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ARTICLE

The mutation spectrum in *RECQL4* diseases

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Mutations in the *RECQL4* gene can lead to three clinical phenotypes with overlapping features. All these syndromes, Rothmund–Thomson (RTS), RAPADILINO and Baller–Gerold (BGS), are characterized by growth retardation and radial defects, but RAPADILINO syndrome lacks the main dermal manifestation, poikiloderma that is a hallmark feature in both RTS and BGS. It has been previously shown that RTS patients with *RECQL4* mutations are at increased risk of osteosarcoma, but the precise incidence of cancer in RAPADILINO and BGS has not been determined. Here, we report that RAPADILINO patients identified as carriers of the c.1390 + 2delT mutation (p.Ala420_Ala463del) are at increased risk to develop lymphoma or osteosarcoma (6 out of 15 patients). We also summarize all the published *RECQL4* mutations and their associated cancer cases and provide an update of 14 novel *RECQL4* mutations with accompanying clinical data.
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Introduction

Mutations in the *RECQL4* gene are known to cause three different autosomal recessive syndromes. These mutations were first found in a subgroup of Rothmund–Thomson syndrome (RTS, MIM 268400) patients[1] and subsequently in patients diagnosed with RAPADILINO syndrome (MIM 266280)[2] as well as in some Baller–Gerold syndrome (BGS, MIM 218600) patients.[3] Before this study, a total of 35 *RECQL4* mutations have been published (Table 1, Supplementary Table 1).[1–12]

In 1868, Rothmund[22] described patients with poikiloderma, growth retardation and juvenile cataracts. In 1936, Thomson[23] published a clinical description of patients with poikiloderma and growth retardation without juvenile cataracts. Later, Taylor[24] suggested that these patients may have related disorders and coined the eponym RTS. In addition to the features described above, other clinical features also include skeletal dysplasias, gastrointestinal disturbances, sparse scalp hair and sparse eyebrows or lashes.[25] Thus far, over 250 RTS patients have been reported in the literature and re-evaluation of the distinctive features of RTS has led to the creation of two subclasses. Rothmund–Thomson syndrome type I is defined by the characteristic poikiloderma and lack of *RECQL4* mutations. This group also includes patients with juvenile cataracts. Rothmund–Thomson syndrome type II patients have poikiloderma as well, but in addition they have a high risk of osteosarcoma, which seems to be related to mutations in *RECQL4*.[9] In molecular studies, which focused on patients with the clinical diagnosis of RTS *RECQL4* mutations were found in ~40–66% of RTS cases.[1,9] Although there are a significant number of RTS patients without known mutations, no other causative genes have yet been identified for RTS.

RAPADILINO syndrome was first described by Kääriäinen *et al* in 1989.[17] These patients have overlapping features with RTS patients, namely intrauterine and postnatal growth retardation and bone malformations, especially radial defects, such as hypoplasia and aplasia of thumbs and radius. However, poikilodermatous rash has never been observed in RAPADILINO patients. In addition, patients with RAPADILINO syndrome do not have alopecia or the absence of eyebrows and eyelashes, features that are usually encountered in RTS. Thus far, 15 RAPADILINO patients have been identified in Finland where RAPADILINO syndrome is overrepresented because of the enrichment of a founder mutation (c.1390 + 2delT/p.Ala420_Ala463del).[2] As only a few RAPADILINO cases have been described in other populations,[26,27] RAPADILINO syndrome is considered to be genetically homogenous.

Baller–Gerold syndrome is genetically heterogeneous, and mutations have been identified in the *RECQL4*, *FGFR2* and *TWIST* genes in patients with the BGS phenotype.[3,28,29] Baller–Gerold syndrome has overlapping clinical features with RTS and RAPADILINO, but the narrow definition of BGS is craniosynostosis with radial aplasia.[30] However, craniosynostosis has also been reported in patients diagnosed as RTS and, for instance, the London Medical Databases (www.lmdatabases.com/) list it as one of the features of both RTS and BGS syndromes. On account of the phenotypic and genotypic overlap between BGS and other syndromes the existence of BGS as a separate entity has been debated.[30,31]

RECQL4 belongs to the *RecQ* gene family of DNA helicases, other members being *RECQL1*, *BLM*, *WRN* and *RECQL5*.[32] The function of these helicases is to maintain the genomic stability that is needed in all eukaryotic organisms.[33,34] In addition, defects in the *BLM* and *WRN* genes lead to severe inherited diseases (Bloom syndrome (MIM 210900) and Werner syndrome (MIM 277700)) having overlapping features with *RECQL4* syndromes, such as cancer predisposition, growth retardation and developmental abnormalities.[35]

The strongest expression of *RECQL4* in human tissues was observed in the thymus and testis[32] whereas the most prominent expression was seen in developing bone, cartilage and intestine when studying expression in the mouse embryos (E15.5 and E18.5).[2] Three knockout mouse models were created for *Recql4* to gain new information about the phenotype and the function of the gene and protein. The first mouse model lacking exons 5–8 was embryologically lethal and thus it could not be used as a model for the *RECQL4* syndromes.[36] Hoki *et al*[37] created the second mouse model by deleting exon 13 of the *Recql4* gene and thus disrupting the helicase domain. Only 5% of *Recql4*-deficient mice survived more than 2 weeks. The mice represented several symptoms similar to human *RECQL4* diseases, such as growth retardation, developmental defects and skin abnormalities, but they did not develop any malignancies. In the third mouse model, most of the helicase domain was deleted (exons 9–13); however, 84% of the knockout mice survived until adulthood.[38] These mice displayed skin and skeleton defects as well as palatal defects all of which have been seen in the *RECQL4* patients.

The exact role of RECQL4 is unclear, but recent studies have provided some insights into its function. It has been shown that RECQL4 has a DNA strand-annealing activity and ssDNA can activate an ATPase function of RECQL4,[39] but in contrast to other RecQ helicases RECQL4 does not possess a DNA helicase activity.[39,40] A study using *Xenopus* oocyte extracts has shown that RECQL4 is crucial for the initiation of DNA replication.[41] A search for the interacting partners of RECQL4 has led to the identification of ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway, but the implication of this interaction is not yet known.[40] Burks *et al*[42] have shown that RECQL4 has a nuclear targeting signal in the *N*-terminus (amino acids 363–492), but localization studies of RECQL4 have shown both nuclear and cytoplasmic localization.[1,40,42,43] Additional localization studies in various human cells have shown that RECQL4 forms discrete nuclear foci and it

Table 1 Reported patients with *RECQL4* mutations

Patient IDs	*Syndrome*	*Exon/ intron*	*Mutation 1*	*Effect of mutation 1 on protein*	*Exon/ intron*	*Mutation 2*	*Effect of mutation 2 on protein*	*Age at onset of osteosarcoma (years)*	*Age at onset of lymphoma (years)*	*References*
A. *Patients with malignancies*										
II-3	RTS	ex10	c.1650del7	p.Ala551TyrfsX5	ex14	c.2269C>T	p.Gln757X	31	—	1, (13)
& II-6								15	—	
II-1	RTS	int7	c.1391−1G>A	Missplicing	ex9	c.1573delT	p.Cys525AlafsX33	21	—	5
& (II-2)								7	—	
FCP-102	RTS	int8	c.1483+25del11	Missplicing	int8	c.1483+25del11	Missplicing	11	—	7,9
& -102 sibling								12	—	
FCP-114	RTS	ex15	c.2547−2548delGT	p.Phe850ProfsX33	—	[a]	[a]	13	—	9
FCP-125	RTS	ex14	c.2269C>T	p.Gln757X	ex14	c.2269C>T	p.Gln757X	9	—	9, (14)
FCP-129	RTS	ex9	c.1573delT	p.Cys525AlafsX33	ex14	c.2269C>T	p.Gln757X	4	—	9, (14)
FCP-136	RTS	int11	c.1878+5G>A	Missplicing	ex15	c.2476C>T	p.Arg826X	7	—	9
FCP-153	RTS	int7	c.1391−1G>A	Missplicing	ex9	c.1573delT	p.Cys525AlafsX33	20	—	9
& -153 sibling								9	—	
FCP-191	RTS	ex15	c.2492−2493delAT	p.His831ArgfsX52	—	—	—	19	—	9
FCP-203	RTS	ex18	c.3072_3073delAG	p.Val1026AlafsX6	ex19	c.3276delG	p.Asp1093MetfsX57	3	—	9
FCP-210	RTS	ex11	c.1718delA	p.Gln573GlyX9	int11	c.1878+32del24	Missplicing	8	—	9
IV-4	RTS	int8	c.1483+27del11	Missplicing	int8	c.1483+27del11	Missplicing	14	—	4, (15,16)
& IV-5								15	—	
AS517	RTS	ex9	c.1568delG	p.Ser523ThrfsX35	ex14	c.2269C>T	p.Gln757X	12	—	12
Patient 8	RTS	ex12	c.1913T>C	p.Leu638Pro	ex14	**c.2419ins5**	**Arg807ProfsX38**	—	2	
r504	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	15	—	2
Patient 7	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	10	—	
r903	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	—	21	2
& r904								—	25	
r704	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	ex5	c.806G>A	p.Trp269X	—	24	2, (17)
Patient 6	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	ex21	**c.3599_3600delCG**	**p.Thr1200ArgfsX26**	—	33	(17)
B. *Patients without malignancies*										
AG05013	RTS	int12	c.2059−1G>T	Missplicing	ex15	c.2492−2493delAT	p.His831ArgfsX52	—	—	1
RTS1	RTS	ex9	c.1573delT	p.Cys525AlafsX33	int12	c.2059−1G>T	Missplicing	—	—	6
FCP-144 & -145	RTS	int14	c.2464−1G>C	Missplicing	int14	c.2464−1G>C	Missplicing	—	—	9
FCP-157	RTS	ex9	c.1573delT	p.Cys525AlafsX33	ex14	c.2269C>T	p.Gln757X	—	—	9
FCP-167	RTS	ex9	c.1573delT	p.Cys525AlafsX33	ex19	c.3270delG	p.Glu1090AspfsX60	—	—	9
FCP-168	RTS	ex15	c.2552delC	p.Pro851GlnfsX97	—	—	—	—	—	9
FCP-175	RTS	ex9	c.1573delT	p.Cys525AlafsX33	ex21	c.3523C>T	p.Gln1175X	—	—	9
FCP-185	RTS	ex14	c.2428C>T	p.Gln810X	ex14	c.2428C>T	p.Gln810X	—	—	9
FCP-195	RTS	ex5	c.1048_1049delAG	p.Arg350GlyfsX21	ex14	c.2269C>T	p.Gln757X	—	—	9
FCP-207	RTS	ex10	c.1704G>A	Missplicing	—	—	—	—	—	9
FCP-219	RTS	ex14	c.2207insC	p.Lys738GlnfsX71	—	—	—	—	—	9
FCP-240	RTS	ex15	c.2476C>T	p.Arg826X	ex15	c.2476C>T	p.Arg826X	—	—	9
FCP-242	RTS	ex14	c.2269C>T	p.Gln757X	ex18	c.3072_3073delAG	p.Val1026AlafsX6	—	—	9
Patient 1	RTS	ex9	c.1573delT	p.Cys525AlafsX33	ex18	c.3061C>T	p.Arg1021Trp	—	—	11
Patient 1	RTS	int2	c.118+27del25	Missplicing	int16	c.2886−2A>T	Missplicing	—	—	8
Patient 1	RTS	int10	c.1705−1G>A	Missplicing	ex12	c.1913T>C	p.Leu638Pro	—	—	10
AS518	RTS	ex9	c.1568delG	p.Ser523ThrfsX35	ex14	c.2269C>T	p.Gln757X	—	—	12
AS287	RTS	ex9	c.1568delG	p.Ser523ThrfsX35	ex16	c.2780T>G	p.Leu927Arg	—	—	12
Patient 9	RTS	ex9	c.1573delT	p.Cys525AlafsX33	int1	**c.84+6del16**	**Missplicing**	—	—	(18,19)
Patient 10	RTS	ex5	c.1048_1049delAG	p.Arg350GlyfsX21	ex14	c.2269C>T	p.Gln757X	—	—	(19)
Patient 11	RTS	ex9	c.1573delT	p.Cys525AlafsX33	ex14	**c.2461C>T**	**p.Gln821X**	—	—	
Patient 12	RTS	int7	c.1391−1G>A	Missplicing	int14	c.2464−1G>C	Missplicing	—	—	
Patient 13	RTS	ex5	c.1048_1049delAG	p.Arg350GlyfsX21	ex14	c.2398C>T	p.Gln800X	—	—	
F1, patients 1–4	BGS	ex9	c.1573delT	p.Cys525AlafsX33	ex18	**c.3061C>T**	**p.Arg1021Trp**	—	—	3, (20)
F2, patient 1	BGS	int17	c.3056−2A>C	Missplicing	int17	c.3056−2A>C	Missplicing	—	—	3, (21)
Patient 14	BGS	ex14	**c.2335del22**	**p.Asp779CysfsX57**	ex14	**c.2335del22**	**p.Asp779CysfsX57**	—	—	
Patient 15 & 16	BGS	ex5	**c.496C>T**	**p.Gln166X**	ex18	**c.3151A>G**	**p.Ile1051Val**	—	—	
r104	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	—	—	2
r203	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	—	—	2
r605 & r606	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	—	—	2
r805	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	—	—	2
r1003	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	int7	c.1390+2delT	p.Ala420_Ala463del	—	—	2
r303 & r304	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	ex19	c.3271C>T	p.Gln1091X	—	—	2, (17)
r405	RAPA	int7	c.1390+2delT	p.Ala420_Ala463del	ex18	c.3214A>T	p.Arg1072X	—	—	2
Patient 1	RAPA	ex9	c.1573delT	p.Cys525AlafsX33	ex13	**c.2091T>G**	**p.Phe697Leu**	—	—	
Patient 2	RAPA	ex12	**c.1910T>C**	**p.Phe637Ser**	ex15	c.2476C>T	p.Arg826X	—	—	
Patient 3	RAPA	ex12	**c.1885del4**	**p.Arg629SerfsX60**	ex14	c.2269C>T	p.Gln757X	—	—	
Patient 4	RAPA	int12	c.2059−1G>A	Missplicing	ex18	**c.3072delA**	**p.Val1026CysfsX18**	—	—	
Patient 5	RAPA	ex8	**c.1397C>T**	p.Pro466Leu	ex12	**c.1887del4**	**p.Glu630AlafsX59**	—	—	

Only patients having at least one deleterious mutation are shown. IDs of the patient studied in this project have been marked in bold as well as novel mutations. Sibling pairs have been marked with &. AS517 and AS518 are also siblings. References without parentheses are the ones in which mutations have been indicated and references in parentheses give additional information about the patients such as clinical descriptions. Patient r704 is patient 2, r303 and r304 are patients 3 and 4 and r203 is patient 5 in Kääriäinen *et al*, 1989.[17] Patient 6 in this study is patient 1 in Kääriäinen *et al*, 1989[17] publication. Patients FCP-129 and FCP-125 in Wang *et al*, 2003[9] are very likely patients 1 and 2 (respectively) in Pujol *et al*, 2000[14] report.

[a]This patient has three amino acid substitutions (p.Arg522Cys, p.Val799Met, p.Pro1170Leu), but none of them was proven to be the pathogenic change. Both p.Arg522Cys and p.Val799Met are found in the SNP database as rs35407712 and rs34293591, respectively. The third change is not found in the SNP database.

Table 2 Clinical data from 16 patients with the *RECQL4* mutations

Clinical features	*1 RAPA*	*2 RAPA*	*3 RAPA*	*4 RAPA*	*5 RAPA*	*6 RAPA*	*7 RAPA*	*8 RTS*	*9 RTS*	*10 RTS*	*11 RTS*	*12 RTS*	*13 RTS*	*14 BGS*	*15 BGS*	*16 BGS*
Short stature >-2SD	+	+	+	+	+	+	+	+	+	+	+	−	+	+	NA	NA
Dermatological changes																
Poikiloderma	−	−	−	−	−	−	−	+[a]	+	+	+	+	+	+	NA	NA
Brownish spots	−	−	+	+	−	+	+	−	−	−	−	+	−	−	NA	NA
Alopecia, loss of eyebrows or eyelashes	−	−	−	−	−	−	−	+	−	−	−	+	+	+	NA	NA
Skeletal abnormalities																
Thumb a-/hypoplasia	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+
Radial a-/hypoplasia	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+
Patellar a-/hypoplasia	+	+	+	+	−	+	−	+	+	+	+	−	+	−	NA	NA
High arched/cleft palate	+	−	+	+	−	+	−	+	+	+	+	−	+	−	NA	−
Joint dislocations	+	−	+	−	−	−	−	+	−	−	−	+	+	+	NA	−
Osteopenia/osteoporosis	−	−	−	−	−	−	−	−	+	+	−	+	−	+	NA	NA
Craniosynostosis	−	−	−	−	−	−	−	−	−	−	−	−	+	+	NA	+
Malignancies																
Osteosarcoma	−	−	−	−	−	−	+	−	−	−	−	−	−	−	NA	NA
Lymphoma	−	−	−	−	−	+	−	+	−	−	−	−	−	−	NA	NA
Diarrhea	+	+	+	+	+	+	+	+	+	+	+	+	−	−	NA	NA
Feeding problems	−	+	+	−	+	−	−	+	+	+	+	+	+	+	NA	NA
Hearing problems, hearing loss	+	−	−	−	−	−	−	−	+	+	−	−	−	+	NA	NA
Gender	M	M	F	M	F	F	M	M	M	F	F	M	F	F	F	F
Age in January 2008 (years)	23	5	5[b]	28	1	35	10	[c]	12	13	11	14	4	3	[d]	[e]

[a]Atypical poikiloderma, the age of onset 2.5 years.
[b]Patient lost for follow-up at the age of 5 years.
[c]Patient died at the age of 3.5 years.
[d]Pregnancy terminated at 11.5 weeks of gestation.
[e]Pregnancy terminated at 23 weeks of gestation.
Brownish spots refer to the brown pigmentation that resembles irregularly shaped café-au-lait spots. This does not include hyperpigmentation seen in poikiloderma.

colocalizes with promyelotic leukemia protein (PML) nuclear bodies as well as with regions of ssDNA. RECQL4 also forms a complex with Rad51 and colocalizes with it in human cells after the induction of DNA double-strand breaks.[43]

The aim of this study was to identify novel *RECQL4* mutations in patients with clinically suspected RTS, RAPADILINO or BGS and to collect and analyze the precise clinical data from the patients with the identified mutations. We also updated the current cancer status of RAPADILINO patients and observed that both lymphoma and osteosarcoma had been diagnosed among RAPADILINO patients again confirming the association between *RECQL4* mutations and cancer risk.

Materials and methods

Subjects, samples and clinical data

This study (collection of samples, the *RECQL4* gene analysis and evaluation of medical records) was approved by the Ethical Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa, Finland. As the phenotype of patients carrying *RECQL4* mutations can be quite variable, we accepted for the study DNA samples from all patients who were suspected to have a clinical diagnosis of RTS, RAPADILINO or BGS. After obtaining the patients' consent, the referring clinicians sent us DNA samples from a total of 35 patients from several different populations. More thorough clinical data were requested only from patients who were found to have *RECQL4* mutations. Clinical data were collected from the medical records of the patients by clinicians who filled out a uniform medical questionnaire documenting the presence of features commonly associated with RTS, RAPADILINO and BGS. These features are listed in Table 2. In addition, we reviewed medical records from 15 Finnish RAPADILINO patients to update their cancer status.

Samples and mutation analysis

The whole *RECQL4* gene was sequenced including all exons and exon–intron boundaries as well as all introns except intron 12 (primer sequences are available upon request).[2] Samples from patients 9 and 10 were sequenced as described by Wang *et al*.[9] The allelic segregation of the mutations was confirmed from both parental samples in all cases except in two families. In the case of patient

8 samples were available only from the patient and his mother. In the case of patient 12, parental samples were not available.

Mutation nomenclature

Mutation positions are given according to the Reference sequence for *RECQL4* (NM_004260). Numbering starts from nucleotide 33, which is the A of the ATG-translation initiation codon.

In silico analyses

Sequences for protein sequence comparisons were retrieved from NCBI (www.ncbi.nlm.nih.gov/) and aligned using the ClustalW program (align.genome.jp/). Protein sequence entities for different species were NP_004251.2 *Homo sapiens*, XP_520023.2 *Pan troglodytes*, NP_478121.2 *Mus musculus*, XP_216973.4 *Rattus norvegicus*, XP_539222.2 *Canis familiaris*, NP_001091506.1 *Bos taurus*, XP_427538.2 *Gallus gallus*, NP_001089101.1 *Xenopus laevis*, NP_652607.1 *Drosophila melanogaster*, XP_315948.4 *Anopheles gambiae*, NP_001053140.1 *Oryza sativa* and NP_174109.2 *Arabidopsis thaliana*.

The effects of the amino acid substitutions were predicted using the PolyPhen (coot.embl.de/PolyPhen/) and SIFT (blocks.fhcrc.org/sift/SIFT.html) programs. Reference sequence NP_004251.2 (gi116812616) was used for human RECQL4.

Results

RECQL4 mutations

On the basis of earlier publications, 35 different mutations in the *RECQL4* gene have been identified. In this study, a molecular change in both alleles of the *RECQL4* gene was found in 16 out of 35 patients analyzed (46%), and 14 of these mutations were novel. All reported patients with deleterious *RECQL4* mutations are presented in Table 1. The Supplementary Table 1 shows all the identified mutations in a structural order from the 5′ end to the 3′ end of *RECQL4* as well as the incidence of each mutation.

Nine of the novel mutations caused an early stop codon or a frameshift, both leading to truncated polypeptides: c.496C>T (p.Gln166X), c.1885del4 (p.Arg629SerfsX60), c.1887del4 (p.Glu630AlafsX59), c.2335del22 (p.Asp779CysfsX57), c.2398C>T (p.Gln800X), c.2419ins5 (Arg807-ProfsX38), c.2461C>T (p.Gln821X), c.3072delA (p.Val626-CysfsX18) and c.3599_3600delCG (p.Thr1200ArgfsX26). Four mutations caused novel amino acid changes: c.1397C>T (p.Pro466Leu), c.1910T>C (p.Phe637Ser), c.2091T>G (p.Phe697Leu) and c.3151A>G (p.Ile1051Val). One of the identified mutations was a 16-base pair deletion in intron 1 (c.84+6del16). As previously described, *RECQL4* has an unusual genomic structure with 13 out of 20 introns being less than 100 bp in length. This intronic deletion results in an intron of 49 bp in length that is probably too small for correct splicing.[4,7–9]

From the sequenced samples, we found a total of 10 amino acid substitutions. Two of these, p.Glu267Asp and p.Arg1005Gln, were frequently detected and they are also reported as common variants in NCBI's SNP database, with their accession numbers and allele frequencies in parentheses: rs4244612 (0.461±0.134) and rs4251691 (0.423±0.181), respectively. Patient 1 had two amino acid substitutions, p.Glu71Gly and p.Phe697Leu, on the same allele. As p.Glu71Gly has also been found in an earlier RTS study from patients with no other mutations in *RECQL4*[9] and from NCBI's SNP database (rs34642881) with allele frequency 0.067±0.170, it could also represent a polymorphism. However, this does not rule out the possibility that the combined effect of these two mutations could be pathogenic. A similar situation was observed in patient 5 who had the p.Arg522His and p.Pro466Leu substitutions in the same allele. p.Arg522His has been reported in NCBI's SNP database (rs35842750) and both heterozygotes and homozygotes were identified in the population studies, thus suggesting this change to be a common variant. Interestingly, patients 1, 9 and 11 with the p.Cys525AlafsX33 mutation had the amino acid substitution p.Ser523Thr in the same allele. This amino acid substitution is not found in the SNP database and may be specifically linked to the p.Cys525AlafsX33 mutation or represent a rare haplotype.[3,5,6]

It is difficult to interpret the effects of the amino acid substitutions because there is no crystallographic model for RECQL4 and the exact physiological role of this protein is unknown. However, bioinformatics tool can be used to predict the significance of the amino acid substitution on the protein. We performed PolyPhen and SIFT analyses for the four novel amino acid substitutions, for the p.Glu71Gly and p.Arg522His changes and for the six previously published amino acid substitutions. In addition, we aligned 12 RECQL4 orthologs from different species to determine conserved amino acids. The results from these analyses are presented in Supplementary Table 2. PolyPhen and SIFT results were indicative, but sometimes conflicting as in the case of p.Pro466Leu and p.Phe697Leu. PolyPhen predicts these changes to be probably damaging in contrast to SIFT's prediction that the changes will be tolerated. These amino acids were well conserved among 12 species. The p.Phe638Pro change found in our and in a previous study[10] is predicted to be damaging by both PolyPhen and SIFT and it is also evolutionarily conserved in all studied species except *Anopheles*. Interestingly, the novel p.Phe637Ser change in an adjacent amino acid found from a RAPADILINO patient is less conserved among species, but yet it is predicted to affect protein function. The p.Ile1051Val substitution is not predicted to affect protein function, even though it is conserved in all six mammals.

It is very likely that at least p.Pro466Leu, p.Phe637Ser and p.Phe697Leu found in this study are the second

pathogenic mutations in these patients even though the possibility of a promoter region mutation that could lead to loss of translation of a specific allele can not be excluded. The effect of p.Ile1051Val remains unsure; however, it was the only change found from siblings 15 and 16 in addition to the p.Gln166X mutation that is clearly pathogenic. This change was not found in SNP database, but further studies will be needed to conclude whether it is pathogenic or a rare benign variant.

Analysis of phenotypes associated with *RECQL4* mutations

Detailed clinical data were collected from 16 patients having *RECQL4* mutations (Table 2). We were interested in clinical features that are frequently described in the literature regarding RTS, RAPADILINO and BGS patients. Short stature was a typical feature for 13 of 14 patients. When evaluating dermatological features 6 out of 14 patients had typical poikiloderma and one patient had atypical poikiloderma. One of these RTS patients also had brownish spots, which were also described in four RAPADILINO patients. Alopecia and loss of eyebrows or eyelashes was found in only four RTS patients. Thumb and radial a-/hypoplasias were diagnosed in 14 out of 16 patients, thus making it the most common feature in this cohort in addition to short stature. Diarrhea was reported in 12 out of 14 patients, but other features were found irregularly. In conclusion, the patients had a minimum of four findings, but none of them actually had all of the features listed in Table 2.

Cancer status

As RTSII patients with *RECQL4* mutations are known to be particularly susceptible to osteosarcoma, we wanted to determine the cancer status among the RAPADILINO patients. On the basis of the previous study of RAPADILINO patients, we knew that patient r504 had osteosarcoma in her teens and that patient r903 had lymphoma in her early twenties.[2] For this study, we collected medical records from all 15 Finnish RAPADILINO patients. Strikingly, we identified one additional osteosarcoma and three new lymphoma cases among these patients. Patient r704 and patient r903's sibling r904 developed the lymphoma in their twenties, and patient 6 in her thirties. Patient 7 developed osteosarcoma at the age of 10 years (Table 1A). Thus, out of 15 Finnish RAPADILINO patients there have been two diagnoses of osteosarcoma and four of lymphoma making the cancer incidence very high among Finnish RAPADILINO patients (40%).

Cancer status was also obtained for the other patients with *RECQL4* mutations in this study. One RTS patient developed lymphoma at the age of 2 years and died of it at the age of 3.5 years (patient 8). This patient had been diagnosed with RTS as he had poikiloderma even though it was atypical. Interestingly, none of the RTS patients in this study had developed osteosarcoma in contrast to the osteosarcoma incidence that was as high as 48% in the most extensive study evaluating cancer status among RTS patients with the *RECQL4* mutations.[9]

Discussion

Mutation screening is a powerful diagnostic tool in syndromes where phenotypic variation is wide, such as the *RECQL4* associated syndromes. At the moment, 64 patients with two *RECQL4* mutations have been identified and in addition, in four patients only one deleterious mutation is known (Table 1). When reviewing all the single mutations identified in *RECQL4* syndromes it can be concluded that the majority of mutations has been found in only one patient, but there are three mutations that are more prevalent (Supplementary Table 1). The most common *RECQL4* mutation is c.1390+2delT (p.Ala420_Ala463del) which is enriched in the isolated Finnish population. All the Finnish RAPADILINO patients are at least compound heterozygotes for this mutation and therefore have at least one gene copy that encodes a RECQL4 protein from which 44 amino acids are missing. In addition, the c.1573delT mutation (p.Cys525AlafsX33) has been found in a total of 12 alleles and interestingly from patients with all three syndromes. The c.2269C>T (p.Gln757X) mutation has been found in 10 alleles and from RTS and RAPADILINO patients. *RECQL4* mutations are typically predicted to be truncating caused by either an early stop codon, missplicing or a frameshift. Over half of these mutations (Supplementary Table 1) are predicted to destroy the reading frame before or in the helicase domain (encoded by exons 8–14) that is thought to be critical for the function of RECQL4 even if the DNA helicase activity of RECQL4 has not been shown.[39,40] The four amino acid substitutions located in the helicase domain may also disturb the functioning of the protein.

There is no clear genotype–phenotype correlation when comparing the phenotype conveyed by specific mutations. Truncating mutations in both alleles usually strongly suggest RTSII or BGS; however, a few RAPADILINO patients have two truncating mutations as well. In addition, amino acid substitutions have been found in patients with all three syndromes (Supplementary Table 1).

On the basis of this and previous studies, we conclude that clinical features of patients with *RECQL4* mutations can be quite variable. However, it seems that approximately 85% of the patients have short stature and skeletal abnormalities, such as thumb, radial and/or patellar a-/hypoplasias. This is complicated by the fact that there is clinical variability even between siblings who carry the same mutations. When evaluating the symptoms of three brother–sister sibling pairs with RAPADILINO it was noted that the clinical picture of the brothers was

significantly milder than their sisters' and it would have been difficult to suspect the RAPADILINO diagnosis without the sister with typical features.[2]

When evaluating differences among *RECQL4* syndromes it seems that a poikilodermatous rash is a distinguishing feature between RTS and RAPADILINO. Thorough examination of the skin is important as the onset and distribution of poikiloderma can be atypical. Usually, poikiloderma in RTS appears at the age of 3–6 months and starts spreading from the cheeks to extremities usually sparing the trunk and abdomen. If the patient develops poikiloderma at an early age and with typical pattern of spread, this fulfills the criteria for a diagnosis of RTS.[25] If the patient has *RECQL4* mutations, but no evidence of poikiloderma, the diagnosis is more likely RAPADILINO syndrome. As seen in Table 1 most patients (63%) have the RTS diagnosis whereas approximately 30% of cases are RAPADILINO patients and fewer than 10% of cases have BGS.

From the reported patients with the *RECQL4* mutations 37% have developed malignancies (Table 1). Interestingly, in six out of seven sibling pairs both siblings have developed malignancies thus suggesting that genetic background has a high impact on cancer risk. Osteosarcomas are typical for RTS patients with *RECQL4* mutations, whereas emphasized here RAPADILINO patients are at risk for both lymphomas and osteosarcomas. Although the number of patients is small, given the low incidence of osteosarcoma and lymphoma in the general population the finding of two cases of osteosarcoma and four cases of lymphoma in 15 patients demonstrate a clear susceptibility to these malignancies. On the basis of the existing data from the function of RECQL4 it is not possible to explain why the Finnish RAPADILINO patients are susceptible to developing both lymphoma and osteosarcoma. However, there may be a connection between the cancer and an abnormal localization of the RECQL4 protein encoded by the most common *RECQL4* mutation (c.1390 + 2delT/p.Ala420_Ala463del). On account of the mutation, the domain that is needed for a nuclear retention of RECQL4 is missing and probably because of this the localization of defective RECQL4 is cytoplasmic.[42] It is also possible that other genetic loci may modify cancer risk, but these questions remain open.

Interestingly, among the 100 knockout mice (lacking exons 9–13) five developed cancers of which three were lymphomas and two were osteosarcomas. In addition, the $Recql4^{-/-}$, $Apc^{Min/+}$ mice had a two-fold increase in the multiplicity of macroadenomas locating in the GI tract and large intestine and macroadenomas were also larger in size.[38] Additional analyses of these mice might shed light on cancers developed in human *RECQL4* defective patients.

In conclusion, the identification of *RECQL4* mutations is significant as it clarifies the risk of the recurrence in the family and reveals the increased cancer risk. The parents of patients with *RECQL4* mutations need to be advised to pursue counseling and regular follow-up sessions for their children. It is very important to note that the follow-up needs to be long-term as the age at onset of cancer can be very variable being from 2 to 33 years among the reported patients with *RECQL4* mutations (Table 1). Clinicians should be aware of both osteosarcoma and lymphoma risk when following patients with the *RECQL4* mutations and counsel their patients accordingly until more experience accumulates.

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