Association Between Osteosarcoma and Deleterious Mutations in the RECQL4 Gene in Rothmund-Thomson Syndrome

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Background: Rothmund-Thomson syndrome (RTS) is an autosomal recessive disorder associated with an increased predisposition to osteosarcoma. Children with RTS typically present with a characteristic skin rash (poikiloderma), small stature, and skeletal dysplasias. Mutations in the RECQL4 gene, which encodes a RecQ DNA helicase, have been reported in a few RTS patients. We examined whether a predisposition to developing osteosarcoma among an international cohort of RTS patients was associated with a distinctive pattern of mutations in the RECQL4 gene. Methods: We obtained clinical information about and biologic samples from 33 RTS patients (age range = 1–30 years). Eleven patients were diagnosed with osteosarcoma. All 21 exons and 13 short introns of the RECQL4 gene were sequenced from the genomic DNA of all subjects. Kaplan-Meier survival analysis was used to estimate the incidence of osteosarcoma among patients with and without mutations predicted to produce a truncated RECQL4 protein. Results: Twenty-three RTS patients, including all 11 osteosarcoma patients, carried at least one of 19 truncating mutations in their RECQL4 genes. The incidence of osteosarcoma was 0.00 per year in truncating mutation-negative patients (100 person-years of observation) and 0.05 per year in truncating mutation-positive patients (230 person-years of observation) (P = .037; two-sided log-rank test). Conclusions: Mutations predicted to result in the loss of RECQL4 protein function occurred in approximately two-thirds of RTS patients and are associated with risk of osteosarcoma. Molecular diagnosis has the potential to identify those children with RTS who are at high risk of this cancer. [J Natl Cancer Inst 2003;95:669–74]

Rothmund-Thomson syndrome (RTS; Mendelian Inheritance of Man No. 268400 (1)) is an autosomal recessive disorder with a heterogeneous clinical profile that includes a characteristic skin rash (poikiloderma); small stature; sparse hair; skeletal, dental, and nail abnormalities; cataracts; and an increased risk of cancer, particularly osteosarcoma, a malignant primary bone tumor (2). The diagnosis of RTS is based primarily on the appearance and pattern of poikiloderma because no conclusive laboratory test or cellular assay for this disorder is currently available. Individuals with RTS may have few or many of the other clinical features. The original description of this syndrome by Rothmund in 1868 (3) and in other early European literature (4) emphasized the classic rash and the early onset of rapidly progressive bilateral juvenile cataracts. However, evaluation of an international cohort of 41 RTS probands revealed a different clinical profile, which included a prevalence of osteosarcoma at approximately 0.30 (2). Currently no clinical or molecular marker predicts which RTS patients will develop osteosarcoma, a malignancy that carries a substantial mortality rate despite available surgery and chemotherapy (5).

In 1999, Kitao et al. (6) used a pure candidate gene approach to show that mutations in the RECQL4 gene, which is located on human chromosome 8q24.3, occurred in two of the six RTS kindreds they examined. The RECQL4 protein belongs to the RecQ family of DNA helicases, which includes proteins encoded by genes that are disrupted in Bloom syndrome and Werner syndrome, two clinically related cancer predisposition syndromes (7). DNA helicases are enzymes that unwind DNA and are involved in many basic cellular processes; interruption of their functions may reduce genomic stability and thus contribute to tumorigenesis (8,9). No complementation or linkage studies have been reported that might indicate whether mutations in more than one gene (termed genetic heterogeneity) are responsible for RTS, and no studies of RECQL4 gene mutations in sporadic osteosarcoma have been reported. We performed comprehensive DNA sequence analysis of the RECQL4 gene from 33 RTS patients to examine the spectrum of RECQL4 mutations in RTS and to assess whether RTS patients with osteosarcoma have a distinctive pattern of mutation.

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See “Notes” following “References.”

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PATIENTS AND METHODS

Human Subjects

RTS patients were ascertained through referrals from regional, national, and international physicians and medical personnel. The patients' physicians made the initial diagnoses of RTS and osteosarcoma on clinical grounds. All patients or their parents provided informed written consent to participate in our research protocol, which was approved by the Institutional Review Board for Human Subjects Research of Baylor College of Medicine (Houston, TX). The protocol also included enrolling both affected and unaffected first- and second-degree relatives of the RTS patients whenever possible. The patients or their parents provided family history information, and a pedigree was constructed for each kindred. We obtained peripheral blood samples and skin punch biopsy samples (when feasible) from each subject. Skin biopsy samples were transported in α-modified Eagle medium (α-MEM) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Blood samples obtained in acid-citrate–dextrose collection tubes were used to establish Epstein–Barr virus-transformed lymphoblastoid cell lines (LCLs) as previously described (10). Three-millimeter dermal biopsy punch samples were manually cut into five or six pieces and cultured in α-MEM medium with 10% fetal bovine serum in 5% CO2 at 37°C to establish fibroblast cultures. Lymphoblastoid cell lines were propagated in RPMI medium (Invitrogen) with 10% fetal bovine serum. Fibroblast cultures were propagated in α-MEM with 10% fetal bovine serum. Requests for reagents should be addressed to Dr. Sharon Plon.

Genomic DNA Extraction and Amplification

We isolated genomic DNA from peripheral blood, LCLs, or primary fibroblast cell lines with the use of a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). For two of the sibling pairs, DNA was available from only one sibling in each pair. DNA from each subject was amplified by polymerase chain reaction (PCR) in seven overlapping fragments that spanned the entire RECQL4 gene (GenBank accession number AB026546) with the use of AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA) and an Advantage-GC complementary DNA (cDNA) PCR kit (Clontech, Palo Alto, CA) according to conditions adapted from Kitat et al. (6). PCR amplification was performed with the use of an Omni Gene Temperature Cycler (Hybaid, Franklin, MA). Data for each of the seven fragments including primer sequences, and conditions for PCR amplification have been deposited in GenBank under accession numbers BV005748, BV005749, BV005750, BV005751, BV005752, BV007440, and BV007442.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from LCLs and/or primary fibroblast cell lines with the use of an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse-transcribed by using oligo (dT) priming and the Superscript II reverse transcriptase supplied with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. Total cDNA was then used with the three following primer sets and an Advantage-GC cDNA PCR kit to amplify portions of the RECQL4 cDNA from healthy control subjects and from patient samples for determination of the splicing pattern: LW1 (5’-TCACAATTTCGTACCTCCCTGAG-3’) and LW6 (5’-ACCAGTGCTGCTAGGTCAG-3’); LW13 (5’-TCTGGCCTGCAAGCCCTCC-3’) and LW30 (5’-GTCCTTCTCCTCAGCGGTCAG-3’).}

DNA Sequence Analysis

Efficiency of the PCR reaction was confirmed by analysis of PCR products by gel electrophoresis on a 1% agarose gel. The products were then purified with the use of a Qiaprep PCR Purification Kit (Qiagen). Purified PCR products were sequenced by the dideoxy nucleotide termination method on automated ABI 377 and ABI 3700 DNA Sequencers (Applied Biosystems). DNA sequences were analyzed for mutations by visual inspection and by aligning the chromatograms with the use of Sequencher version 4.0 software (Gene Codes Corporation, Ann Arbor, MI). Mutations were defined by their position in the genomic sequence; e.g., g.4470G>A is the substitution of an adenine for a guanine at position 4470 in the gene. For those mutations predicted to result in a change in amino acid, this information is also provided; e.g., V799M is a substitution of a methionine for a valine at amino acid 799 (11).

Polymorphisms detected during DNA sequence analysis have been submitted to the Single Nucleotide Polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/SNP/) under accession numbers ss5607369 through ss5607380. Polymorphisms were defined as genetic changes in nonconserved portions of introns, in exons with the resulting codon predicted to encode the same amino acid, or in exons with the resulting codon predicted to encode a different amino acid (missense) if the polymorphism did not segregate with the diagnosis of RTS in the affected families or the change was found on the same allele as was a truncating mutation.

Definition of Mutant Alleles

For five kindreds that included seven affected subjects with apparently homozygous mutations in RECQL4, we confirmed by DNA sequencing or restriction analysis that one or both parents were heterozygous for the mutation. For compound heterozygous patients, we were able to assign alleles by sequencing genomic DNA from both parents in five patients, by restriction analysis in one patient, and by sequencing cloned PCR products from another patient. Products were cloned with the use of a TOPO cloning kit (Invitrogen). For three compound heterozygous patients, parental DNA was not available, and cloning of PCR products could not distinguish the alleles because the mutations were found in different parts of the gene; therefore, in these three cases, we assumed that each truncating mutation was carried on a separate allele.

DNA Restriction and Southern Blot Analyses

Genomic DNA (10 μg) was digested with DNA restriction endonucleases SplI and BamHI (New England Biolabs, Beverly, MA), separated on a 0.7% agarose gel, and transferred to Gene Screen Plus membranes (PerkinElmer Life Sciences, Boston, MA). The immobilized DNA was hybridized with a [32P]-labeled RECQL4 probe, which consisted of a 1.9-kb fragment of the RECQL4 gene amplified by PCR from genomic DNA, as described in Genbank accession number BV005749 that
spanned exons 5–10 of the RECQL4 gene. Hybridization was performed in 50% formamide, 1 M NaCl, 10% sodium dodecyl sulfate, and 10% dextran sulfate at 42 °C. Membranes were exposed to a storage phosphor screen and analyzed with a Storm 860 PhosphorImager and ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

**Statistical Analysis**

We used the Kaplan–Meier method of survival analysis and the two-sided log-rank test for equality to estimate the time to occurrence of osteosarcoma among RTS patients with and without truncating RECQL4 mutations. The time to outcome was defined as the age at which a patient was diagnosed with osteosarcoma or, for patients without osteosarcoma, the age of the patient at last contact. The dataset was thus censored.

**RESULTS**

**RTS Patients**

We studied 33 RTS patients from 29 kindreds that included four affected sibling pairs. There were 23 males and 10 females (age range = 1–30 years). On the basis of direct physical examinations or review of medical records, we classified 30 patients as “definite RTS” because they displayed within the first year of life the poikilodermatous rash that is characteristic of RTS patients (2). Three patients were classified as “probable RTS” because they displayed a rash with atypical features or had a later time of onset for RTS but still had at least two other features associated with RTS, including thumb and radius bone anomalies, loss of hair or eyelashes, cataracts, osteosarcoma, or skeletal dysplasias (2). Eleven patients were diagnosed with osteosarcoma (median age at diagnosis = 9 years, range = 4–20 years); three of those 11 patients have died from metastatic disease. Except for one patient who developed Hodgkin’s lymphoma 8 years after receiving chemotherapy for osteosarcoma, no other patient in this cohort was diagnosed with any other cancer.

**RECQL4 Gene Mutations in RTS Patients**

Comprehensive mutation analysis by direct DNA sequencing of all 21 exons and 13 short introns demonstrated that 23 of the 33 RTS patients carried at least one deleterious mutation in the RECQL4 gene; 18 patients had two mutations and five patients had one mutation (Table 1). We identified 19 different deleterious mutations that are predicted to disrupt splicing or translation and result in an absent or truncated RECQL4 protein. These mutations spanned exons 5–21, tended to cluster in the helicase domain (i.e., the region of the gene that is conserved among helicase-encoding genes), and included four of the six previously reported mutations in this gene (Fig. 1) (6,12). The mutations consisted of nine frameshift mutations, four nonsense mutations, and six mutations that were predicted to affect mRNA splicing. We used reverse transcription–polymerase chain reaction (RT–PCR) analysis of total RNA isolated from LCLs or primary fibroblasts that were established from a patient carrying each mutation to confirm that the six putative splicing mutations caused missplicing of RECQL4 messenger RNA. Two of the six splicing mutations consisted of different intronic deletions that did not involve splice site junctions but resulted in introns that were too small to splice appropriately (13). One frameshift mutation, in exon 21, is predicted to cause the loss of the last 30 amino acids of the RECQL4 protein. The functional importance of the carboxyl terminus of the RECQL4 protein is suggested by its high conservation throughout evolution.

One of the five patients with a single truncating mutation (i.e., patient FCP-114) had a nonsense mutation on the paternal allele and three different missense mutations on the maternal allele. Two of these missense mutations — g.4470G>A/V799M and g.6205C>T/P1170L — disrupt evolutionarily conserved residues in the RECQL4 protein and thus may alter RECQL4 protein function. To date, we have not identified a second mutation in the RECQL4 genes of the four other patients that had a single truncating mutation.

We detected no truncating mutations in the RECQL4 genes of 10 RTS patients. Eight of these carried the diagnosis of “definite RTS,” suggesting that the absence of truncating mutations is not due to misdiagnosis of the disorder. Five of the 10 patients without a truncating mutation carried a missense mutation in one allele, the g.454A>G/E71G mutation found in two patients and the g.4470G>A/V799M mutation found in one patient affect an amino acid that is conserved among RECQL4 proteins, the g.773C>T/P103L mutation affects a semi-conserved amino acid, and the g.5436G>A/R1021Q mutation does not affect a conserved amino acid. The functional consequences of these missense mutations are not known because a functional assay for RECQL4 activity is not available. Among these 10 RTS patients with no truncating mutation, six also had no missense mutations detected in RECQL4.

To increase the sensitivity of our mutation analysis, we examined further the biologic samples from 14 of the 15 patients that had only one or no truncating mutation. We performed Southern blotting of genomic DNA (for 12 patients) and RT–PCR analysis of RNA (for nine patients) to examine whether any of these patients had RECQL4 gene rearrangements or genetic changes that could result in cryptic splicing errors (e.g., sequence changes within an intron that were not detected by DNA sequence analysis but that could generate a splicing error). We found no evidence for RECQL4 gene rearrangements. The predicted RT–PCR products encompassing the 5‘ and 3‘ ends of the RECQL4 mRNA were detected in all nine patients tested (data not shown), suggesting that none of these patients had cryptic splicing errors.

**Genetic Heterogeneity in RTS**

The absence of detectable RECQL4 gene mutations in 10 RTS patients led us to examine whether their disease was associated with mutations in another gene(s). In one family with an affected sibling pair, we were able to test the possibility of genetic heterogeneity by genotyping several intragenic RECQL4 SNPs that we found during our sequence analysis (Fig. 2). Neither brother had osteosarcoma, but both had the skin rash that is characteristic of RTS (2), small stature, and alopecia. We analyzed the genotypes of the parents and of these brothers for these SNPs to determine the haplotypes at the RECQL4 gene for this family. This analysis demonstrated that the two affected brothers inherited the same allele from their mother but different RECQL4 alleles from their father, thus excluding RECQL4 as a recessive locus for RTS in this family and arguing strongly for genetic heterogeneity in RTS.

**Osteosarcoma and Truncating Mutations in the RECQL4 Gene**

We compared the pattern of mutations among RTS patients that had and had not been diagnosed with osteosarcoma. All 11
patients diagnosed with osteosarcoma had at least one truncating mutation in RECQL4. Nine of these osteosarcoma patients had two truncating mutations, and the other two had one truncating mutation. One of the osteosarcoma patients with a single truncating mutation also carried three missense mutations on the other allele, as described above. In the other patient with a single truncating mutation, a second mutation has not been identified to date. These findings suggest that RTS patients who carry truncating mutations in their RECQL4 genes have an increased risk of osteosarcoma. To further examine this possibility, we used Kaplan–Meier survival analysis to estimate the time to outcome (i.e., a diagnosis of osteosarcoma) among RTS patients with and without at least one truncating mutation (Fig. 3). The incidence of osteosarcoma was 0.00 per year among patients with no truncating mutations (100 person-years of observation) and 0.05 per year among patients with one or two truncating mutations (230 person-years of observation). The two-sided log-rank test for equality of osteosarcoma functions showed that this difference was statistically significant ($P = .037$). We obtained similar results when we used only one proband per family in this analy-

### Table 1. Mutation and osteosarcoma (OS) status in Rothmund–Thomson syndrome (RTS) probands

<table>
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<th>Proband</th>
<th>Sex</th>
<th>RTS status†</th>
<th>OS</th>
<th>Age at analysis, y‡</th>
<th>Allele 1 mutation§</th>
<th>Allele 2 mutations§</th>
<th>No. of truncating mutations</th>
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*FCP-xxx = Familial Cancer Predisposition (FCP) Study number; M = male; F = female; + = diagnosis of osteosarcoma; − = no diagnosis of osteosarcoma.
†RTS status D = diagnostic criteria (2) for definite RTS; RTS status P = diagnostic criteria (2) for probable RTS.
‡Denotes age at diagnosis of osteosarcoma or age when last known to be unaffected with osteosarcoma.
§Base numbers of mutations are based on genomic sequence of the RECQL4 gene (6).
∥Sibling pair.
¶Mutation disrupts splicing.
#Mutation not directly determined (DNA not available). Mutations assumed to be the same as those in affected sibling.
**Sibling pair.
††Sibling pair.
‡‡Sibling pair.

**Fig. 1.** Distribution of RECQL4 mutations detected among 29 Rothmund–Thomson syndrome kindreds as determined by polymerase chain reaction amplification and DNA sequencing of all exons and small introns. Nineteen truncating mutations (i.e., frameshift, nonsense, and deletion mutations; shown above the gene diagram) and eight missense mutations (shown below) were detected. Diamonds denote previously published mutations. Exons are indicated by numbered black and white boxes; white boxes indicate the region of the gene that is conserved among DNA helicases. Common single nucleotide polymorphisms are not indicated.
The two-sided log-rank test. The number of patients available for the analysis at 5-year intervals is indicated adjacent to each curve.

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Half-filled symbols delineate the unaffected obligate heterozygous parents. Neither sibling had detectable truncating or missense mutations in RECQL4. The brothers are discordant for RECQL4 intragenic single nucleotide polymorphisms. The base position in the RECQL4 gene for each single nucleotide polymorphism is shown with the sequence at that position for each member of the family. Boxes delineate the deduced haplotypes at RECQL4 found in this kindred. Each haplotype is indicated by a roman numeral as well as whether an open, shaded, or striped box.

Fig. 2. Evidence for genetic heterogeneity in Rothmund–Thomson syndrome. Pedigree of a kindred that includes two brothers (FCP-221 and FCP-222) with classic Rothmund–Thomson syndrome phenotype (filled squares and arrows). Half-filled symbols delineate the unaffected obligate heterozygous parents. Neither sibling had detectable truncating or missense mutations in RECQL4. The brothers are discordant for RECQL4 intragenic single nucleotide polymorphisms. The base position in the RECQL4 gene for each single nucleotide polymorphism is shown with the sequence at that position for each member of the family. Boxes delineate the deduced haplotypes at RECQL4 found in this kindred. Each haplotype is indicated by a roman numeral as well as whether an open, shaded, or striped box.

Fig. 3. Kaplan–Meier analysis of RECQL4 mutation status and time to diagnosis of osteosarcoma (OS) in Rothmund–Thomson syndrome (RTS). Incidence of OS in patients with no truncating mutations was 0.00 per year (100 person-years of observation), and the incidence of OS in patients with one or two truncating mutations was 0.05 per year (230 person-years of observation) \(* P = .037\) using the two-sided log-rank test. The number of patients available for the analysis at 5-year intervals is indicated adjacent to each curve.

Discrepancies and when we excluded patients with “probable RTS.” Thus, this analysis confirms that the presence of truncating mutations in the RECQL4 gene was associated with the development of osteosarcoma in these RTS patients.

DISCUSSION

We describe the spectrum of mutations that we detected in the RECQL4 gene in a cohort of patients with the clinical diagnosis of RTS. We found that approximately two-thirds of these patients had deleterious mutations in the RECQL4 gene that are predicted to result in truncated RECQL4 proteins. The truncating mutations were distributed across the entire gene and many were clustered around the helicase region. Mutations were detected in the coding regions, at splice-site junctions, and within the short introns of this gene. We detected no single mutation in more than five of the 29 families in our study, suggesting that unlike the Bloom and Werner syndromes, RTS is not associated with a specific founder mutation. This absence of founder mutations in our cohort is consistent with the fact that the cohort consisted of families with different ethnicities and ancestries. Our results should encourage the use of RECQL4 mutation testing, both to support the clinical diagnosis of RTS and to facilitate prenatal testing for families that have defined mutations in this gene.

Of the 10 RTS patients without truncating mutations, six patients had no detectable mutations in RECQL4, and four patients had single missense mutations of unclear functional significance. Consistent with this finding, Kitao et al. \((6)\) previously detected RECQL4 mutations in only two of six screened RTS kindreds. RECQL4 was identified by a pure candidate gene approach, and no linkage data or complementation analyses have suggested how many genes might be involved in RTS. In addition to the absence of detectable mutations in approximately one-third of the patients in our study, we found further evidence for genetic heterogeneity in one family with the classic RTS phenotype based on discordant RECQL4 intragenic SNPs. The finding of genetic heterogeneity in RTS contrasts with Bloom syndrome and, to a lesser degree, with Werner syndrome, in which nearly all affected individuals have mutations in the BLM or WRN genes, respectively. This apparent genetic heterogeneity in RTS may explain why a single cellular phenotype associated with RTS is not consistently reported in the literature. For example, some studies \((14,15)\) report that cells from RTS patients have increased sensitivity to UV or gamma irradiation, whereas other studies \((16)\) report no such sensitivity. Similarly, genetic heterogeneity may also help explain the differences in photosensitivity that are reported by different RTS patients.

On the basis of our analysis of the clinical and molecular spectra of RTS, we suggest that there may be at least two forms of this disorder: Type I RTS, which is associated with the characteristic poikiloderma but not with osteosarcoma, and Type II RTS, which is characterized by poikiloderma as well as an increased risk of osteosarcoma and deleterious mutations in RECQL4. The disease originally described by Rothmund was primarily a skin disorder that was accompanied by bilateral juvenile cataracts \((3)\). Rothmund did not report any cancers in patients with the disease nor did subsequent authors who re-described the original families from his studies many years later \((17)\). We believe that Rothmund may have described the Type I form of this disorder. Few of the case reports describing RTS associated with osteosarcoma describe European patients. Of the 10 patients without truncating mutations (and osteosarcoma) in our cohort, eight are of European Caucasian descent, which may help explain the decreased reporting of osteosarcoma in papers by European investigators \((4)\).

Our genotype/phenotype analysis shows clearly that development of osteosarcoma in RTS is associated with the presence of mutations that are predicted to result in the loss of RECQL4 protein function and, conversely, that the presence of truncating mutations in RECQL4 are associated with a high risk of developing osteosarcoma among RTS patients. However, because our cohort included predominantly children and young adults, we
cannot predict whether older individuals with RTS with or without RECQL4 mutations have an increased risk for other malignancies.

When possible, we have obtained family cancer history data on the RTS probands in our study. To date, no obvious cancer phenotype is apparent in the siblings, obligate heterozygous parents, or grandparents. However, this question needs to be formally addressed.

Although other genetic disorders (i.e., Li–Fraumeni syndrome, retinoblastoma, and Werner syndrome) have been associated with an increased risk of osteosarcoma (18–20), none has such a specific and substantial predisposition to osteosarcoma as is seen in patients with RTS. At present we do not know why RTS patients are predisposed so uniquely to osteosarcoma. RTS patients often have skeletal defects; perhaps RECQL4 plays a specific role in bone differentiation or development and/or maintenance of genomic stability in osteoblasts. As more clinical and molecular data become available, we will learn more about the biology of osteosarcoma in RTS and be able to extrapolate those findings to allow us to understand and treat osteosarcoma among the general population. Studies are currently under way to examine the role of RECQL4 mutations in sporadic osteosarcoma in the general population.

Our findings suggest the importance of informing parents of a child with RTS and RECQL4 mutations that their child has an increased risk for osteosarcoma and the need for careful surveillance. Our results also raise the hope that future surveillance and prevention trials will be designed for those young children diagnosed with RTS who have deleterious mutations in RECQL4 and are thus at high risk of developing osteosarcoma.

References

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Notes

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