Are Shwachman-diamond syndrome patients radiosensitive?

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Abstract
Mutations in the Schwachman-Bodian diamond syndrome (*SBDS*) gene—involved in ribosome biogenesis—cause Shwachman-diamond syndrome (SDS), a known bone marrow failure disorder. A dysfunctional ribosome biogenesis is postulated as a cause of phenotypes seen in SDS patients. Recently, lymphocytes from SDS patients with hypomorphic SBDS expression were shown to harbor significantly increased DNA damage and γH2AX foci in response to X-rays or gamma rays. Additionally, SBDS knockdown in cells increases ROS (reactive oxygen species) levels and enhances proliferation defects in a p53 dependent manner. These new reports suggest that SBDS may have a novel and a yet unexplored role in DNA repair and damage response pathways. In this short opinion article, I will discuss these recent observations and delineate hypothesis to explain the potential new roles of SBDS.
Key words: Shwachman-diamond syndrome (SDS), bone marrow failure, SBDS, radiosensitivity, DNA repair

What is Shwachman-diamond syndrome (SDS)?

SDS (SDS, OMIM 260400) is an autosomal recessive disorder characterized by exocrine pancreatic insufficiency, skeletal deformities, bone marrow failure and leukemic predisposition [1,2]. It is a multi-system disorder that occurs in about 1/75,000 individuals, with a male to female ratio of 1.7:1 [3,4]. Over 95% of SDS patients harbor biallelic mutations in the *SBDS* gene located on chromosome 7q11 [5]. The SBDS protein belongs to a conserved protein family with orthologs from Archae to vertebrates and plants [6]. SDS patients exhibit reduced expression level of SBDS, which plays an important role in ribosome assembly [7]. Defective ribosome biogenesis is proposed to cause the diverse clinical features of SDS patients, although an exact mechanism is not known. In light of the new phenotypes observed for SBDS, the underlying cause of SDS pathophysiology may encompass more causative factors than just the defective ribosome biogenesis. I will explore some of these hypotheses in the following sections.

Phenotypes associated with SDS patients

SDS patients present characteristic physical manifestations such as short stature, skeletal deformities, exocrine pancreatic insufficiency and hematological deformities. Primary diagnosis of SDS occurs during childhood, where some patients experience learning disabilities. Summarized details of some important physical symptoms in SDS patients are discussed below:

Skeletal deformities: About half of the SDS patients present with metaphyseal dystosis (developmental delay of long bones) involving the hips and the femur [8]. Another one third to one half of patients report rib-cage abnormalities, which includes short ribs with flared ends, narrow rib-cage and a chostochondral thickening (pertaining to costal cartilages) leading to respiratory failure [2,9]. Most skeletal problems seen in patients associate with delayed appearance of the secondary ossification centers, excluding, clinodactyly (curved little finger), syndactyly (webbing between digits), osteopenia (decreased bone density), kyphosis (forward bent spine), scoliosis (lateral curved spine), vertebral collapse, slipped femoral epiphyses (rounded end of

long bone) and supernumerary (extra) thumb [1,10].

Exocrine pancreatic insufficiency: SDS patients are deficient in pancreatic digestive enzymes such as trypsinogen, isoamylase, etc., causing mal-absorption of fats and nutrients [11]. Mal-absorption of nutrients results in vitamin deficiency, characteristic loose stool, weight loss, edema and a general failure to thrive. While the serum level of pancreatic enzymes serves as a diagnostic tool for SDS patients, the ultra-sound and other imaging techniques can additionally confirm the presence of fatty pancreas in patients [1,11].

Hematological abnormalities: Neutropenia or abnormal-low levels of neutrophils are a characteristic abnormality seen in SDS patients and its onset can be as early as the neonatal stage [8,12]. However, neutropenia fluctuates from low to normal levels during the lifetime of the patient [13,14]. Neutrophils of SDS patients additionally exhibit impaired mobility, migration, chemotaxis and an altered cytoskeletal function [15-17]. Low-levels of neutrophils thus increase patients' susceptibility to recurrent bacterial, fungal and viral infections, including respiratory and skin infections [18]. Although multiple B and T- cell defects reported in patients could also contribute to immune dysfunction [19,20].

Other blood cell irregularities seen in SDS patients include, anemia (from low reticulocyte counts), elevated fetal-hemoglobin, thrombocytopenia (low platelet counts) and sometimes even the trilineage cytopenia (reduction in all blood lineages—red cells, white cells and platelets) [1,21,22]. Patients with the observed cytopenias may evolve into aplastic anemia, myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) and may require transfusions [1,13,23-25]. The bone marrow of SDS patients also display decreased frequencies of $CD34^+$ progenitor stem-cells, reducing the *in vitro* generation of hematopoietic colonies of all cell lineages [26].

Genetics of Shwachman-diamond syndrome

That mutations in *SBDS*—a novel gene—associate with SDS is a fairly recent discovery [5,27]. *SBDS* spans 305 kb on chromosome 7 and is distally located to its unprocessed pseudogene, *SBDSP*, which 97% identical to *SBDS* [5]. About 96% of SDS patients carry mutations in exon 2. The most prominent of these mutations are: 183-184TA→CT (introduces an in-frame stop codon, K62X), 258+2T→C (disrupts donor splice site of intron 2 causing a 8-bp deletion after an upstream cryptic splice donor site is used, which causes premature truncation of the encoded protein), and the complex 183-184TA→CT+258+2T→C mutation, amongst the others reported in SDS patients [5]. Most of these mutations arise from recombination between *SBDS* and the *SBDSP* by gene conversion events. *SBDS* is ubiquitously expressed and is an essential gene [28]. Since homozygous allele mutations are not known in SDS patients, SBDS may also be essential in humans. It is unknown how these mutations correlate with the pleiotropic phenotypes in SDS patients. Because SDS patient cells acquire chromosomal changes during their lifetime, absence of SBDS is speculated to cause the leukemia transformation in patients [29].

The first crystal structure of the *SBDS* homolog, AF0491 (*Archaeoglobus fulgidus*), revealed it to comprise three well-folded domains: N-terminal domain, central domain and the C-terminal domain [30]. Likewise, the human SBDS protein contains the same three domains, except for the flexible connecting loop between the N-terminal domain and the central domain [31]. Patient-associated mutations in *SBDS* localize at the N-terminal domain [32]. In human cells, SBDS localizes to the nucleolus during G1 and G2 phases of the cell cycle and depends on active transcription [33,34].

Known functions of SBDS

Defective Hematopoiesis: Hematopoietic progenitors from SDS patients have reduced colony formation *in vitro* [21]. siRNA knockdown of *SBDS* in $CD34^+$ stem cells, early progenitors and K562 cells also impairs erythroid differentiation [35]. Similarly, siRNA knockdown of *Sbds* in murine hematopoietic cells causes defective granulocytic differentiation and myeloid progenitor generation, and reduced B-lymphocyte numbers [36]. These examples associate the loss of SBDS with the phenotypes seen in SDS patients. However the molecular mechanism linking SBDS to hematopoiesis remains unknown.

Ribosome biogenesis: SDS is recognized as a ribosomopathy, a condition where a defective assembly of ribosomes leads to growth and cell proliferation defects [37-39]. Evidence of the role of SBDS protein in ribosome biogenesis first came from the proteome and transcriptional microarray data that showed binding of yeast ortholog, YLR022c, to phospholipids and RNA processing enzymes [40,41]. For ribosome biogenesis, SBDS protein takes part with EFL1 (elongation factor–like 1) GTPase to release eIF6 (eukaryotic initiation factor 6) from the 60S ribosomal subunit [42]. The eIF6 protein is a critical factor for late maturation of 60S ribosomes in the cytoplasm. eIF6 associates with the 60S ribosome and prevents its binding to the 40S ribosome subunit by steric hindrances [43,44]. SBDS stimulates the GTP hydrolysis of EFL1 to release eIF6 from the 60S ribosome [42,45]. The exact mechanism of EFL1 and SBDS-mediated removal of eIF6 is also unknown. In the absence of SBDS, eIF6 is not released from the 60S subunit, which fails to reconstitute with the 40S to produce the 80S ribosomes. Such ribosome joining defects can be profiled in a sucrose gradient, which then shows altered 60:80 ratios as is seen in SBDS deficient SDS lymphocytes or stromal cells, mouse cells, or even amoeba (*Dictyostellium discoideum)* [42,46,47]. Because of the reduced formation of the 80S ribosomes, SBDS mutant cells exhibit translational inefficiency and reduced global protein synthesis [47]. Altered ribosome biogenesis is thus associated with growth impairment, which is also postulated as a cause of hematopoiesis.

Other SBDS functions: Besides the defective ribosome biogenesis phenotype observed in SBDS-deficient SDS cells, other novel SBDS phenotypes have now come to light. For example SBDS depletion: 1) in HeLa cells exhibit accelerated Fas-ligand-induced apoptosis, 2) in human skin fibroblasts results in increased mitotic abnormalities and aneuploidy that was also seen in SDS cells, 3) in SDS neutrophils causes chemotaxis defect, 4) in murine monocytes formed reduced number and size of osteoclasts, 5) in SDS bone marrow shows p53 overexpression, 6) in myelocytes activate p53 and hence apoptosis, 7) in lymphoblastoid cells alters translation of CCAAT enhancer binding proteins C/EBPα and C/EBPβ that affects granulocyte differentiation, 8) in B-cells and SDS leukocytes hyperactivation of mTOR (mechanistic target of rapamycin) and STAT3 (Signal transducer and activator of transcription 3), 9) in HeLa and myeloid cells increases ROS levels, 10) in SDS lymphocytes causes radiosensitization [45,48-57]. All these phenotypes are ascribed as causative factors in enhanced cell death or skeletal deformities or as leukemogenesis facilitators causing SDS phenotypes.

The most intriguing aspect of these new functions of SBDS is that they reflect a common theme of genomic instability, increased cell death via apoptosis, p53 activation and suppressed translation. While the ribosomopathies such as the DBA (Diamond-Blackfan anemia) are also characterized by increased p53 activation via RPL11 (60S ribosomal protein L11) and RPL5 (60S ribosomal protein L5) with a dysfunctional translation, but genomic

instability is not associated with DBA or other ribosomopathies [39]. Likewise a similar p53 induction pathway in SDS is unknown, making SDS a unique ribosomopathy. Because a lack of SBDS results in both ribosome biogenesis and genomic instability, the compelling questions is whether these phenotypes are caused by independent functions of SBDS. Could it be possible that absence of SBDS results in dysfunctional ribosome biogenesis, which then causes the genomic instability phenotype or *vice versa*? Since SBDS knockdown causes genomic instability, does SBDS have a role in DNA repair? And finally, if the SDS lymphocytes accumulated DNA damage, can SDS be categorized as a radiosensitive disorder?

What are radiosensitivity disorders?

As many as 40 DNA-repair-defective disorders are associated with radiosensitivity [58-60]. The most prominent of these are the autosomal recessive A-T (Ataxia-telangiectasia with mutations in the ATM gene), NBS (Nijmegen breakage syndrome with NBS1 gene mutations), FA (Fanconi Anemia with mutations in about 1 of its 21 genes), LIG4 (DNA Ligase IV deficiency disorder with mutations in LIG4 gene), SCID (Severe combined immunodeficiency disease with mutations in Artemis, LIG IV, DNA-PKcs [DNA-dependent protein kinase catalytic subunit] and Cernunnos-XLF). Each of these disorders is characterized by homozygous mutations in genes involved in DNA repair. And in all cases, patients exhibit enhanced sensitivity to radiation, except for the heterozygous individuals who have a higher radiosensitvity threshold. Patients with these disorders, similar to SDS patients, present with immunodeficiencies, growth retardation and cancer predisposition. In addition, accidental radiation exposure poses a high risk to these radiosensitive patients and radiotherapy in particular becomes a challenging treatment regimen to treat cancers [59,61]. Interestingly, radiosensitivity phenotype can also be conferred by mutations in genes, which have functions beyond DSBR, such as SMARCAL1 that is associated with Schimke immuno-osseous dysplasia (SIOD) [62,63].

Whether SDS patients' lymphoblastoid or other cells are also radiosensitive, as would be in a radiosensitivity (colony survival assay (CSA)) assay is not known [60]. Recently, Morini et.al., showed that two SDS patient lymphocytes carried more oxidative DNA damage and possible single strand breaks via a COMET assay [57]. The authors also observed an enrichment of γH2AX foci formation in two of these SDS cell lines compared to controls, which indicate either a BER (base excision repair) or a DSBR (double strand break repair) deficiency. However, a confirmed radiosensitivity phenotype in SDS cells couldn't be established from this study. By including more patient data, testing patient's cells in the CSA assay; and assessing both the BER and DSBR repair capacity in patient cells, can allow researchers clarify this assertion. Nevertheless, other independent studies have also hinted at the possibility of SDS as a radiosensitivity disorder.

First, in the murine *Sbds* disease model—which is embryonically lethal—the senescence phenotype seen in the embryonic pancreas is p53 dependent [64]. The authors also observed that p53 ablation surprisingly rescued the embryonic lethality. p53 is a global genome regulator and plays an important role in different DNA repair pathways, including excision and double strand break repair (DSBR) [65]. In the context of DSBR—associated with γH2AX foci formation—deficiency of DNA repair factors such as XRCC4, LIG3 (Non-homologous end joining repair), etc., causes p53 upregulation and apoptosis [65,66]. Additionally, p53 regulates RAD51 expression which is an important factor for homologous recombination dependent DSBR [67]. These examples suggest that the

increase in p53 expression in SBDS deficient cells could result from persistent double strand breaks because SBDS protein may have an important role in DSBR.

Second, SBDS was shown to functionally bind to diverse proteins including DNA-PK and RPA, involved in the DSB repair [68]. Strikingly, the authors observed that knockdown of SBDS sensitized cells to ER (endoplasmic reticulum) stressor thapsigragin, with an increased phosphorylation of ER stress marker eIF2α [68]. Here, ribosome disassembly and translational insufficiency may contribute to an increased ER stress in cells. In a separate study, increased peIF2α was shown to suppress RAD51 expression, suggesting that ER stress response suppresses DSBR [69]. It thus seems that SBDS deficient cells, such as in SDS, may have multiple pathways to compromise its DSB repair capacity and become radiosensitive (Figure 1).

Concluding remarks and future perspective

In this short opinion, I have delineated that SDS is caused by mutations in SBDS, which is known to be involved in ribosome biogenesis. However, current literature review suggests that the exact mechanism by which defects in ribosome assembly led to patient phenotypes is difficult to ascertain. Recent studies show that SBDS is associated with new functions, including maintenance of DNA repair in cells and regulation of p53 activation. Consequently, SDS cells have increased DNA damage and unresolved phosphorylated H2AX foci, which are hallmarks of the radiosensitivity disorders. In order to conclusively ascertain that SDS exhibit features of radiosensitivity, SDS patient cells (lymphoblastoid or hematopoietic progenitor) must be tested for radiosensitivity in a CSA assay. If SDS cells were radiosensitive, then the RAD51 expression must be checked to determine if the patients lacked DSBR capacity due to lower levels of RAD51 [70]? Or, establish expression levels of proteins for the BER pathway, which also seems to be implicated. Additionally, SDS cells should also be tested for activated ER stress response, since SBDS knockdown results in ER stress activation. By exploring these non-ribosomal functions of SBDS we can improve our understanding of SDS and potentially open up new lines of treatment and or management for patients with SDS or similar disorders.

Acknowledgements

I acknowledge Dr. Graham Walker and the Walker lab members for their continued support.

Figure 1: Hypothesis to describe the cause of radiosensitivity in SDS cells. First, in SDS cells, lack of SBDS causes failure to stimulate the GTPase activity of ELF1 and prevents the release of eIF6 from the 60S ribosome, resulting in defective ribosome biogenesis and translational insufficiency. Second, SDS cells exhibit an activated p53 response, which could arise from the ribosomal stress or from some unknown regulation from the SBDS protein. Activated p53 regulates DSBR proteins, such as RAD51, and may suppress DSBR activity in cells. A suppressed DSBR protein expression can also cause p53 activation. In SDS cells, the exact source of p53 activation is yet to be determined. Third, lack of SBDS causes ER stress in cells, which can also suppress DSBR by suppressing RAD51 expression or abundance. ER stress can also arise from translational insufficiency or from an unknown interaction of SBDS with the ER stress machinery. A suppressed DSBR capacity can cause radiosensitivity of SDS cells.

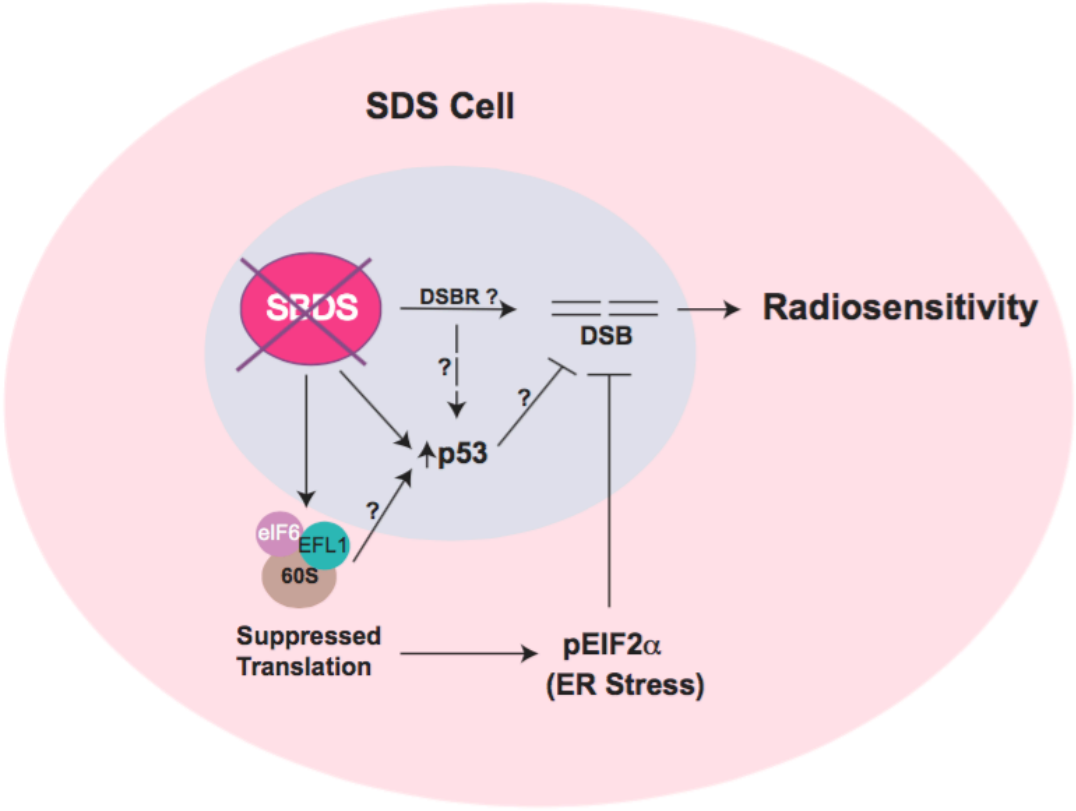

Figure 1

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