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Research Article

Mutation Screening of Elongation Factor 2 in Shwachman-Diamond Syndrome Patients Lacking Mutations in the *SBDS* Gene

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Shwachman-Diamond syndrome is an autosomal recessive disorder characterized by bone marrow failure, pancreatic insufficiency, and skeletal abnormalities. Mutations in *SBDS* gene explain, by literature, 90% of SDS cases. The Italian experience shows that only the 5% of individuals diagnosed as affected by SDS on clinical and hematological grounds lack mutations in the *SBDS* gene. It is well established that SBDS protein is essential for the assembly of mature ribosomes. The yeast SBDS ortholog functions within a pathway containing elongation factor-like 1, homologous to human GTPase elongation factor-2, to promote the release and recycling of the nucleolar shuttling factor Tif6 from cytoplasmic pre-60S subunits in a cascade targeted to form the active ribosome. We considered that mutations of genes that disrupt pathways shared by SBDS may result in disease with comparable clinical features. EEF2 was evaluated as a candidate gene by mutation screening in clinically defined SDS which lack mutations in the *SBDS* gene. To date, no deleterious mutations were found in EEF2 in four Italian patients without *SBDS* mutations, but with a clinical diagnosis of SDS.

1. Introduction

Shwachman-Diamond-syndrome- (SDS-)associated mutations were described in a gene-designed Shwachman-Bodian-Diamond syndrome (*SBDS*) [1] that encodes a member of a highly conserved protein family, with orthologues in diverse species including archaea, plants, and eukaryotes. Structural studies of the *Archaeoglobus fulgidus* SBDS ortholog [2] revealed the presence of three domains. The N-terminal domain is identical to the single domain yeast protein Yhr087wp that is implicated in RNA metabolism. The protein that is most closely structurally related to the second domain is the C-terminal domain of *E. coli,* RuvA, that is involved in Holliday junction recognition during the recombination event. The closest structural homologue to C-terminal third domain of *Archaeoglobus fulgidus* SBDS ortholog is the fifth domain of yeast *S. cerevisiae* elongation factor 2 [2]. The *SBDS* mRNA is widely expressed throughout the human tissue [1]; furthermore, immunofluorecence studies showed that the SBDS protein is localized to both the nucleus and the cytoplasm, but is particularly concentrated within the nucleolus [3]. The nucleolus is best known as a site of ribosome biogenesis. An interesting discovery from proteomic studies of the nucleolus [4–6] was that approximately 30% of nucleolar proteins constituted either novel or uncharacterized proteins. It is known that RNA processing factors are localized in the nucleolus, which is intriguing since the SBDS protein has been postulated to play a role in RNA processing based on data from SBDS orthologues. Consistent with an essential cellular function, loss of the mouse SBDS ortholog results in early embryonic lethality [7]. Menne et al. [8] demonstrated a critical function for the yeast SBDS ortholog, called Sdo1, in late maturation of cytoplasmic pre-60S ribosomes. In the eukaryotic cells 60S, is one of the two subunits of the polyribosome 80S, the important "machinery" involved in the translation from mRNA to protein. Menne and colleagues showed that Sdo1 functions within a pathway containing elongation factor-like

Table 1: Primers sequences and amplicon size of the 11 fragments used for the analysis of the *EEF2* gene.

Exon	Forward primer	Reverse primer	Amplicon length
1	cctataaaaagctgagtgttgacgtc	caccagcgaggcagggt	142
2	gtgccatgctgtgttcctggaaat	tcgctggactgaacctcactcatt	425
3-4	atgaatcttgggaccaagtcggtg	ctgtcacccaacattcctggcaaa	872
5-6	gtctactgaagaaagcctgcgtct	tccagatcttaagagaggagccctga	765
7	tgggctttcttgtttcttggtggg	tgtcagagcatccggaaacagca	211
8-9	tggcttcaagagaggaagcgtgta	atgctccttacttctagctcccga	649
10	tgaagaggatgtttcctgacagcc	agtctccatttacagccacagcca	582
11	ttcccagcaccttccctgaaatct	cacatgcctgtggaagactgcaaa	396
12	aggatgcgtctgtgtgtaaggtca	atgaggtccctctagagcctggaa	481
13-14	acttggcaggtggagggcaa	tcaggacgcctcctttaacacctt	563
15	tgagctcctgacaggactttccttctgccct	ccctgcgaagacaccgtgaaa	803

1 (Efl-1) to promote the release and recycling of the nucleolar shuttling factor Tif6 from cytoplasmic pre-60S subunits. 60S, free from Tif6, is able to assemble to subunit 40S and to form the active ribosome. Efl-1 is a cytoplasmic GTPase that is highly homologous to human GTPase elongation factor-2 (EEF2, formerly known as EF-2), and it promotes a GTP-dependent dissociation of Tif6 from 60S ribosomes *in vitro* [9]. The structural homology between Efl1 and the ribosomal translocase EEF2 [9] indicates that they bind to a similar site at the GTPase center of the ribosome [10, 11]. Structural homology between the archaeal SBDS protein and the fifth domain of EEF 2 [2] supports the hypothesis that Sdo1 may also bind in the vicinity of the GTPase center of the 60S subunit. Recently, Sezgin et al. [12] demonstrated that the knockdown of SBDS leads to growth inhibition and defects in ribosome maturation, suggesting a role for wildtype SBDS in nuclear export of pre-60S subunits. It is known that gene products mutated in some inherited bone marrow failure syndromes such as dyskeratosis congenital and Diamond-Blackfan anemia are all involved in different aspects of ribosome synthesis. Diamond-Blackfan anemia is associated with mutations in the *RPS19* [13] and *RPS24* [14] genes that encode structural components of the 40S ribosomal subunit. The human Cbf5 homolog dyskerin, DKC1, is mutated in the bone marrow failure disorder dyskeratosis congenita [15]. The early embryonic lethality associated with defective epiblast formation in SBDS-deficient mice, taken together with the high levels of SBDS expression in rapidly proliferating tissues [6], is entirely consistent with a critical requirement for SBDS in the *de novo* protein synthesis during the development. The SDS phenotype may reflect the sensitivity of specific cell types that are unable to dynamically upregulate ribosome synthesis at critical points during the development as a consequence of SBDS deficiency. Sdo1 is therefore a new component of a critical conserved pathway that is required for translational activation of ribosomes and links defective 60S subunit maturation to an inherited bone marrow failure syndrome associate with leukaemia predisposition. It has been reported [1] that about 10% of individuals with SDS lack mutations in the *SBDS* gene. Thereby, EEF2 was evaluated as a candidate gene by mutation screening in clinically defined SDS patients who lack mutations in the *SBDS* gene.

2. Methods and Materials

EEF 2 gene is composed of 3164 bp, with a coding sequence of 2577 bp, divided into 15 exons. Screening has been performed in the coding and flanking region of a selected subgroup of SDS patients classified on rigorous clinical criteria [16] but were negative for *SBDS* mutations.

2.1. Patient Enrollment. The subgroup of SDS patients was ascertained based on an objective clinical evidence of bone marrow hypoplasia (neutropenia) and exocrine pancreatic dysfunction (low serum trypsinogen and isoamylase, abnormal 72 hr fecal fat study, fatty pancreas) as well as confirmation of the absence of *SBDS* mutation on both alleles. Four over 80 Italian patients were selected (5%).

2.2. DNA Extraction. Peripheral blood samples from the subgroup of SDS patients were collected (4–8 mL) by venipuncture in EDTA-vacutainer tubes. Genomic DNA was extracted from peripheral blood samples using the salting out method [17].

2.3. EEF 2 Sequencing Analysis. We characterized the genotype of the subgroup of SDS patients by direct sequencing. The primers used for the amplification and sequencing of the 15 exons and their flanking intron/exon junctions were chosen by the Primer Express Software (Applied Biosystems) (see Table 1).

PCRs were carried out in a 50 μl reaction volume containing 350 ng of genomic DNA, 0.5 μM primers, 200 μM dNTP, 5 μL 10× PCR buffer, and 2U FastStart Taq DNA Polymerase (Roche, Mannheim, Germany). PCR products were checked by 2% agarose gel electrophoresis. The amplicons were then purified by a spin column purification method (GENOMED GmbH, Löhne, Germany) and prepared for the sequencing analysis following the BMR Genomics Service instructions (BMR Genomics, Padua, Italy).

3. Results and Discussion

SDS is a rare genetic syndrome: Minelli et al. [18] shows that the number of mutation proven SDS cases in Italy is 1/168,000. Consequently, the carrier incidence expected in the Italian population is 1/205. In the present study, we identified 4 out of 80 Italian patients, classified based on rigorous clinical criteria as having SDS but lacking mutations in the *SBDS* gene. Our diagnosis criteria are well described in the guidelines by Dror et al. [16], and it allowed us to identify a small group of SDS-like patients (5%), a smaller group, and then the one indicated by literature (10%); as the clinical diagnosis of SDS is usually difficult to make due to the complexity of the clinical phenotype, rarity of the disease, and the paucity of large studies, we suppose that an overestimation of SDS diagnosis is the reason of such discrepancy. To date, no deleterious mutations were found in EEF2 in four Italian patients without *SBDS* mutations. The known SNP rs36525 in intron 8, with a reported MAF of 0.43, was detected in one patient consistent with this polymorphism having no clinical significance. We point out that the present work aims to perform mutation screening of the coding and flanking regions of EEF2 gene; causing disaese variations might be present in noncoding regions of the EEF2 gene or be due to copy number variations. Why SDS-like patients with no *SBDS* mutations are observed could be due at least to 2 possibilities. It is possible that patients with normal *SBDS* alleles have been misclassified and actually have a distinct clinical syndrome. Alternatively, SDS may be a genetically heterogenous disorder. Mutations of a gene or genes that disrupt a pathway shared by *SBDS* may result in diseases with identical clinical features. We are now working on the screening of other genes with the same characteristics.

Conflict of Interests

The authors declare that they do not have any conflict of interests in the submitted paper; in particular, they do not have any direct financial relation with the commercial identity mentioned in their paper that might lead to a conflict of interests.

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