternative splicing variants, T1, T2 and T3 have been reported for human **SESN1** (8). The cloned zebrafish **sesn1** cDNA corresponds to the human **SESN1**-T2 variant. On the basis of data mining of EST traces and zebrafish cDNA library screening, no evidence for alternative splicing of **sesn1** in zebrafish was found. Early expression of **sesn1** was shown by RT–PCR in all examined zebrafish embryonic stages from 2.5 h post fertilization (h.p.f.) until adult stage (data not shown). This observation is consistent with a function of S sesn1 in mediating Nodal signaling.

**Zebrafish sesn1-knockdown model**

To abrogate Sesn1 function in the embryo, we designed an antisense morpholino oligonucleotide (MO) directed against the exon 2 splice donor site of **sesn1**: **sesn1**-MO (Supplementary Material, Figs S2–S4). An MO containing the same sequence as the **sesn1**-MO except for five nucleotides was used as a control: 5-mis MO. **sesn1**-MO-injected embryos showed developmental defects that were MO-dose-dependent. Therefore, we classified the knockdown phenotypes into three categories (class I–III) with increasing severity resulting from the injection of 1.7, 3.4 and 8.3 ng of **sesn1**-MO, respectively (Fig. 2, Supplementary Material, Figs S5 and S6). Details on developmental anomalies observed in **sesn1**-MO-injected embryos can be found as supplementary information (Supplementary Material, Figs S5 and S6). The specificity of the **sesn1**-MO effects was shown in three experiments. First, the injection of the control MO (5-mis MO) never caused developmental anomalies (Fig. 2D–H). Secondly, the specificity was shown in a rescue experiment by the coinjection of 1.7 ng **sesn1**-MO with 50 pg mRNA encoding the full-length zebrafish Sesn1. This coinjection restored the normal phenotype (Supplementary Material, Table S2). Additionally, a second MO directed against the exon 3 splice donor site of **sesn1** was designed (Supplementary Material, Figs S2–S4). Injection of this MO resulted in a phenotype that was
indistinguishable from the phenotype obtained by the injection of the first MO (data not shown).

Laterality defects in the zebrafish sesn1-knockdown model

To investigate a possible role for Sesn1 in left–right axis determination, we studied organ laterality in class I sesn1-knockdown embryos. During zebrafish development, the heart tube shifts or ‘jogs’ to the left of the dorsal midline (9) (Fig. 3A) and subsequently acquires a rightward or D-loop. Jogging was studied in 40 h.p.f. embryos by in situ hybridization for the gene nkx2.5, which marks the position of the heart tube (Fig. 3A–D) (10). Looping was morphologically assessed by live analysis at 3.5 days p.f. In the sesn1-knockdown embryos, the direction of the jogging was randomized with half of the embryos displaying no- or a rightward jog (Fig. 3B–D and M). Looping of the heart tube was absent in most of the sesn1-knockdown larvae (Fig. 3M). Laterality of the gut was assessed by the position of the pancreas. Defective left–right determination causes the pancreas to be mispositioned to the left side or to remain in an early embryonic midline position (11). Injections of 1.7 ng sesn1-MO and 1.7 ng control 5-mis MO were done in embryos of a transgenic zebrafish strain expressing green fluorescent protein (GFP) in the pancreas (Peers B, unpublished) (Fig. 3E–H). In parallel with this experiment, class I sesn1-knockdown embryos and control 5-mis MO embryos were stained by in situ hybridization with a preproinsulin (ins) probe as a marker for the endocrine pancreas (Fig. 3I–L) (12). Both experiments showed that Sesn1-knockdown led to a misplacement of the pancreas either to the left side or to the midline in half of the embryos (Fig. 3E–M). To further assess the specific effect of Sesn1-knockdown on the position of the pancreas, the GFP and the preproinsulin probes were used (Fig. 3E–M).
experiment were repeated with the second MO (MO against the exon 3 splice donor site). These experiments also showed a misplacement of the pancreas in half of the embryos (data not shown). These data clearly demonstrate an essential role of Sesn1 in left–right patterning in zebrafish.

Expression pattern of sesn1 during zebrafish embryogenesis

To further study the mechanism of Sesn1 involvement in left–right asymmetry, we analyzed the expression pattern by whole-mount in situ hybridization at different stages of zebrafish embryogenesis. Distinct expression was present in the anterior axial hypoblast, the somites and the posterior LPM (Figs 2A–C, 4A and B, Supplementary Material, Fig. S7). Details on the expression pattern can be found as supplementary information.

Effect of sesn1 on asymmetric gene expression

A possible effect of Sesn1 on asymmetrically expressed genes was examined. To this purpose, we studied spaw, pitx2, lft1 and lft2 expression in the sesn1-knockdown class I embryos (Fig. 4, Supplementary Material, Figs S8–S10).

In wild-type embryos, spaw expression is initiated at the left LPM at 10–12 somite stage and spreads in a posterior-to-anterior fashion (Fig. 4C, G and K). In the more anterior left LPM, Spaw induces the expression of pitx2, lft1 and lft2.
(Fig. 1A) (13), as part of a conserved cascade that directs the left-specific pattern of morphogenesis in mouse, chicken, frog and fish (14,15). Forty-one percent of the class I sesn1-knockdown embryos showed no spaw expression in the posterior left LPM (Fig. 4E), whereas embryos that did express spaw in this region showed poor maintenance of the expression and no- or very limited progression towards the anterior (Fig. 4D, H and I). spaw expression near the tailbud (asterisk) remains unaffected in sesn1-MO knockdown embryos (D–F and I–N). Since spaw expression in the LPM depends on a positive auto-regulatory feedback loop (Fig. 1A and B), whereas expression near the tailbud is independent of this mechanism, these results suggest a requirement for Sesn1 in Spaw (Nodal) auto-activation (13).

The left-sided expression domains of the genes pitx2, lft1 and lft2 that are induced by Spaw (Nodal)-signaling in wild-type embryos, were abolished in most sesn1-knockdown class I embryos (Supplementary Material, Table S3 and Figs S8–S10). Symmetrical expression domains of pitx2, which are Spaw-independent, like the Rohon–Beard cells, the ventral CNS and the hatching gland (13), remained unaltered (Supplementary Material, Fig. S8). Lft1 expression in the posterior notochord was present in most sesn1-knockdown embryos (D–F and L–N). Since spaw expression in the LPM depends on a positive auto-regulatory feedback loop (Fig. 1A and B), whereas expression near the tailbud is independent of this mechanism, these results suggest a requirement for Sesn1 in Spaw (Nodal) auto-activation (13).

The expression of sesn1 in wild type embryos (A and B) and effect of sesn1-MO knockdown on spaw expression (C–O). (A and B) In situ hybridization with a sesn1 probe on wild-type embryos (WT). Posterior view with dorsal towards the top. Sesn1 is bilaterally expressed in the posterior LPM (black arrows). (C–O) In situ hybridization with a spaw probe on wild-type embryos (WT) (C, G, K) and on sesn1-MO knockdown embryos (MO) (D–F, H–I and L–N). Dorsal view with anterior towards the top (C–J) and lateral view with dorsal towards the right (K–N). White arrows indicate spaw expression in the LPM (C, G and K). White arrowheads point towards the anterior margin of spaw expression in the LPM (L) and lateral (N). Sesn1-knockdown causes absence of spaw expression in the LPM (E, I and M), poor maintenance and limited extention of Spaw towards the anterior (D, H and I). Spaw expression near the tailbud (asterisk) remains unaffected in sesn1-MO knockdown embryos (D–F and I–N). (O) The number of embryos with a particular pattern of spaw expression. n, number of embryos that were examined; %L, %A, %B, %R, % of the examined embryos presenting left-, absent-, bilateral- or right-sided spaw expression, respectively, in the LPM.
zygotic Oep and mutants lacking maternal and zygotic Sur (Fig. 1B) (16–18). These observations suggest that Sesn1 is required for mediating Nodal signaling during gastrulation.

Interaction of sesn1 with foxh1

Several studies in different vertebrate models demonstrate the requirement of Nodal auto-activation for stable nodal expression in a subset of expression domains (13,19,20). The Nodal auto-activation in the LPM is mediated by the forkhead transcription factor FoxH1 (also known as Sur in zebrafish) (Fig. 1B) (18–21). Since our data from the zebrafish experiments were consistent with a role of Sesn1 in FoxH1-mediated Spaw (Nodal) auto-activation, we investigated a possible interaction of Sesn1 with FoxH1. Indirect immunofluorescence data indicated that Sesn1 and FoxH1 were both present in the nucleus, suggesting that these proteins could meet in this compartment (Fig. 6A). A possible interaction was verified by co-immunoprecipitation experiments. This showed that HA-tagged Sesn1 co-immunoprecipitated with Myc-tagged FoxH1 and vice versa (Fig. 6B) (data not shown). We therefore conclude that Sesn1 is able to bind to FoxH1 or to a complex that contains FoxH1.

Mutation analysis

We found no mutations in the coding sequence and in the intron–exon boundaries of SESNJ in a panel of 194 patients with isolated heterotaxia.

DISCUSSION

We previously identified SESNJ (also known as PA26) as a candidate gene for heterotaxia (OMIM606325) by positional cloning of the breakpoint regions of a de novo translocation in a heterotaxia patient (7). The aim of this study was to validate the function of SESNJ both by functional analysis in a zebrafish model and by mutation analysis in patients with isolated heterotaxia.

Zebrafish sesn1-knockdown embryos showed laterality disturbances both at the level of the heart and the gut and thus provided convincing evidence for the requirement of Sesn1 in normal left–right asymmetry. The generation of morphological left–right asymmetry in vertebrates can be divided into three phases (15,22–26). First, there is the breaking of global embryonic bilateral symmetry and stabilizing this information in an organizing or signaling center. In the second step, left–right information is propagated and transmitted to more outlying tissue and a cascade of asymmetric gene expression in the left LPM is triggered (Fig. 1A). The third step is the recognition of this left–right information by organ primordia and the translation into asymmetric organ morphology. To study the precise function of Sesn1 in one of these phases, further analysis of this gene in zebrafish was performed.

The expression pattern of Sesn1 during zebrafish embryo-genesis did not provide obvious clues to a possible mechanism since sesn1 expression appeared symmetrical at all stages of development and since no expression was detected in midline structures or in possible left–right signaling centers. However, the bilateral expression of sesn1 within the posterior part of the LPM (Fig. 4A and B) was particularly interesting because in this region, shortly after the initiation of sesn1 expression, the earliest known asymmetric gene expression (spaw, a nodal-related gene) occurs (Figs 1A and 4C) (13). Because of the crucial role of spaw in this region in triggering the conserved cascade of asymmetric gene expression, a possible effect of Sesn1 on spaw expression was examined. In the sesn1-knockdown embryos either spaw expression was absent in the posterior LPM or the expression did not make the normal progression towards the more anterior region of the LPM. For other nodal-related genes, it is known that both

Figure 5. Effect of sesn1-MO knockdown on the expression of ntl and gsc. Dorsal view with animal pole up on wild-type embryos (A and C) showing normal expression of ntl (A) and gsc (C) and on sesn1-MO knockdown class III embryos (B and D) showing absence of the expression of ntl in the dorsal margin of the blastoderm (B) and reduced expression of gsc (D) in the same region.

Figure 6. Interaction of Sesn1 with FoxH1. (A) Sesn1 and FoxH1 colocalize in the nucleus, as demonstrated by indirect immunofluorescence on transfected COS-1 cells. (B) Sesn1 can interact with FoxH1 or a FoxH1 containing complex. Proteins from HEK293T cell extracts, transiently expressing combinations of HA-tagged Sen1 and Myc-tagged FOXH1 with or without constitutive active (ca) ALK4, were immunoprecipitated with α-Myc antibody and precipitates were analyzed by immunoblotting using anti-HA antibody.
the maintenance of the expression and the progression occurs by Nodal auto-activation through Nodal signaling. Therefore the specific alterations in spaw expression in sesn1-knockdown embryos provided evidence for the requirement of Sesn1 in Spaw (Nodal) auto-activation and thus in Nodal signaling in the LPM. Three zebrafish models with a defect in Nodal signaling in the LPM display left–right anomalies. These are (Fig. 1B) (i) loss of the zygotic function of Oep, an EGF-CFC-family protein (11), (ii) mutations in the gene sur, encoding the transcription factor FoxH1 (also known as FoxH1) (18–21) and (iii) spaw-morpholino knockdown embryos (13). The current interpretation in each is that Nodal auto-activation is blocked so that the cascade of asymmetric gene expression in the left LPM (pitx2, lft1 and lft2) is not triggered (11,13,27). Consistent with the findings in these models, the left-sided expression domains of these genes pitx2, lft1 and lft2 were abolished in most sesn1-knockdown class I embryos (Supplementary Material, Table S3 and Figs S8–S10). Interestingly, similar to the observations in sur mutants, bilateral expression domains of pitx2 and expression in the notochord of lft1 were present in all sesn1-knockdown embryos (Supplementary Material, Figs S8 and S9). In this, both sur mutants and sesn1-knockdown embryos differ from oep mutants, where virtually all pitx2 and lft1 expression is abolished, coincident with the loss of most mesodermal tissue due to a more general impairment of Nodal signaling (27,28). We conclude that Sesn1 is required for only a subset of Nodal-signaling events including Nodal auto-activation in the LPM.

The sesn1-knockdown embryos resembled Nodal-signaling mutants not only in the absence of asymmetric gene expression but also in morphologically visible developmental defects. Compared the mutants sur, cyc:sqt, cyc, and oep (Fig. 1B), similar features were: defects in the prechordal plate, the floorplate and the notochord, inward turned eyes, a reduced dorsal axis extension and a ventral curvature of the body (16–18,29,30). A discussion on midline defects and laterality disorders in sesn1-knockdown embryos can be found as supplementary information. Most similarities were found between the class I sesn1-knockdown embryos and the mutant sur. In sur, the FoxH1 gene is disrupted resulting in an impairment of the FoxH1-dependent Nodal signaling (Fig. 1B). Since certain Nodal signaling events are independent of FoxH1, sur mutant embryos present with a less severe phenotype than mutants with a complete loss of Nodal signaling (18). The main differences are mesendodermal structures, being present in both sur mutants and sesn1-knockdown embryos, but absent in severe Nodal-signaling mutants like cyc:sqt double mutants and mutants lacking maternal and zygotic Oep (16,17). We conclude that not only the expression data but also the developmental defects of the sesn1-knockdown embryos are consistent with a role of sesn1 in a subset of Nodal-signaling events.

Class III sesn1-knockdown embryos, typically resulting from the injection of a high dose of sesn1-MO arrested in their development and died during gastrulation (Fig. 2S–U). Interestingly, they showed very similar developmental defects as the severe Nodal-signaling mutants: cyc:sqt double mutants and mutants lacking maternal and zygotic Oep (16,17). To confirm the hypothesis that the observed gastrulation defects in class III sesn1-knockdown embryos could also be due to insufficient Nodal signaling, two genes that are regulated by Nodal signaling at gastrulation stage were studied in sesn1-knockdown class III embryos. Both genes Nil and gsc showed similar alterations of expression as seen in the severe Nodal-signaling mutants (16–18). Although we were mainly interested in the function of Sesn1 in the LPM because there the molecular basis of laterality is established, these altered expressions of Nil and gsc during gastrulation provided additional evidence for a role of sesn1 in mediating Nodal signaling. On the basis of molecular and morphological evidence, we conclude that both the observed gastrulation defects in class III knockdown embryos and the laterality disturbances in class I embryos can be explained by insufficient Nodal signaling.

In the class I sesn1-knockdown embryos, it was clear that Spaw (Nodal) auto-activation was impaired in the LPM. Nodal auto-activation in the LPM is mediated by the forkhead transcription factor FoxH1 (also known as Sur in zebrafish) (Fig. 1B) (18–21). Consistent with a role of Sesn1 in Nodal auto-activation, we found that Sesn1 can interact with FoxH1 or a FoxH1 containing complex in vitro.

SESN1 (also known as PA26) has initially been identified as a target of the p53 tumor suppressor (8). It appears to be a p53 target gene in adult tissues, with properties common to the GADD family of growth arrest and DNA damage-inducible stress-response genes. It is thought to be a potential regulator of cellular growth. More recently, SESN1 was shown to be required for regeneration of peroxiredoxins. Thus SESN1 is proposed as a modulator of the peroxide signaling and antioxidant defense (31). A link between this function and the role of SESN1 in left–right asymmetry during embryogenesis remains unclear.

Previously, we failed to detect mutations in the coding sequence and in the intron–exon boundaries of SESN1 in a panel of 40 patients with isolated heterotaxia (7). Extending this study, we equally found no mutations in an additional group of 194 patients. This is in line with the fact that mutations in LEFTYA, CFC1 and ACVR2B, three other components of Nodal signaling, are very rare causes of human heterotaxia (3–6). A possible explanation might be that mutations in Nodal signaling components would cause lethal developmental defects in the majority of cases. This hypothesis is consistent with the early crucial role of Nodal signaling in all vertebrate animal models during gastrulation, long before this Nodal signaling is reemployed in the LPM to establish left–right asymmetry. In this light, the approach used in this study illustrates the power of functional studies in zebrafish to validate a candidate gene for a human developmental disorder. In particular, this applies to etiologically highly heterogeneous disorders, where animal studies can reveal the function of genes that are indispensable in certain underlying human embryonic mechanisms but that are only exceptional causes of the disorder.

**MATERIALS AND METHODS**

**Identification of sestrin coding sequences in zebrafish**

We BLAST-searched the full-length cDNA sequences of the human sestrins against zebrafish EST traces. The zebrafish
EST clones were purchased from Research Genetics and were sequenced. Sequencing of the IMAGE clones 4759505 (NCBI BI473420) and 2601412 (NCBI AW134248 and AW116571) allowed for the identification of the cDNA sequences of zebrafish *sesn2* and *sesn3*, respectively. The cDNA sequence of zebrafish *sesn1* was obtained by screening a zebrafish cDNA library (Library LLKmp964 and LLKmp964PP2 purchased from RZPD), sequencing of the obtained clone LLKmp964L1310Q2 and performing 5’RACE (RLM-RACE Kit Ambion). A PCR primerset for the library screen was chosen on the basis of a TBLASTN search of the human SESN1 against the sangerZFdb (Zebrafish Genome Browser Sanger Institute). Zebrafish sestrin cDNA sequences were confirmed by the sequencing of RT–PCR products. Zebrafish mRNA was extracted by guanidinium isothiocyanate (Trisol Reagent, Life Technologies, Gibco-BRL) from adult zebrafish with the AB genetic background. A zebrafish cDNA pool was made by the use of Superscript First Stand Synthesis System for RT–PCR (Invitrogen life technology) with random hexamers.

**MO and mRNA injections**

MOs were obtained from Gene Tools and resuspended in 1× Danieu’s solution. MO sequences: *sesn1*-MO directed against the exon 2 splice donor site 5’-TGTTAACGCTACCTCTGTCCTCTCTGG, 5-mis MO 5’-TGTTAACGCTACCTCTGTCCTCTTTGT TCTCG, *sesn1*-MO directed against the exon 3 splice donor site 5’-CCGTCCTGACACCTACCATATCC. GFP mRNA was added to each sample as a control for the injections. Injections were performed into the cell cytoplasm at a one or two cell stage. Embryos were incubated at 28.5°C. After 6 h, dead and unfertilized embryos and embryos that were not expressing GFP homogeneously, were removed.

**Plasmids and RNA synthesis**

Zebrafish *sesn1* cDNA was introduced in the pGEM-TEasy vector (Promega) to generate probes for *in situ* analysis. A vector containing spaw cDNA was provided by M. Rebagliati (University of Iowa, Iowa city, USA). Plasmids to generate probes for Pitx2, Lft1 and Lft2 were provided by H.J. Yost (University of Utah, UT, USA). For overexpression and rescue studies, zebrafish *sesn1* cDNA was introduced into the pCS2+ vector (RZPD). Capped mRNA was synthesized in vitro from linearized DNA template plasmids, using the mMESSAGE mMACHINE SP6 kit (Ambion). mRNA was diluted in 1× Danieu’s solution.

**Whole-mount in situ hybridization**

*In situ* hybridization was performed as described by Jowett (32). Stained embryos were mounted in 80% glycerol (in PBST) and analyzed using a Leica Fluo Combi stereomicroscope.

**Experiments in mammalian cells**

Mouse cDNA encoding Sns1 was amplified by PCR using IMAGE clone 6414521 as a template. The resulting PCR fragments were cloned downstream of- and in frame with- an HA or Flag tag in pcDNA3. Indirect immunofluorescence and co-immunoprecipitations were carried out on COS1 or HeK293T cells, respectively, which transiently express Flag- or HA-tagged Sns1 and Myc-tagged FoxH1 (33). Immunofluorescence was done using polyclonal anti-Myc (A14, Santa Cruz) or monoclonal anti-Flag (M2, Sigma) primary antibodies and CY2 or CY3 labeled goat anti mouse IgG or goat anti rabbit IgG secondary antibodies. Western blot analysis was carried out using a monoclonal anti-Myc antibody (9E10, Santa Cruz) or monoclonal anti HA antibody (12CA5) as primary antibodies and HRP-conjugated rabbit anti mouse IgG secondary antibodies (Jackson) followed by detection with the Western Lightning kit of Perkin Elmer.

**Mutation analysis**

All exons of *SESN1* were amplified by PCR using intronic primers (primer sequences and annealing temperatures are available on request). PCR fragments were analyzed by denaturing high-performance liquid chromatography on the Transgenic WAVE system. Direct sequencing for a subset of fragments was performed. PCR products were sequenced in both directions with the primers used in the PCR reaction and by the use of the BigDye v3.1 ET terminator cycle sequencing kit from Applied Biosystems. Sequencing reactions were loaded onto an Applied Biosystems Prism 3100 Genetic Analyzer.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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