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Analysis of genomic instability using multiple assays in a patient with Rothmund–Thomson syndrome

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Abstract

We report on a patient with Rothmund–Thomson syndrome (RTS) whose cytogenetic evaluation showed a normal karyotype with no evidence of trisomy mosaicism or chromosomal rearrangements. Cultured lymphocytes from the patient, her mother, and a control exposed to mitomycin C and diepoxybutane did not show increased sensitivity to the dialkylating agents. Unlike some previous reports, we found no evidence of a deficiency in nucleotide excision repair, as measured with the functional unscheduled DNA synthesis assay. Glycophorin A analysis of red blood cells for somatic mutation revealed suspiciously high frequencies of both allele loss and loss-and-duplication variants in the blood of the patient, a pattern consistent with observations in other *RecQ*-related human diseases, and evidence for clonal expansion of a mutant clone in the mother. Discrepant results in the literature may reflect true heterogeneity in the disease or the fact that a consistent set of tests has not been applied to RTS patients.

Keywords

induced chromosome breakage; sister chromatid exchange; somatic mutation; unscheduled DNA synthesis

Rothmund–Thomson syndrome (RTS) is a rare autosomal recessive disorder with poikiloderma of the face and extremities beginning in infancy. Variable features include skeletal abnormalities, hypogonadism, juvenile cataracts, alopecia, retarded physical development, and a high incidence of cutaneous and noncutaneous malignancies (1). It has been postulated that a primary defect in DNA repair is responsible for the premature aging and cancer susceptibility associated with this syndrome, although no definitive test results have been reported. Conflicting results for chromosomal instability and sensitivity to

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ultraviolet (UV)-B irradiation have been reported (1). Mutations in a newly identified *RecQ* helicase gene at 8q24.3 have recently been identified in 3 of 7 RTS families (2, 3). We report on a patient with RTS who was analyzed using a variety of methodologies to investigate evidence of genomic instability.

Materials and methods

Case report

The patient is the only child born to her 23-year-old nonconsanguineous Puerto Rican parents. She has a healthy maternal half-brother. At delivery, she was small for 42 weeks gestation, with a birth weight of 2.03 kg and a length of 44.5 cm. Bilateral absence of the radii and thumbs and an umbilical hernia were noted at birth. Chromosome analysis was 46,XX. Medical complications during infancy included severe gastroesophageal reflux, recurrent infections, chronic microcytic hypochromic anemia requiring transfusions on three different occasions, and multiple orthopedic surgeries on her extremities. At 4 months of age, she developed pigmentary changes in her skin. At 1 year of age, the patient began to develop a reticular pattern of hyperpigmentation over her elbows and legs. The poikilodermal changes were consistent with RTS. Ophthalmologic examination and renal and abdominal ultrasounds were normal.

On physical examination at 4 years and 9 months (Fig. 1), her height was 82 cm (50th percentile for 18 months) and her weight was 11.6 kg, both well below two standard deviations of the mean for her age. Her head circumference was 49 cm, at the 25th percentile for her age. She had a flat nasal bridge, normal fundi, radial ray defects, and partial 2–3 syndactyly of the toes. Her skin showed poikilodermal changes over her entire body, including the face. The patient's motor and speech development was delayed, but her social development was normal.

Cytogenetic evaluation

Human subject research was conducted under IRB protocol. Peripheral blood from the patient, her mother, and a normal control were cultured in Roswell Park Memorial Institute culture medium (RPMI) 1640 supplemented with 15% fetal bovine serum (FBS), 1% antibiotic, and 2% phytohemagglutinin for 72 h. Culture conditions included no treatment with and without 7.5 μg bromodeoxyuridine (BrdU) for 72 h, 50 ng/ml mitomycin C (MMC) for 72 h with and without BrdU (4, 5), and 0.1 μg/ml diepoxybutane (DEB) (6) for the last 48 h of culture. One hundred GTG-banded cells were analyzed for chromosome abnormalities from the untreated culture. One hundred Giemsa solid-stained metaphases were scored for chromosome breakage for each culture. Cultures treated with BrdU were stained for sister chromatid exchange (SCE) using fluorescence plus Giemsa methodology (5).

Unscheduled DNA synthesis (UDS)

Nucleotide excision repair (NER) capacity was measured using autoradiographic analysis of UDS (7). The protocol has been described previously (8) with modifications. Lymphocytes were extracted from whole blood samples by Ficoll–Paque gradient (Pharmacia, Piscataway,

NJ, USA) and placed into culture on Matrigel (Becton Dickinson, Bedford, MA, USA) in RPMI medium containing 15% FBS and antibiotics. Primary cultures of human foreskin fibroblasts (FF) were run as standards for comparison in every experiment. After 3 days in culture without passaging, cells were irradiated for 12 s with 254 nm UV light at a fluence of 1.2 J/m^2 per s, for a total dose of 14 J/m^2. Mean grains per nucleus for each experimental sample were divided by the mean grain counts for the FF as a percentage of FF activity, the accepted standard.

Glycophorin A (GPA) assay

Both patient and maternal blood samples were confirmed as heterozygous for the M/N blood type. Samples were processed as previously described (9). The frequency per million of each class of variant (V_f) was calculated as the total number of events falling within each defined region of the histogram (N/Ø and N/N, respectively) divided by the total number of cells analyzed.

Results

Among 100 banded metaphase cells from the patient, four chromatid or chromosome breaks, one trisomy 8 cell, one trisomy 11 cell, and one endoreduplicated cell were identified. Chromosome breakage and SCE were similar for patient, mother, and control for untreated and/or DEB-treated cultures, and they were similarly elevated in MMC-exposed cultures (Table 1).

The patient's and mother's lymphocytes exhibited 14.0 and 12.3% of average normal FF NER capacity, respectively. Because levels of NER vary with tissues (8), a comparison was made with the level of NER measured in lymphocytes from 33 normal individuals (9.4±7.4% FF; Fig. 2). Thus, the patient actually exhibited 150% of average normal lymphocyte NER activity, and her mother exhibited 130%, indicating no NER deficiency. The slightly lower NER capacity of the 27-year-old mother's lymphocytes is consistent with the observation that NER capacity decreases with age in normal individuals (10).

The frequency of GPA variant cells (V_f) for the patient and her mother's sample were similar to previous estimates of 'normal' V_f for the GPA assay (Fig. 3; Table 2). Several studies with the GPA assay have demonstrated an age-dependence for somatic mutation (13, 14), with frequencies of both types of variant cells significantly increasing with age. It is therefore significant that the patient's GPA V_f are both higher than her mother, who is older, and the best control in terms of sharing such factors as genetic background and environmental exposures with the patient. By comparison to pediatric controls (Table 2; Fig. 4), our patient's results are in the high normal range for both types of mutations, as well as for total mutation. In terms of cumulative rank order for the two types of mutants, the patient is the highest of all the pediatric samples tested. The flow histogram of the GPA analysis of the mother's blood sample reveals a distinct N-allele loss peak to the left of the main M/N peak containing an average of 1.46×10^{-4} variant cells, well above our threshold for an 'outlier' (Fig. 3), which occurs in ~2–5% of normal young adult individuals (11, 12, 15).

Discussion

Our finding of one trisomy 8 cell may be relevant since trisomy 8 mosaicism has been a reported finding for some patients in cultured lymphocytes and/or fibroblasts (16–19). Evidence for *in vivo* trisomy 8 mosaicism was identified in two sibs in buccal mucosa and unstimulated lymphocytes (18). Other spontaneous abnormalities seen in patients have included isochromosomes involving 2q, 7q, 8q, and 21q, as well as balanced and unbalanced translocations (16–18, 20). Our patient did not show evidence of increased spontaneous or induced chromosomal breakage with exposure to MMC or DEB, and there was no increase in SCE, either spontaneously or after treatment with MMC.

Prior published results for chromosome instability and UDS have shown variable responses (Table 3), suggesting heterogeneity for RTS. The recent discovery of mutations in the *RecQL4* gene in cell lines from some RTS patients implicates this helicase gene in the etiology of the disease (2, 3). Contradictory pieces of evidence suggest that RTS may be a heterogeneous disease, and that some cases of RTS are due to NER deficiency and that others are due to mutations in *RecQL4* (or other *RecQ*-related genes). Another possibility is that *RecQL4* is a previously undescribed component of the NER system itself; several of the known genes in the pathway code for DNA helicases, and there is a precedent for mutations in single NER genes to either affect DNA repair, producing a xeroderma pigmentosum (XP) phenotype, or to affect transcription, resulting in the Cockayne syndrome phenotype (31).

Our results with the UDS assay suggest that the patient has normal NER capacity. Historically, most reports that evaluate NER in single patients are compared to a single concurrently analyzed normal control. These data are then reported as absolute grain counts or scintillation counts, which will vary with a number of factors, including the fluence of exposure, the radioactivity of the label, the time of development, the sensitivity of the emulsion, etc. Therefore, data from one experiment cannot be directly compared with that of another, precluding the accumulation of a database of comparable assay data.

Although previous studies have utilized lymphocytes, the vast majority of diagnostic applications of the UDS assay have been performed on cultured skin fibroblasts (32), since the obvious effects of UV exposure in XP patients are in the skin. Some studies, including that by Vasseur et al. (23), have observed similar effects in both skin fibroblasts and peripheral blood lymphocytes. Not only does the skin have a higher innate capacity for NER (making it easier to detect deficiencies), but it is far more likely to be exposed to UV radiation than bone marrow or circulating lymphocytes (33). Thus, for the fairly subtle deficiencies that have been reported for this disease (residual activity 20–70% of normal), it may well be that it is too difficult to document such decreases in lymphocytes to use them as a reliable basis for diagnostic assay.

A number of syndromes associated with premature aging and/or the increased incidence of cancer have been found to have underlying deficits in DNA repair, resulting in abnormally high frequencies of somatic mutation. The GPA assay has previously been used to demonstrate characteristically high frequencies of mutations in Bloom syndrome (BS) (34,

35), Fanconi anemia (FA) (36), ataxia telangiectasia (AT) (9, 37), and Werner syndrome (WS) (38, 39).

Although the GPA V_f observed for the patient and her mother are superficially normal, they are actually unusual enough to suggest an underlying defect in DNA repair. The patient exhibits high V_f relative to her mother and a population of pediatric controls (all older than our patient) for both the allele loss and loss-and-duplication classes of variants, although they are at the high range of the normal population. The other two known human diseases associated with mutations in human analogs of the *RecQ* helicase gene are BS and WS. They both show similarly increased levels of N/Ø and N/N, although very high (~100-fold) elevations in V_f were typically observed in patients with BS (34, 35) and relatively small (~2-fold) elevations were seen in the WS population (38). Thus, whereas the actual level of mutational increase may vary, the *RecQ* helicase-related diseases may be characterized by similar patterns of increased frequencies of both allele loss and loss-and-duplication variants.

The unusual GPA-labeling flow profile in the patient's mother has been seen in sporadic unexplained outliers in normal populations (15) due to the clonal expansion of a hematopoietic stem cell carrying a single GPA mutation, as has been directly demonstrated for an *HPRT*-deficiency mutation in the lymphoid lineage (40). It is interesting to speculate that this clone may have undergone a loss of heterozygosity at the RTS locus, rendering it hypersensitive to further mutational events. This effect has been reported for BS and FA homozygotes where intragenic mitotic recombination has resulted in a population of revertant cells (41, 42).

We have presented a new case of RTS with normal levels of NER, suggesting that a deficiency of this type of DNA repair is not a causative factor in this particular patient, and that it may have subtly increased levels of somatic mutation, consistent with the pattern observed in other *RecQ*-related human diseases. With this continued evidence of possible heterogeneity within RTS, it is clear that a more standardized set of studies should be applied to individuals diagnosed with this disease, and that appropriate samples should be analyzed by experts in different aspects of the phenotype and correlated to the gene mutation in the gene or genes responsible for this disease.

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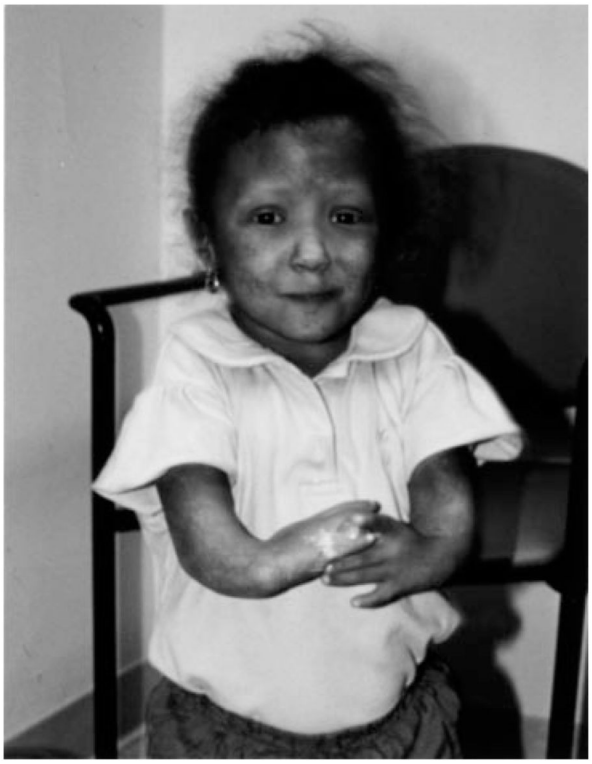

Fig. 1.
The patient at 5.25 years of age.

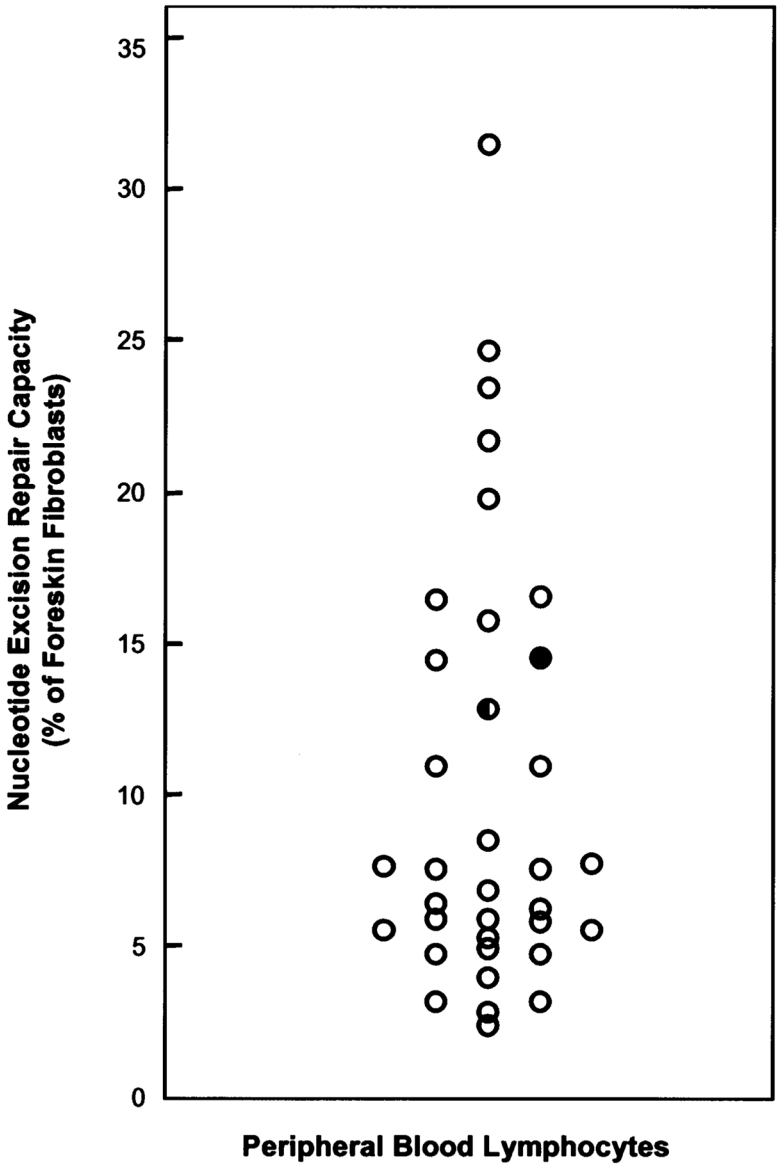

Fig. 2.

Comparison of the UDS capacities of the patient (filled symbol) and her mother (half-filled symbol) with a population of 33 normal individuals (age 32±11 years) with a range of values of 0.8–30.1% (mean 8.9±6.9%) (open symbols).

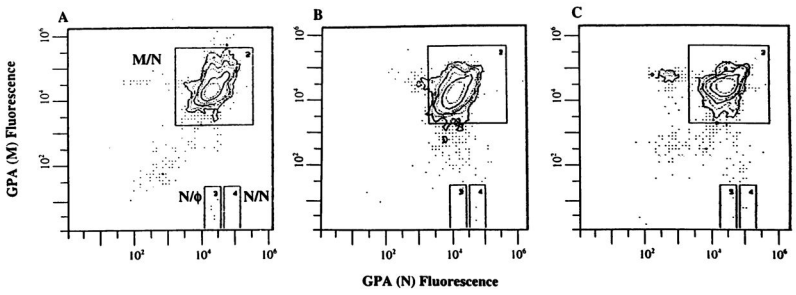

Fig. 3.

Flow distributions of labeling intensities from the analysis of 1×10^6 erythrocytes via the GPA assay from the concurrent normal control (A), the patient (B), and her mother (C). The vast majority of cells have the normal M/N phenotype and exhibit equal labeling with the M-specific and N-specific monoclonal antibodies. Directly below this main peak (at less than 1% labeling intensity) lies the N/Ø (allele loss) window, and to the immediate right, the N/N (loss-and-duplication) window (note log scales). The mother's flow profile also exhibits a distinct peak of cells to the left of the normal peak (at ~1/3 intensity) corresponding to cells that have lost expression of the N-allele.

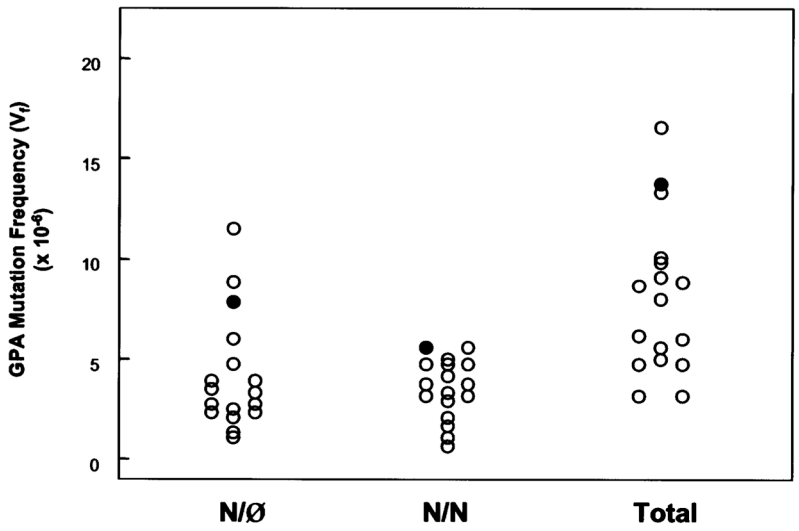

Fig. 4.

Comparison of the GPA V_f of the patient (filled symbol) with a population of 16 pediatric controls (age 11±3 years) (open symbol). From left to right, N/Ø, N/N, and total V_f (N/Ø + N/N). Our patient shares the highest N/N V_f and has the third highest N/Ø V_f for a cumulative rank of 4; the next highest individuals, the child with the highest N/Ø and the other child with the highest N/N V_f, had cumulative rankings of 5 and 7, respectively.

Table 1

Cytogenetic findings

	% Breaks			Average SCE	
	Untreated	**MMC**	**DEB**	**Untreated**	**MMC**
Control	8	26	6	9.0 (6–14)	50.5 (22–71)
Patient	4	18	7	10.1 (6–15)	44.2 (16–73)
Mother	7	21	3	8.1 (3–14)	53.0 (24–80)

Percentage of breakage indicates chromatid or chromosome breaks among 100 cells. SCE was scored among 25 cells and averaged (ranges in parentheses).

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Table 2

Frequency of somatic mutation at GPA N-allele

Individual or population	n	Mean (±SD)	N/Ø V_f ($\times 10^{-6}$) Median	Range	n	Mean±SD	N/N V_f ($\times 10^{-6}$) median	Range
RTS patient	—	8.0	—	—	—	5.8	—	—
RTS mother	—	4.6	—	—	—	3.2	—	—
Local controls	72	6.9±8.4	4.8	0.2–68.4	72	5.5±6.0	4.0	0.2–35.2
	71[a]	6.0±4.0	4.8	0.2–23.4				
Pediatric controls	16	4.2±2.7	3.3	1.4–11.6	16	3.7±1.4	3.8	1.0–5.8
Jensen *et al*., 1991 (11)	99	9.4±12.1	6.4	0.5–101.3	99	14.9±22.6	7.0	0.1–168.7
	97[b]	8.0±6.3	6.2	0.5–33.5	92[c]	10.1±8.7	6.4	0.1–37.2
Manchester *et al*., 1995 (12)	106	9.6±15.7	7.2	0.8–150.8	106	8.0±12.9	5.4	0.4–124.8
	104[d]	7.7±4.2	7.0	0.8–25.4	105[e]	6.9±5.9	5.4	0.4–34.2

[a] Excluding outlier with V_f of 68.4×10^{-6}.

[b] Excluding outliers with V_f of 52.0 and 101.3×10^{-6}.

[c] Excluding outliers with V_f of 41.8, 44.8, 47.4, 57.2, 60.2, 119.0, and 168.7×10^{-6}.

[d] Excluding outliers with V_f of 70.9 and 150.8×10^{-6}.

[e] Excluding outlier with V_f of 124.8×10^{-6}.

Table 3

Assays of genomic instability and DNA repair capacity in RTS*

	Induced DNA repair			Chromosome instability				SCE		Somatic mutation
	Ultraviolet light	**Ionizing radiation**	**Spontaneous**	**MMC**	**Diepoxybutane**	**Bleomycin**	**Ionizing radiation**	**Spontaneous**	**MMC**	**Spontaneous**
Literature	Decreased [4] (21–23)	Increased [2] (26)	Increased [1] (19) (fibroblasts)	Increased [1] (27)		Normal [2] (18)	Increased [2] (28)	Increased [1] (29)		Normal [1] (30) (*HPRT*)
	Normal [2] (24, 25)	Normal [2] (26)	Normal [4] (18, 19) (lymphocytes)	Normal [2] (18)				Normal [1] (18)		
Our patient	Normal		Normal	Normal	Normal			Normal	Normal	Normal [?] (GPA)

* Numbers of patients are in square brackets and reference numbers are in round brackets.