Mutations of the SBDS gene are present in most patients with Shwachman-Diamond Syndrome

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ABSTRACT

Shwachman-Diamond Syndrome (SDS) is a rare multisystem disorder characterized by exocrine pancreatic insufficiency, bone marrow dysfunction, and metaphyseal chondrodysplasia. Recent studies show that mutations of SBDS, a gene of unknown function, are present in the majority of patients with SDS. In the present study, we show that most, but not all, patients classified based on rigorous clinical criteria as having SDS had compound heterozygous mutations of SBDS. Full length SBDS protein was not detected in leukocytes of SDS patients with the most common SBDS mutations, consistent with a loss-of-function mechanism. In contrast, SBDS protein was expressed at normal levels in SDS patients without SBDS mutations. These data confirm the absence of SBDS mutations in this subgroup of patients and suggest that SDS is a genetically heterogeneous disorder. The presence (or absence) of SBDS mutations may define subgroups of patients with SDS that share distinct clinical features or natural history.
INTRODUCTION
Shwachman-Diamond Syndrome (SDS) is a rare multisystem disorder characterized by exocrine pancreatic insufficiency, bone marrow dysfunction, and metaphyseal chondrodysplasia.\textsuperscript{1-5} SDS is the second most common cause of congenital exocrine pancreatic insufficiency after cystic fibrosis. Bone marrow dysfunction is present in nearly all patients with SDS.\textsuperscript{3-6} In the largest published series (88 patients), chronic or intermittent neutropenia was present in 97%.\textsuperscript{5} Anemia and thrombocytopenia were present in more than a third of patients. Patients with SDS have a marked propensity to develop myelodysplasia or acute myeloid leukemia.\textsuperscript{6-8}

SDS is inherited in an autosomal recessive fashion.\textsuperscript{9} Recently, Boocock et al. reported that compound heterozygous mutations of the \textit{SBDS} (Shwachman-Bodian-Diamond syndrome) gene on chromosome 7 were present in the majority of patients with SDS.\textsuperscript{10} Most of these mutations resulted from gene conversion with a neighboring pseudogene (\textit{SBDSP}). Similar data were reported in a smaller series of patients of Japanese ancestry.\textsuperscript{11} Most of the \textit{SBDS} mutations are predicted to truncate a substantial portion of the SBDS protein, suggesting that they act in a loss-of-function manner. Herein, we show that most, but not all, patients classified prospectively based on clinical criteria as having probable SDS had \textit{SBDS} gene mutations. Moreover, a strong correlation between \textit{SBDS} genotype and expression of full length SBDS protein was observed.

MATERIAL AND METHODS
\textbf{Human subjects.} 33 families who attended the Second International Conference on Shwachman-Diamond Syndrome in St. Louis in 1999 were invited to participate in a study to elucidate the genetic basis of SDS. After obtaining informed consent, a thorough history, physical examination, review of medical records, and selected laboratory testing were performed. In addition, genomic DNA was extracted from peripheral blood leukocytes using standard protocols. The diagnosis of SDS was based on the presence of the following two criteria,\textsuperscript{12} bone marrow failure (neutrophils $< 1500 \times 10^6$/l, hemoglobin $< 10$ g/dl, or platelets $< 150 \times 10^9$/l) or exocrine pancreatic insufficiency (serum trypsinogen $< 16.7$ µg/l, abnormal pancreatic stimulation test, low fat soluble vitamin levels, or abnormal 72-hour fecal fat study together with a characteristic pancreatic abnormality in an imaging study). A diagnosis of SDS was considered probable when both criteria were documented in the medical record or by the
on site evaluation. A diagnosis of SDS was considered possible if only one criterion was
documented and a second was suggested by an answer in the questionnaire completed
by families. As controls, genomic DNA from 48 ethnically matched healthy volunteers
was sequenced. The human studies committee at Washington University approved this
study.

**Sequence analysis.** A PCR-based strategy to sequence all 5 exons, splice junctions,
and 500 base pairs of 5’- and 3’-untranslated regions of the *SBDS* gene was developed.
Primers were designed to specifically amplify the *SBDS* gene but not the *SBDSP*
pseudogene (supplemental table S1). Five nanograms of genomic DNA were amplified
using AccuTaq (Sigma) and MJ Research (Cambridge, MA) PTC-225 thermal cyclers
with the following parameters: hot start, 96°C for 2 min 30 sec; and 96°C for 20 sec,
60°C for 15 min, for 35 cycles. Amplicons were sequenced with Big Dye version 3.1
chemistry and analyzed using ABI 3730 capillary sequencers. Sequence analysis was
done in a blinded fashion with respect to the clinical diagnosis.

**Immunoblotting.** Rabbits were immunized with a mixture of two peptides from the
extreme carboxy-termini of the murine (VLSLKDVEEGDEKFE) or human
(VLNLKDVEEGDEKFE) SBDS protein (both with a cysteine at their amino-termini). The
resulting antiserum recognized an approximately 29kDa protein from both human and
murine cell lysates (figure 1, and data not shown). Cryopreserved leukocytes were lysed
in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% sodium
deoxycholate, 1% NP40, 0.1% SDS, 0.5 mM PMSF, 0.5 mM diisopropylfluorophosphosphate
and 10 μg/ml aprotinin and leupeptin) and immunblotted with SBDS or actin antiserum
(sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA) or immunoprecipitated with SBDS
or control antiserum.

**RESULTS AND DISCUSSION**
Genomic DNA suitable for sequencing was available for 29 of the 33 families. Clinical
characteristics of the probands in these families at the time of their participation in this
study are shown in table 1. Based on clinical criteria outlined in Methods, 18 of these
families were classified as having one or more affected individuals with probable SDS, 6
with possible SDS, and 3 were judged not to have SDS. In 2 families, insufficient data was available to classify the probands (“unclassified”).

Compound heterozygous mutations of the SBDS gene were detected in the majority of patients with probable or possible SDS (table 1). In agreement with previous reports, the 258+2T>C and 183-184TA>CT mutations were the most common.\(^{10,11}\) The 258+2T>C mutation results in the disruption of the donor splice site of intron 2 and the use of a cryptic splice donor site a position 251-252. The resulting abnormally spliced mRNA results in a frameshift and the premature truncation of the 250 amino acid SBDS.

### Table 1. Patient characteristics and SBDS genotypes

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<th>Sex</th>
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<th>Hgb (gm/dL)</th>
<th>Plt (\times 10^9/L)</th>
<th>ANC (\times 10^9/L)</th>
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\(^1\)Age at time of entry into registry and laboratory evaluation. \(^2\)The absolute neutrophil count (ANC) for patients receiving G-CSF (IRT) is indicated by "(G)". \(^3\)Normal value for immunoreactive serum trypsinogen G-CSF (IRT) is > 16.7 ng/ml.

Not available (na), 183-184CT>TA (183/184), 258+2T>C (258+2), 258+1G>C (258+1)
protein at amino acid 84. A single patient had a 258+1G>C mutation that results in a similar splicing defect. The 183-184TA>CT mutation introduces a premature stop codon resulting in the truncation of the SBDS protein at amino acid 62. Point mutations resulting in single amino acid substitutions were detected in two families. The 505C>T mutation has been reported previously and results in a cysteine for arginine substitution at amino acid 169. The 652C>T mutation is novel and generates a premature stop codon at amino acid 218. None of these mutations were detected in 48 healthy controls. In 18% (5 of 28) of patients classified with probable or possible SDS, SBDS mutations were not detected (table 1). Likewise, no SBDS mutations were detected in 11% (17 of 158) and 29% (2 of 7) of previously studied patients with SDS. Since sequence analysis in all three studies was limited to exons, splice junctions, and the immediate 5' - and 3' - untranslated regions, it is possible that mutations outside of the sequenced regions may have been missed that effect SBDS expression. To address this possibility, SBDS protein expression in blood leukocytes from these patients was examined. In samples from a healthy individual, two bands of approximately 29kDa (the predicted size for the SBDS protein) were detected by immunoblotting with the SBDS antiserum. Immunoprecipitation with the SBDS antiserum showed that the upper band represents the SBDS protein (figure 1A). The SBDS protein was detected in samples from family members with normal SBDS alleles (figure 1B, lanes 1 and 2) but not in patients containing the most common SBDS genotype (compound heterozygous mutations for 183-184TA>CT plus 258+2T>C). The SBDS antiserum was raised against a carboxy-terminal peptide, thus these data do not exclude the presence of an aminoterminal protein fragment. A preliminary study by Leary et al. also showed the absence of detectable SBDS protein in cell lines derived from patients with SDS who had SBDS mutations. Reduced SBDS protein expression in the sample containing the 258+2T>C and 505C>T mutations was observed; based on densitometry, the relative intensity of the SBDS band in the 505C>T sample was 40% of that observed in normal samples, suggesting that the 505C>T allele results in the production of full length SBDS protein (lane 6). Unfortunately, no protein was available from the two patients with probable SDS and normal SBDS alleles. However, SBDS protein was readily detected in two patients classified as having possible SDS with normal SBDS alleles, (lanes 7 and 8). Of note, SBDS protein expression in family members heterozygous for a SBDS mutation was comparable to family members with normal SBDS alleles (figure 1C).
Figure 1. SBDS Western. A. Proteins extracted from normal cryopreserved blood leukocytes were analyzed by immunoblotting with the SBDS antiserum either directly (None) or after immunoprecipitation with SBDS or control antiserum. Two bands of approximately 29kDa are detected in whole cell extracts. The upper band but not lower band is efficiently immunoprecipitated by the SBDS antiserum, suggesting that the upper band represents the SBDS protein. B & C. Protein extracts of cryopreserved blood leukocytes from family members in the registry were analyzed by immunoblotting with the SBDS antiserum (upper panels) or an actin antibody (lower panels). SBDS genotypes: lane 1, 2, and 9 (normal), lanes 3-5 and 10 (183-184TA>CT x 258+2T>C), lane 6 (258+2T>C x 505C>T), lanes 7 and 8 (normal with clinical diagnosis of possible SDS), lane 11 and 13 (normal x 258+2T>C), and lane 12 (normal x 183-184TA>CT). The relative densitometry signal for the SBDS band compared to the actin band is shown for lanes 9-13. Molecular size markers are indicated at the left in kDa. This data is representative of two independent experiments using the same protein extracts.
Together with the sequencing data, the SBDS protein expression data provide strong evidence that there is a subgroup of patients with clinical features of SDS who do not have SBDS mutations. These data suggest at least two 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. Mutation of gene(s) that disrupt a pathway shared by SBDS may result in disease with identical clinical features. Distinguishing between these possibilities will require the elucidation of the biological functions of SBDS.

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SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.
REFERENCES

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