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Short Research Communication

Development of Soft Tissue Sarcomas in Ribosomal Proteins L5 and S24 Heterozygous Mice

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Abstract

Diamond-Blackfan anemia (DBA) is an inherited bone marrow failure syndrome associated with ribosomal protein (RP) gene mutations. Recent studies have also demonstrated an increased risk of cancer predisposition among DBA patients. In this study, we report the formation of soft tissue sarcoma in the *Rpl5* and *Rps24* heterozygous mice. Our observation suggests that even though one wild-type allele of the *Rpl5* or *Rps24* gene prevents anemia in these mice, it still predisposes them to cancer development.

Key words: Ribosomal proteins RPL5 and RPS24; Diamond-Blackfan anemia; Soft tissue sarcoma; *Rpl5* and *Rps24* heterozygous mice

Introduction

Diamond-Blackfan anemia (DBA) is a hereditary red blood cell aplasia that usually presents within the first year of life. Heterozygous point mutations and large deletions in 16 ribosomal protein (RP) genes, *RPS19, RPS24, RPS17, RPL5, RPL11, RPL35A, RPS7, RPS10, RPS26, RPL26, RPL15, RPS28, RPS29, RPL31, RPS27*, and *RPL27* have been considered the underlying cause of disease in about 65% of patients [1-5]. DBA is also associated with physical abnormalities with varying severity such as craniofacial, upper limb, heart, and urinary-system defects in about 30-50% of patients [3, 6]. However, the severity of these abnormalities varies among patients [3]. A potential link between ribosomal protein deficiency and risk of tumor formation has also been demonstrated in human and zebrafish [7-10]. Vlachos et al. reported that a percentage of patients registered in the Diamond-Blackfan Anemia Registry of North America developed a variety of cancer including acute myeloid leukemia, colon carcinoma, osteogenic and soft tissue sarcomas at the median age of 41 [11]. The risk of cancer in these patients was 5.4 fold higher than that of the general population [11]. Current therapeutic approaches for DBA have been focused on increasing the level of red blood cells through bone marrow transplantation, glucocorticoids, and red blood cell transfusion in steroids-resistant patients [6, 12]. L-leucine has also been considered as an alternative therapy [6, 12].

Over the past decade, many research teams have established both *in vitro* and *in vivo* models of DBA to better understand the pathological and molecular mechanisms of ribosomal protein deficiency [3, 13, 14]. To date, very few approaches have been taken to

address the mechanism of increased cancer predisposition associated with this disease. Recently, we reported the detection of *Rps24* and *RpL5* mutations in patients with DBA [15, 16], and have generated mouse models to further address the effect of these mutations in developing anemia and cancer. In this study, we characterized the *Rpl5*+/- and *Rps24*+/- mice models for DBA. Even though these mice did not exhibit anemia, two *Rpl5*+/- and one *Rps24*+/- mice developed soft tissue sarcoma. By taking advantage of our murine models, we hope to gain insight into the molecular mechanism of ribosomal protein deficiency and cancer development.

Results and Discussion

Generation of *Rps24* and *Rpl5* Heterozygous mice

Generation of the C57BL/6 *Rpl5+/- and Rps24*+/- mice was carried out by InGenious Targeting Laboratory (iTL; Ronkonkoma, NY, USA), and all animal studies were approved by Boston Children's Hospital's Institutional Animal Care and Use Committee. A pGK-gb2 loxP/FRT-flanked Neomycin cassette was inserted in embryonic stem cells to replace 383 bp of the *Rps24* gene, including exons 2-3, or 8.11 kb of the *Rpl5* gene, including exons 1-8. These cells were injected into C57BL/6 blastocysts, and the chimeric animals were mated to generate heterozygous mice. All the *Rps24-/-* and *Rpl5-/-* mice died by E11-12 despite there being a normal Mendelian distribution of heterozygous, homozygous, and wild-type blastocysts. Heterozygous mice were born at the expected frequency of about two-thirds (given the embryonic lethality of homozygous KO mice) and appeared clinically normal. In particular, heterozygotes of both genotypes did not develop hematological phenotypes that have been detected in patients with DBA, such as anemia. No changes were detected in the complete blood cell count (CBC) (Table S1) as well as the *in vitro* colony forming unit-erythroid (CFU-E), burst forming unit-erythroid (BFU-E) and colony forming unit-granulocyte /macrophage (CFU-GM) assays (Figure S1). Real-time PCR and immunoblot analysis also showed similar expression levels of *Rpl5* and *Rps24* mRNA, and RPL5 and RPS24 proteins in both heterozygous and wild-type mice (Table S2 and Figure S2). These observations are similar to previously reported findings for *Rps19*+/- mice [17] suggesting that one wild-type copy of these ribosomal proteins is sufficient to prevent the development of anemia.

Detection of Tumors in *Rps24* and *Rpl5* Heterozygous Mice

We also investigated the risk of cancer development in aging *Rpl5*+/- and *Rps24*+/- male and female mice by monitoring them for up to 36 months after birth (Table S3). Out of 21 *Rpl5*+/- mice monitored between 15 to 36 months of age, two male mice each developed a 0.5 cm tumor at 22 months of age (Figure S3). Similarly, out of 23 *Rps24*+/- mice (between15 and 30 months of age), one female mouse developed a 2 cm tumor spanning from upper left ear to the head/neck region at 17 months of age (Figure S3). However, none of the 31 control wild-type mice, ranging from 18 to 30 months of age, developed any type of tumor. We preformed statistical analysis, and the development of sarcoma was not statistically significant. The low number of cancer occurrence in mice matches the low incident of cancer in patients with DBA. One possible explanation for late occurrence of cancer development in mice (around 2 years) compared to patients with DBA (median age of 41) is that mice compensate for the loss of one *Rpl5* or *Rps24* allele possibly by either producing more *Rpl5* and *Rps24* mRNA from a single allele or increasing the stability of *Rpl5* and *Rps24* mRNA. However, over time, having only one allele may activate a new set of signaling pathways that may reduce the effect of the compensatory pathway and promote the development of late onset cancer. In contrast, ribosomal protein gene mutations in zebrafish have resulted in developmental defects of varying degrees similar to the symptoms detected in patients with DBA [18-20]. Similar to mice, some of these mutations have predisposed fish to cancer development by 2 years of age, which is considered late in the life span of zebrafish [9]. Together, these observations further support the possibility that prolonged ribosomal protein deficiency accumulation can increase the risk of cancer, as has been observed in patients [11].

Histological and Immunohistochemical Analysis of Tumors Isolated from *Rps24* and *Rpl5* Heterozygous Mice

To determine the nature of tumors, we performed histological and immunohistochemical studies on tumor and normal skin tissues from *Rpl5*+/- and *Rps24*+/- mice as well as normal skin tissue of wild-type mice (Figure 1). Histological sections from all tumors showed that the tumors involved the dermis, were densely cellular, and composed of predominantly atypical spindle cells arranged in intersecting fascicles with brisk mitotic activity (Figures 1C-1D and 1F- 1I). The overlying epidermis showed no evidence of dysplasia and was not associated with the tumors (Figure 1). Immunohistochemical studies of tumor cells showed strong cytoplasmic reactivity for vimentin, a mesenchymal marker commonly expressed in sarcomas, and negative staining for

pan-keratin, LCA, and S100, which excludes diagnosis of carcinoma, lymphoma, and melanoma, respectively (Figure 2). These overall findings were consistent with the characteristics of a high-grade spindle cell soft tissue sarcoma. In all the studies, wild-type, *Rpl5*+/-, and *Rps24*+/- normal skin tissues were histologically unremarkable (Figure 1). Due to the low incident of sarcoma formation in our mouse model, we performed PubMed and two public databases searches and to our knowledge, there are no records of spontaneous sarcoma formation specifically on the C57BL/6 wild-type mice [21, 22]. Also, we did not find any records indicating a correlation between the age of the mouse and the formation of sarcoma in C57BL/6 strain [21, 22].

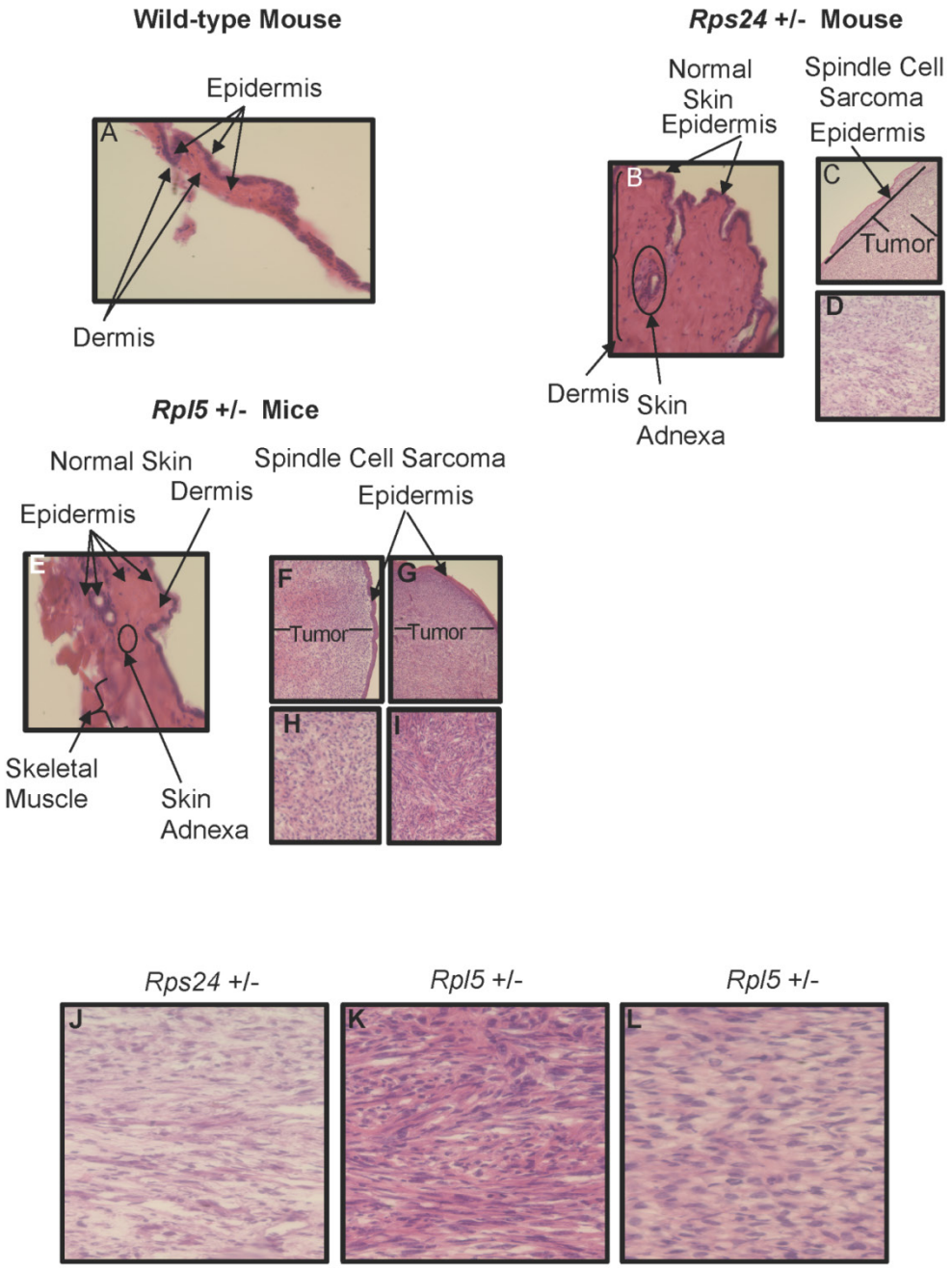

Figure 1. Histology of Wild-type, *Rps24*+/-, and *Rpl5*+/- Normal Skin and Spindle Cell Sarcoma Tissues. Hematoxylin and Eosin staining showed normal epidermis of wild-type (**A**), *Rps24*+/- (**B**), and *Rpl5*+/- (**E**). However, there was a uniform localization of spindle tumor cells beneath the epidermis from *Rps24*+/- mouse (**C** and **D**) and *Rpl5*+/- mice (**F**, **H**, **G**, and **I**) with *Rps24*+/- tumor cells (**Figure S3** and **J**) and tumor cells from *Rpl5*+/- with smaller tumor (**Figure S3** and **K**) having very similar morphological appearances. In contrast, tumor cells from the *Rpl5*+/- mouse with a larger tumor (**Figure S3** and **L**) were more rounded and had clear vacuoles, lesser degree of fascicular architecture and nuclear pleomorphism. All images are at 40X magnification.

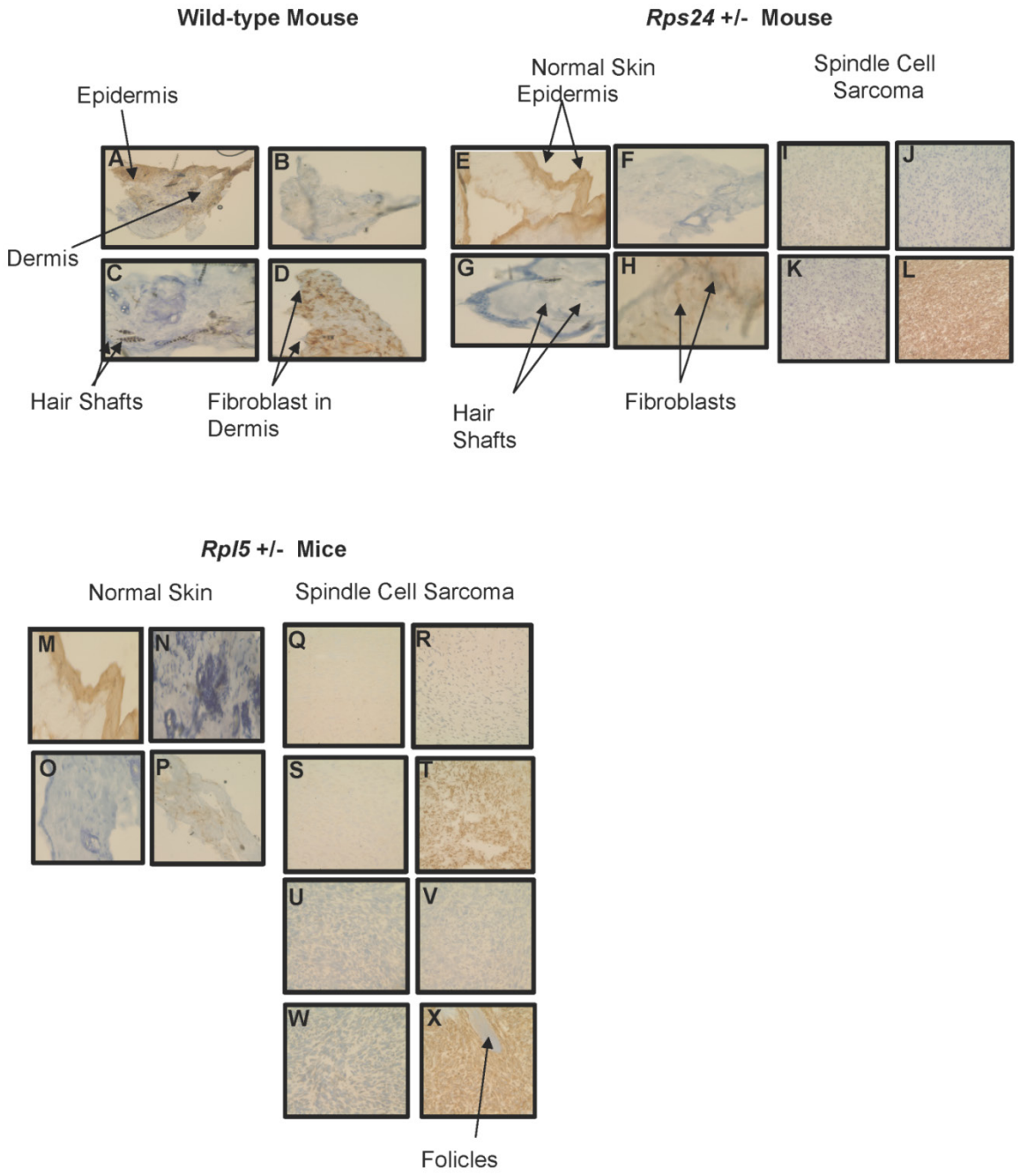

Figure 2. Immunohistochemical Comparison of Wild-type, *Rps24*+/-, and *Rpl5*+/- Normal Skin with Tumor Tissues. Pan-keratin staining was detected throughout the epidermis with no detectable staining in the dermis of wild-type (**A**), *Rps24*+/- (**E**), and *Rpl5*+/- (**M**) skin sections, and was also negative in all the tumor tissues (**I**, **Q**, and **U**). Negative staining for both LCA (**B, F, J, N, R, V**) and S100 (**C, G, K, O, S, W**) was observed in the dermis of all tissue sections. Vimentin staining throughout the dermis in wild-type (**D**) and *Rps24*+/- (**H**) normal skin tissues corresponded to fibroblasts. In contrast, all the tumor tissues stained very strongly for vimentin (**L**, **T**, and **X**). All images are taken at 40X magnification.

The proposed mechanisms for sarcoma development are either a mutation in the *p53* gene, which is considered to be a low incidence in sarcoma, or an overexpression of one of the p53 inhibitors such as MDM2 [23-26]. Recent *in vitro* and *in vivo* models of ribosomal protein deficiencies have also demonstrated the role of ribosomal proteins in regulating p53 stabilization and activity [10, 27, 28]. According to these studies, MDM2 interaction with ribosomal proteins dissociates it from p53, resulting in an increase in p53 expression and activation. To investigate if the presence of a mutation in *p53* gene or a change in p53 expression level was the potential mechanism for sarcoma formation in our mouse model, we performed DNA sequencing of the *Tp53* gene in DNA isolated from tumors and normal skin (control). However, no mutations were detected. This could be due to the low number of tumors studied in this ex-

periment or a low incidence of p53 mutations in sarcomas. Moreover, there was a similar fold change in the expression level of p53 protein in *Rpl5*+/- tumor compared with *Rpl5*+/- normal skin and wild-type skin tissues (Figure S4). In contrast, the level of p53 protein was lower in *Rps24*+/- tumors and normal skin compared with *Rpl5*+/- tumor and normal skin and wild-type skin tissues (Figure S4). As it has been reported previously, ribosomal proteins use different signaling pathways to regulate p53 expression and activity, which may account for the differences detected in the p53 expression between the *Rps24*+/- and *Rpl5*+/- tissues [19, 27, 28]. Another possibility is that these ribosomal proteins exert different functions depending on cell types and tissues. Interestingly, in their recent article, Wang et al. also reported that knock-down of *RPS24* in human colon cancer cells *in vitro* significantly decreased cell proliferation and migration and induced cell cycle arrest, which suggested the possible role of RPS24 in cell growth possibly through regulating the cell cycle [29]. Therefore, further experiments are required to investigate the effect of RPS24 and RPL5 proteins on p53 expression in our mouse models. In conclusion, even though *Rpl5*+/- and *Rps24*+/- mice did not have anemia, they became more susceptible to cancer development when compared with wild-type mice.

Supplementary Material

Supplementary methods, supplementary tables and figures. http://www.jcancer.org/v07p0032s1.pdf

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Authors' Contribution

SK and PD performed the experiments, analyzed the data, and wrote the manuscript. DY, RG, MJ, MAJ, and HZ performed the experiments and edited the manuscript. AHB and HTG edited the manuscript and advised with experiments.

Competing Interests

The authors have declared that no competing interest exists.

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