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Short Report

Successful umbilical cord blood stem cell transplantation in a patient with Rothmund–Thomson syndrome and combined immunodeficiency

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The ATP-dependent DNA helicase Q4 (RECQL4) belongs to a family of conserved RECQ helicases that are felt to be important in maintaining chromosomal integrity (Kitao et al.,1998, Genomics: 54 (3): 443–452; (6)). Deletions in the *RECQL4* gene located on chromosome 8 region q24.3 have been associated with Rothmund–Thomson syndrome (RTS, OMIM 268400), a condition characterized by poikiloderma, sparse hair, small stature, skeletal abnormalities, cataracts and an increased risk of malignancy (1–2, 5). We present a patient with a molecularly confirmed diagnosis of RTS with two unique genetic alterations in *RECQL4* (IVS16-2A>T and IVS2+27_51del25), who at the age of 7 months nearly succumbed to *Pneumocystis carinii* pneumonia. Evaluation of his immune system demonstrated a $T^{-}B^{+}NK^{-}$ phenotype with agammaglobulinemia consistent with combined immunodeficiency (CID). Studies to evaluate for known genetic causes of CID were not revealing. The patient received an umbilical cord blood (UCB) transplant with complete immune reconstitution. This report represents the first description of a CID phenotype and UCB transplantation in a patient with RTS.

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Rothmund–Thomson Syndrome (RTS) is a rare autosomal recessive genodermatosis that is associated with genetic instability and predisposition to malignancy. Patients with RTS have a wide spectrum of clinical findings including skeletal abnormalities, growth retardation, juvenile cataracts, sparse hair/eyelashes and an increased incidence of tumors, particularly osteosarcoma (1, 2). The most characteristic feature is cutaneous poilkiloderma (reticulated pigmentation, telangiectases and dermal atrophy). Diagnosis is based primarily on the clinical features. Genotyping is available to confirm a clinical diagnosis, or for prenatal diagnosis in those families with a known mutation.

Using ATP-dependent DNA helicase (RECQ) genes as candidate genes, Kitao et al showed that mutations in *RECQL4* occurred in two of the six

RTS kindreds evaluated (3). Subsequent analyses have shown *RECQL4* mutations occur in approximately 66% of patients with the RTS phenotype (4). *RECQL4* is located on chromosome 8q24.3, and its gene product is in the ATP-dependent DNA helicase family (5). RECQ DNA helicases are involved in maintenance of chromosomal integrity (6, 7) and belong to a family of conserved proteins that all share a DNA helicase motif, of which there are five such proteins in humans.

Most patients with RTS who underwent evaluation have been shown to have intact immunologic function (8). Although some authors have alluded to increased infections among certain patients with RTS and rare immunologic abnormalities (9, 10), no patients have been described with clinically significant immunodeficiency. Here, we describe a boy with molecularly confirmed RTS and a $T^-B^+NK^-$ phenotype with agammaglobulinemia consistent with combined immunodeficiency (CID). He received an unrelated umbilical cord blood (UCB) transplant following a reduced intensity preparative regimen with complete immune reconstitution and has remained free of opportunistic infections.

Case report

At 6 months of age, the proband was admitted to a local hospital with tachypnea and hypoxemia that progressed to respiratory failure. Bronchoalveolar lavage demonstrated *Pneumocystis carinii* and *Enterobacter cloacae* and was negative for viruses. *P. carinii* pneumonia (PCP) was treated with trimethoprim sulfamethoxazole and prednisone for 21 days and *E. cloacae* with ceftriaxone and gentamicin for 10 days. He responded well and was extubated on day 7 of treatment. Upon stabilization, he was transferred to our institution for evaluation of a probable immunodeficiency state.

Physical examination at presentation was notable for a small male infant with microcephaly, hypertelorism, low-set posteriorly rotated ears, bilateral absent thumbs with forearm contractures and club feet (Fig. 1a). An erythematous, macular rash was noted on his face and the extensor surfaces of the upper and lower extremities, sparing his trunk (Fig. 1b). Radiographs confirmed bilateral radial aplasia. The patient had a normal heart and kidneys on ultrasound examination.

Results

Immunologic evaluation

The admission chest radiograph revealed an absent thymic shadow. Repeated blood counts (Table 1) showed lymphopenia (638–817 cells/mm^3), and flow cytometry demonstrated decreased numbers of CD3+ T cells (76–421 cells/mm^3; normal 2400–6900 cells/mm^3) decreased CD56+ NK cells (0–32 cells/mm^3; normal 200–1200 cells/mm^3) and normal CD20+ B cells (98–211 cells/mm^3; normal 20–2300 cells/mm^3). CD4+ and CD8+ T cells were proportionately decreased. The percentages of naïve CD4+CD45RA+ T cells were decreased, 39–50% (normal 64–93%), and the percentages of memory CD4+CD45RO+ T cells was increased, 50–55% (normal 5–18%), indicative of decreased thymopoiesis. Lymphocyte proliferation studies revealed absent responses to *Candida albicans*, tetanus toxoid and alloantigens. Lymphoproliferative responses to polyclonal mitogens although markedly decreased were not absent. Natural killer cell cytotoxicity as measured by *in vitro* lysis was approximately 10% of normal controls. The patient had agammaglobulinemia, and IVIG therapy was begun. The immune phenotype was consistent with a $T^-B^+NK^-$ form of CID.

Tests to evaluate for known causes of $T^-B^+NK^-$ CID were performed. Adenosine deaminase and purine nucleoside phosphorylase red blood cell enzyme activities were normal (performed by M. Hershfield). Gene analysis for common gamma chain (Cγ) was normal (performed by J. Puck). Jak3 protein levels measured by immunoassay and FACS analysis levels were normal (performed by A. Knutsen). HIV antibody titers on both the patient and the mother were negative. Given the unusual erythematous rash in a patient with CID, maternal engraftment and Omenn syndrome were considered. Microchimerism studies demonstrated no maternal cells, while polymerase chain reaction (PCR) demonstrated a polyclonal pattern of TCR-Vβ rearrangement.

Cytogenetic studies

Cytogenetic analysis from cultured skin fibroblasts demonstrated a 46XY karyotype with a high rate of spontaneous chromosomal breaks. Testing showed a total of 22 breaks in 37 analyzed cells (0.6 breaks/cell; normal 0–0.05 breaks/cell). Sixteen cells had multiple chromosomal breakages. A control fibroblast culture set up and harvest during the same period using the same methods yielded zero breaks. None of these cultures were induced for chromosome breakage. The testing offered no evidence for the diagnosis of Fanconi anemia. The number of chromosomal breaks was not significantly enhanced by Diepoxybutane (performed by

Table 1. Immunologic studies in a child with $T–B^+NK^-$ CID and Rothmund–Thomson syndrome

	Patient		
Study	Pre-transplant	Post-transplant	Normal for age
ALC	638–817[c]	3834[a]	3800–9900
CD3, cells/mm^3	76–421	2952	1900–5900
%	10–66	77	49–76
CD4, cells/mm^3	91–297	1955	1400–4300
%	12–44	51	31–56
CD8, cells/mm^3	8–147	997	500–1700
%	1–23	26	12–24
CD20, cells/mm^3	98–211	652	20–2300
%	12–88	17	1–80
CD56, cells/mm^3	0–32	230	160–950
%	0–5	6	2–13
CD4+CD45RA+, %	39–50	48	64–93
CD4+CD45RO+, %	50–55	8	5–18
PHA, cpm	38684–85891	511414	257,076 ÷/× 2.6
%NR	9.6–21.1	163.0	≥50
Con A, cpm	2092–9533	223774	163,503 ÷/× 3.1
%NR	1.0–2.1	58.7	≥50
PWM, cpm	20116–80660	144424	116,647 ÷/× 2.9
%NR	10.9–56.1	99.8	≥50
Candida, cpm	95–1526	2914	16,846
SI	1.4–2.4	1.5	≥3
Tetanus, cpm	56–2716	170779	17,482
SI	1.2–4.2	30.3	≥3
Alloantigens, cpm	157–3816	69612	119,893 ÷/× 1.1
%NR	0.1–1.4	43.9	≥30
NK cytotoxicity, % lysis	6.3	42.8	41±3
IgG, mg/dl	<33	348[b]	399–1068
IgA, mg/dl	<7	18	15–95
IgM, mg/dl	11	72	49–202
IgE, IU/ml		<2	3–29
Anti-Hib, mcg/ml		>9.0	>1.0
Anti-Tetanus toxoid, mcg/ml		>7.0	>0.5
4		4.2	>2.0
6B		3.3	>2.0
9N		2.8	>2.0
14		12.5	>2.0
18C		6.4	>2.0
19F		12.5	>2.0
23F		12.5	>2.0

Con A, concanavalin A; MLC, mixed lymphocyte culture to B-cell alloantigens; NR, normal response; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SI, stimulation index.
Normal lymphocyte population for 6 month-old patients is expressed as the range for 5τh and 95τh percentiles (10). Normal lymphoproliferative responses are expressed as geometric mean ÷/× SE in adult control mean (14, 17). Normal immunoglobulin serum concentrations for 2-year-old patients are expressed as the range for 5τh and 95τh percentiles.
[a]Post-transplantation T-cell studies performed 11 months and [b]B-cell studies 24 months post-transplant.
[c]Pre-transplantation studies were performed at 6.7, 7.2, 7.4 and 7.9 month-old prior to UCB transplantation; studies represent range of values.

S. Olson), mitomycin C or UV radiation (performed by M. Shah). Subsequently, Nijmegen chromosome breakage was analyzed and was negative. In addition to breakages and gaps, structural rearrangements were observed in 10 metaphase cells. Three cells had either an isochromosome 7q or deletion of 7q. Two cells had Robertsonian translocations t(14;15)(q10;q10), three cells had a dicentric formation of chromosome 16, and two cells had a ring of chromosome 12 and a radial formation involving 2q and 6q.

UCB stem cell transplantation

The patient received an unrelated UCB transplant at 8 months of age. The UCB unit was a 4 of 6 HLA match to the patient with single mismatches at both HLA-A and HLA-B loci. The total nucleated and CD34+ cell dose was 1.9×10^8 and 1.2×10^6/kg, respectively. The patient received a preparative regimen consisting of cyclophosphamide (80 mg/kg), fludarabine (120 mg/M^2) and antithymocyte globulin [ATG; (150 mg/kg)]. Graft *vs* host disease

(GvHD) prophylaxis consisted of cyclosporine A and corticosteroids. Donor neutrophil and platelet engraftment occurred at day +11 and day +31, respectively. PCR-based engraftment studies demonstrated 65% donor cells at day +11 with 95% donor cells by day +21. The post-transplant course was complicated by grade IV acute GvHD that required pulse steroids (6 mg/kg/day) and Infliximab (10 mg/kg/week × 2) in addition to cyclosporine for control. The patient was discharged home on day +35 and was slowly tapered from immune suppression with no evidence of chronic GvHD.

Immune recovery post-transplant

Donor T-cell engraftment was evident at 4 and 8 months post-transplantation with measurable CD3+ T cells (518–1460 cells/mm^3) and CD56+ NK cells (78–385 cells/mm^3) in the peripheral blood. By 1 year post-transplantation (Table 1), T- and NK-cell numbers and functions were normal. Lymphoproliferative responses to mitogens, alloantigens and tetanus toxoid were normal. NK cytotoxicity measured by *in vitro* lysis was also normal. Although B cell numbers were normal at 1 year post-transplant, the patient remained hypogammaglobulinemic. IVIG infusions were continued for another 9 months, stopped and B-cell function evaluated. Although IgG and IgA levels remained below the age-appropriate range, 348 mg/dl (normal 399–1068 mg/d) and 18 mg/dl (normal 49–202 mg/dl), respectively, antibody responses to *Hemophilus influenzae* type B, tetanus toxoid and conjugated pneumoccocal vaccines were protective (Table 1).

Diagnosis of RTS

Clinical diagnosis

Although our patient had many clinical features of RTS, the CID phenotype had not been previously described as a component of this syndrome. Post-transplant the patient's rash became more prominent, failing to respond to either systemic or topical immune suppressive medications. On dermatologic evaluation, characteristic poikiloderma was noted in a photodistribution involving the malar face and distal extremities (Fig. 1). Though these characteristic cutaneous features were strongly suggestive of RTS, a skin biopsy was performed to rule out chronic GvHD and establish a clinical diagnosis.

Molecular diagnosis

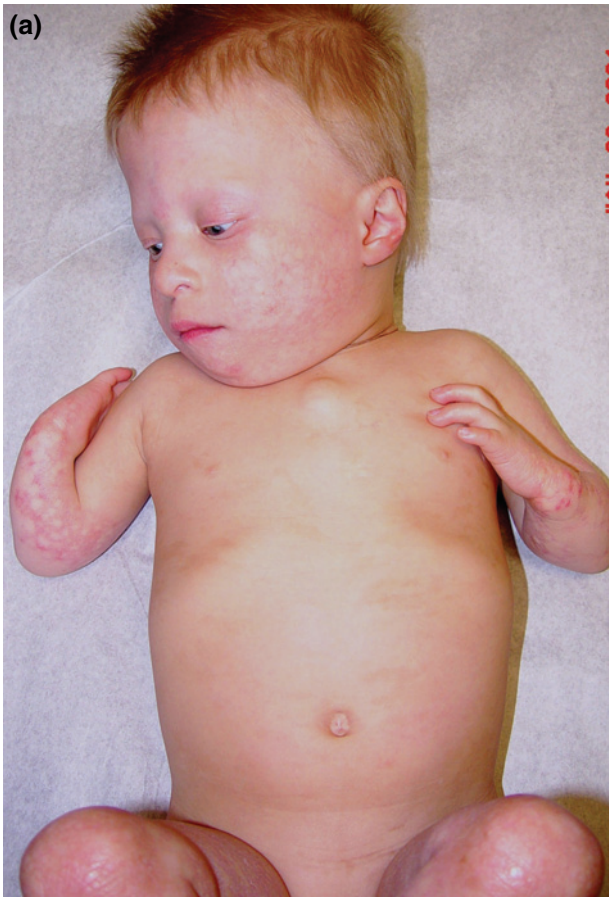
Genetic testing for RTS was conducted by PCR amplification followed by direct sequence analysis (performed by B. Roa) of the *RECQL4* gene. Sequence analysis identified a sequence variant in intron 16 of the *RECQL4* gene – an A to T change was noted at the -2 position, on the junction between intron 16 and exon 17. This is a previously unreported splicing mutation (IVS16-2A>T) (11). Nucleotides at this position are highly conserved and presumed necessary for proper mRNA processing. This particular substitution is predicted to impair normal splicing and is interpreted as a disease-causing mutation (12). Additionally, a new intronic heterozygous sequence variant was found in intron 2: IVS2+27_51del25. This 25 base pair intronic deletion has not been previously reported among patients with RTS.

To determine whether these intronic changes affected splice products of *RECQL4*, we performed RT-PCR on the proband's sample and compared with wildtype sample (performed by S. Otta). Briefly, cytoplasmic RNA was extracted from primary fibroblasts, and cDNA was synthesized using oligo(dT)priming. PCR was performed using specific primers that span the two regions of intronic mutations (sequences available upon request). In both cases, the proband produced bands that differed from wildtype, suggesting that missplicing of *RECQL4* occurred in the patient. In addition, a Western blot (performed by S. Otta) using a C-terminal antibody to *RECQL4* showed absence of a *RECQL4* protein band in the proband compared with wildtype control. (Fig. 3)

Discussion

Immunodeficiency is not a recognized feature of the RTS phenotype. Furthermore, RTS has not been reported as linked to currently known causes of CID. We have described a patient with CID and a classic RTS phenotype who experienced successful immune reconstitution following UCB transplantation.

The existence of such a strong clinical phenotype in our patient led to more refined genetic testing, particularly in light of his CID. Direct sequence analysis demonstrated a splice site mutation (IVS16-2A>T) on one allele, which to our knowledge has not been previously reported. Wang et al. (personal communication), however, have an A>G mutation at this same position. The second allele had a 25 bp deletion located within intron 2 at position 27 (IVS2+27_51del25).



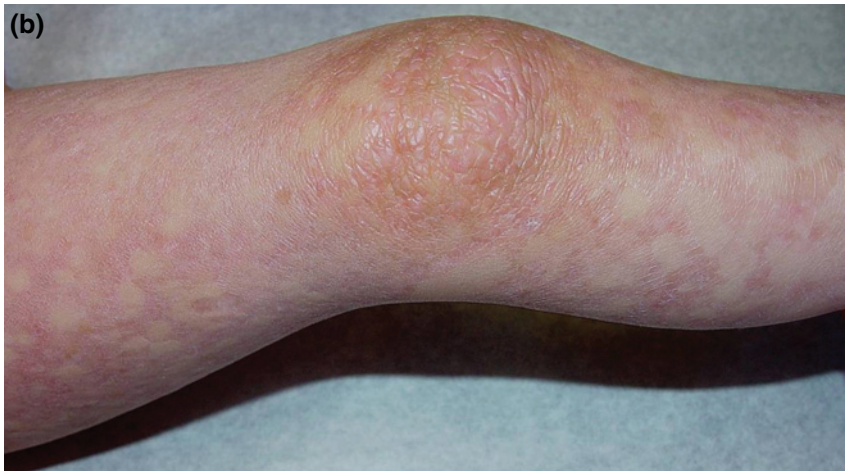


Fig. 1. Phenotypic features of Rothmund–Thomson syndrome (RTS). (a) Microcephaly, bilateral absent thumbs, forearm contractures, photodistributed poikilodermatous skin changes and linear hyperpigmented patches on the trunk, oriented along the lines of Blashchko, in a patient with RTS. (b) Magnified view of the characteristic poikiloderma with telangiectasia, atrophy, pigment change and island sparing.

Introns do not code for functional protein, and thus, genetic alterations are not usually associated with disease. However, intronic deletions and subsequent intronic-size constraints have been postulated as a mechanism for disease in RTS (13) and other diseases (14, 15). The *RECQL4* gene structure is unusual, as it contains 21 exons in only 6 kb of DNA sequence (5). Intronic deletions altering the *RECQL4* gene would likely involve those introns less than 100 bp in length. An 11-bp deletion in intron 8 (IVS8) of the *RECQL4* gene has been described in two independent RTS kindreds (13, 16). This deletion, which did not affect the 3' or 5' consensus splice sites, reduced the size of IVS8 to 66 bp and resulted in aberrant splicing compared with wildtype DNA (13). Wildtype and proband DNA were subcloned into an *in vitro* mammalian expression system, recapitulating the expression pattern seen for the endogenous genes. Restoration of wildtype splicing *in vitro* was obtained by replacing the 11 base pair deletion

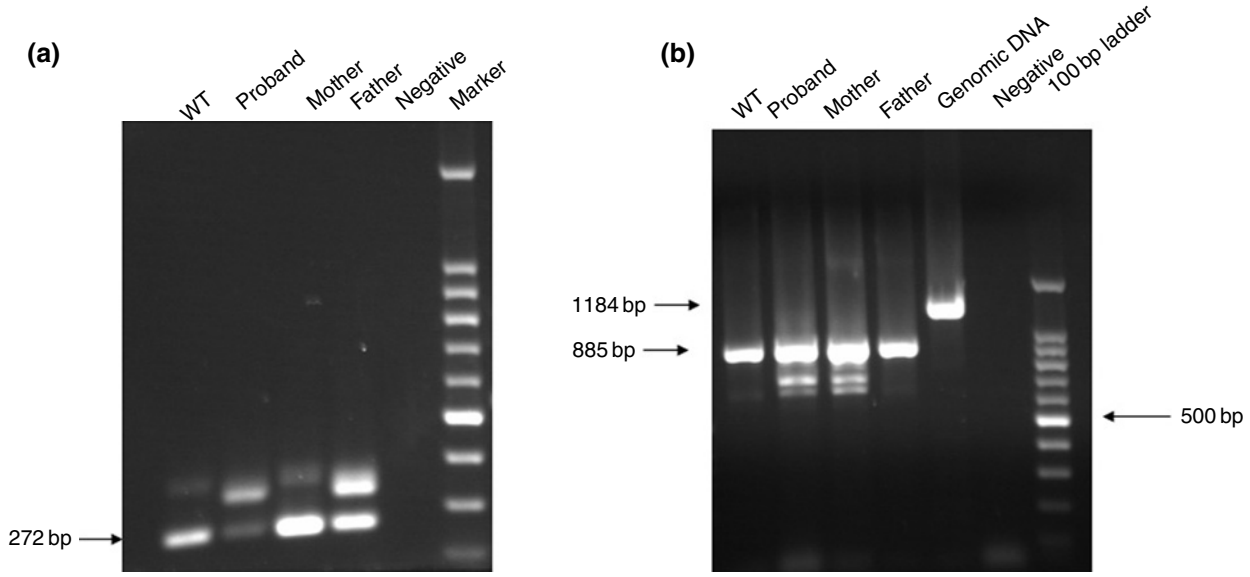


Fig. 2. Reverse-transcriptase polymerase chain reaction (RT-PCR) of intron 2 and intron 16 of the *RECQL4* gene. (a) RT-PCR of intron 2 segment using the primers: Forward – AGCCAGGACGACGTGGAG; Reverse – AGGGTGCCTTTCAG-ATTGGCCTTG. (b) RT-PCR of intron 16 segment using primers: Forward – GGGTACAGCGAGCCTTCAGG; Reverse - GTCCTTCTCCTCAGCGGTCAG.

with either wildtype or an unrelated 11 base pair sequence, leading the authors to conclude that intronic size constraint was the mutational mechanism leading to the RTS phenotype in their patient (13).

The prospect of a previously undescribed form of CID generated questions on the existence and behavior of a murine knockout (KNO) that lacked functional *RECQL4*. One successful model has been reported (18), created by in frame deletion of exon 13 – an exon with a critical role in helicase activity. Interestingly, the KNO mice (–/–) clinically and histopathologically appeared to have RTS – all of them demonstrated significant growth retardation, skin atrophy, bone dysplasia, dystrophic teeth and immunological abnormalities. The *RECQL4*-deficient mice had disproportionately smaller thymi, with a loss in the differentiation of the cortical–medullary boundary. Additionally, there was a disappearance of the white pulp areas of the spleen.

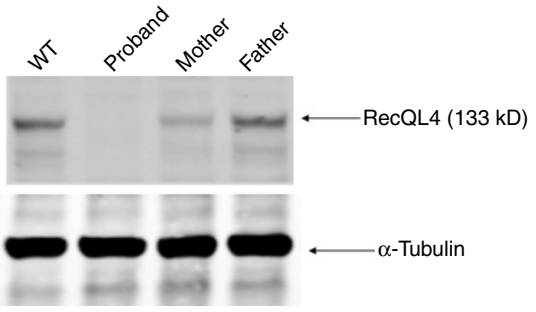


Fig. 3. RecQL4 Western Blot. Forty-five micro gram of fibroblast-derived protein was applied to 8% SDS-PAGE gel followed by transfer to nitrocellulose. Immunoblotting was performed with C-terminal *RECQL4* antibody (rabbit polyclonal) and visualized by electrochemiluminescence.

There is a great deal of phenotypic variability in patients with RTS. Genotype–phenotype analysis has shown the development of osteosarcoma in RTS is associated with truncating mutations within the *RECQL4* gene (4). Other investigators have speculated that unique structural features of the *RECQL4* gene predispose to mRNA splicing defects leading to different levels of aberrantly spliced mRNA which may account in part for the clinical variability observed in RTS patients (16). The possibility that the immune deficiency described in this child with RTS could be a result of unique *RECQL4* gene mutations is intriguing. The *RECQL4* gene product is expressed at high levels in the thymus (18, 19), a pattern consistent with a role in T-lymphocyte development. T-cell development can be affected by intrinsic T-cell defects as well as abnormalities of thymic epithelium. Given the complete immune reconstitution following hematopoietic stem cell transplant in this child, one would speculate a T-lymphocyte-specific *RECQL4* effect.

Few patients with RTS have had formal analysis of immune function, and none has been described with CID. We believe it is likely that CID is part of the phenotypic spectrum of RTS and may have resulted from unique genetic mutations in this patient. The immune system should

be thoroughly evaluated in patients with RTS to assess the frequency of immune deficiencies in this disorder and to aid in phenotype/genotype analyses. Furthermore, the diagnosis of RTS should be included in the evaluation of patients who present with CID and an unusual rash. The patient described in this report demonstrated successful immune reconstitution after umbilical cord transplantation, however, did not show any significant change in his poikiloderma or other physical features of RTS post-transplant. He has done very well post-transplant and will continue to be followed with close surveillance, particularly in light of the increased risk for the development of osteosarcoma.

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