Research Article

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

Elena Nicolis¹ and Marco Cipolli²

¹ Laboratory of Molecular Pathology, University Hospital of Verona, Piazzale Stefani, 37126 Verona, Italy

² Cystic Fibrosis Center, University Hospital of Verona, Piazzale Stefani, 37126 Verona, Italy

Correspondence should be addressed to Elena Nicolis; elenalbius@gmail.com

Received 10 October 2012; Accepted 27 October 2012

Academic Editors: B. Blaumeiser, B.-H. Jeong, J. Moreaux, and M. Romkes

Copyright © 2013 E. Nicolis and M. Cipolli. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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, immunofluorescence

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	gtgccatgctgtgttcttgaaat	tcgctggactgaacctcactcatt	425
3-4	atgaatcttgggaccaagtcggtg	ctgtcacccaacattcctggcaaa	872
5-6	gtctactgaagaaagcctgcgtct	tccagatcttaagaggagccctga	765
7	tgggcttctgtttcttggtggg	tgctcagagcatccggaacagca	211
8-9	tggcttcaagagaggaagcgtgta	atgctccttacttctagctcccga	649
10	tgaagaggatgttctctgacagcc	agtctcatttacagccacagcca	582
11	ttcccagcacttccctgaaatct	cacatgcctgtggaagactgcaaa	396
12	aggatgcgtctgtgtgaaggtca	atgaggtccctcttagagcctggaa	481
13-14	acttggcaggtggagggcaa	tcaggagcctcctttaaaccctt	563
15	tgagctctgacaggacttctctgcct	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 μ L reaction volume containing 350 ng of genomic DNA, 0.5 μ M primers, 200 μ M dNTP, 5 μ L 10 $\times$ 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 disease 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 heterogeneous 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.

Acknowledgments

This work was funded by grants from AISS—Associazione Italiana Sindrome di Shwachman. The authors gratefully acknowledge Professor Cesare Danesino and Dr. Antonella Minelli, Department of Molecular Medicine, University of Pavia, Fondazione IRCCS Policlinico San Matteo Pavia, Italy; Dr. Furio Poli, Cystic Fibrosis Center, IRCCS Burlo Garofolo, Trieste, Italy. Special thanks go to the patients and their families.

References

- [1] G. R. B. Boocock, J. A. Morrison, M. Popovic et al., "Mutations in *SBDS* are associated with Shwachman-Diamond syndrome," *Nature Genetics*, vol. 33, no. 1, pp. 97–101, 2003.
- [2] C. Shammas, T. F. Menne, C. Hilcenko et al., "Structural and mutational analysis of the *SBDS* protein family: insight into the leukemia-associated Shwachman-Diamond Syndrome," *Journal of Biological Chemistry*, vol. 280, no. 19, pp. 19221–19229, 2005.
- [3] K. M. Austin, R. J. Leary, and A. Shimamura, "The Shwachman-Diamond *SBDS* protein localizes to the nucleolus," *Blood*, vol. 106, no. 4, pp. 1253–1258, 2005.
- [4] J. S. Andersen, C. E. Lyon, A. H. Fox et al., "Directed proteomic analysis of the human nucleolus," *Current Biology*, vol. 12, no. 1, pp. 1–11, 2002.
- [5] A. Scherl, Y. Couté, C. Déon et al., "Functional proteomic analysis of human nucleolus," *Molecular Biology of the Cell*, vol. 13, no. 11, pp. 4100–4109, 2002.
- [6] A. K. L. Leung, J. S. Andersen, M. Mann, and A. I. Lamond, "Bioinformatic analysis of the nucleolus," *Biochemical Journal*, vol. 376, no. 3, pp. 553–569, 2003.
- [7] S. Zhang, M. Shi, C. C. Hui, and J. M. Rommens, "Loss of the mouse ortholog of the Shwachman-Diamond syndrome gene (*Sbds*) results in early embryonic lethality," *Molecular and Cellular Biology*, vol. 26, no. 17, pp. 6656–6663, 2006.
- [8] T. F. Menne, B. Goyenechea, N. Sánchez-Puig et al., "The Shwachman-Bodian-Diamond syndrome protein mediates translational activation of ribosomes in yeast," *Nature Genetics*, vol. 39, no. 4, pp. 486–495, 2007.
- [9] B. Senger, D. L. J. Lafontaine, J. S. Graindorge et al., "The nucleolar Tif6p and Efl1p are required for a late cytoplasmic step of ribosome synthesis," *Molecular Cell*, vol. 8, no. 6, pp. 1363–1373, 2001.
- [10] J. S. Graindorge, J. C. Rousselle, B. Senger et al., "Deletion of *EFL1* results in heterogeneity of the 60 S GTPase-associated rRNA conformation," *Journal of Molecular Biology*, vol. 352, no. 2, pp. 355–369, 2005.
- [11] C. M. T. Spahn, M. G. Gomez-Lorenzo, R. A. Grassucci et al., "Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation," *The EMBO Journal*, vol. 23, no. 5, pp. 1008–1019, 2004.
- [12] G. Sezgin, A. L. Henson, A. Nihrane et al., "Impaired growth, hematopoietic colony formation, and ribosome maturation in human cells depleted of Shwachman-Diamond syndrome protein *SBDS*," *Pediatric Blood & Cancer*. In press.
- [13] N. Drapchinskaia, P. Gustavsson, B. Andersson et al., "The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia," *Nature Genetics*, vol. 21, no. 2, pp. 169–175, 1999.
- [14] H. T. Gazda, A. Grabowska, L. B. Merida-Long et al., "Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia," *American Journal of Human Genetics*, vol. 79, no. 6, pp. 1110–1118, 2006.
- [15] S. W. Knight, N. S. Heiss, T. J. Vulliamy et al., "X-linked dyskeratosis congenita is predominantly caused by missense mutations in the *DKC1* gene," *American Journal of Human Genetics*, vol. 65, no. 1, pp. 50–58, 1999.
- [16] Y. Dror, J. Donadieu, J. Kogelmeier et al., "Draft consensus guidelines for diagnosis and treatment of Shwachman-Diamond syndrome," *Annals of the New York Academy of Sciences*, vol. 1242, pp. 40–55, 2011.
- [17] S. A. Miller, D. D. Dykes, and H. F. Polesky, "A simple salting out procedure for extracting DNA from human nucleated cells," *Nucleic Acids Research*, vol. 16, no. 3, p. 1215, 1988.

- [18] A. Minelli, E. Nicolis, Z. Cannioto et al., "Incidence of Shwachman-Diamond syndrome," *Pediatr Blood Cancer*, vol. 59, no. 7, pp. 1334–1335, 2012.