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SPRED1 mutations (Legius syndrome): another clinically useful genotype for dissecting the NF1 phenotype

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Abstract

Objective: Mutations of the *SPRED1* gene, one of a family of Sprouty (Spry)/Spred proteins known to 'down-regulate' mitogen-activated protein kinase (MAPK) signalling, have been identified in patients with a mild neurofibromatosis type1 phenotype **with** pigmentary changes but no neurofibromas (**Legius syndrome**). To ascertain the frequency of *SPRED1* mutations as a cause of this phenotype and to investigate whether other *SPRED/SPRY* genes may be causal, a panel of unrelated mild NF1 patients were screened for mutations of the *SPRED1-3* and the *SPRY1-4* genes

Methods: 85 patients with a mild NF1 phenotype were screened for *SPRED1* mutations. 44 patients negative for both *NF1* and *SPRED1* mutations were then screened for *SPRED2-3* and *SPRY1-4* mutations. Complexity analysis was applied to analyse the flanking sequences surrounding the identified *SPRED1* mutations for the presence of direct and inverted repeats or symmetric sequence elements in order to infer probable mutational mechanism.

Results: *SPRED1* mutations were identified in six cases, 5 were novel and included 3 nonsense (R16X, E73X, R262X), 2 frameshift (c.1048_c1049 delGG, c.1149_1152 del 4bp), and a single missense mutation (V44D). Short direct or inverted repeats detected immediately adjacent to some *SPRED1* mutations may have led to the formation of the microdeletions and base pair substitutions.

Discussion: The identification of *SPRED1* gene mutation in NF1-like patients has major implications for counselling NF1 families.

Key words

NF1, *SPRED1*, *SPROUTY*, *SPRY1*, Mutations

Introduction

Neurofibromatosis type 1 (NF1 [MIM 162200]), is a common autosomal dominantly-inherited complex disorder, with a 1/2500 birth incidence and a 1/5000 prevalence worldwide.¹ This disorder exhibits a high mutation rate, with almost half of all NF1 patients being *de novo* cases of the disease. The cardinal clinical features of NF1 involve pigmentary changes (café-au-lait spots and skinfold freckling), the development of cutaneous neurofibromas and of hamartomas of the iris (Lisch nodules).^{1, 2} Neurofibromas are outgrowths of the peripheral nerve sheath, and the majority of adult NF1 patients will have multiple cutaneous neurofibromas. Much larger plexiform tumours are also characteristic of this disorder. Neurofibromatosis type 1 is caused by inactivating mutations of the 17q11.2-located *NF1* gene that encodes neurofibromin that functions by tightly controlling cellular levels of active Ras proteins. Any inactivation of neurofibromin therefore leads to prolonged activation of functional Ras proteins, resulting in unregulated increases in cell growth, proliferation and an increased risk of malignancy, identifying *NF1* as a tumour suppressor gene.² The *NF1* gene therefore represents one of the key regulatory points in the complex RAS-MAP kinase signalling pathway in the cell.^{3, 4}

A small but significant proportion of patients with the NF1 pigmentary phenotype never develop neurofibromas and have been classified as ‘Autosomal dominant café au lait spots only’ on clinical grounds. The problem is that this diagnosis can only be confidently made in families with two or more generations where the adults have **no** neurofibromas. We previously reported the same c.2970-2972 delAAT (p.990delM) mutation in exon 17 of the *NF1* gene in 21 unrelated probands with this phenotype.⁵ Recently, *SPRED1* gene mutations have been reported with the same clinical picture.⁶ The situation is further complicated in that some of these patients also had learning problems and macrocephaly and so in childhood the phenotype is indistinguishable from NF1. The Spred1 protein is a member of the Sprouty (SPRY) family of proteins that function to inhibit the growth factor mediated MAP-kinase pathway signalling by interaction with both Ras and Raf1 proteins.^{7, 8} The Spred1 protein contains several distinct domains that enable different Spreds to specifically inhibit the MAPK pathway. Spred specifically binds to RhoA, Ras, Raf1 and Caveolin-1, thus preventing their activation and suppressing the Ras/MAPK pathway.⁹

Brems et al⁶ identified 11 different *SPRED1* mutations in 91 unrelated patients with NF1 pigmentary phenotype. **We have screened for *SPRED1* mutations in** 85 unrelated, *NF1* gene mutation-negative individuals, none of whom had cutaneous neurofibromas. Six *SPRED1* germline mutations were identified of which five were novel changes in these patients. Forty-four of these patients, with neither a *SPRED1* nor *NF1* mutation, were further screened for mutations in six biologically-related genes (*SPRED2*, *SPRED3*, *SPRY1*, *SPRY2*, *SPRY3* and *SPRY4*).

Material and methods

Patient samples

Eighty-five unrelated patients referred for *NF1* gene testing and found to be *NF1* mutation-negative and who did not have cutaneous neurofibromas, were screened for *SPRED1* mutations. **Limited amounts of DNA meant that only 44 patients found to be without either an *NF1* or a *SPRED1* mutation were subsequently** screened for the mutations in six other genes (*SPRED2*, *SPRED3*, *SPRY1*, *SPRY2*, *SPRY3* and *SPRY4*).⁹

Lymphocyte DNA was extracted and *SPRED1* PCR carried out using the exon-specific primer sequences reported by Brems et al, 2007.⁶ For PCR amplification of the *SPRED2*,

SPRED3, *SPRY1*, *SPRY2*, *SPRY3* and *SPRY4* genes the M13-tagged primer sequences and the PCR conditions shown in Table1 were used. The mutation screen applied to all amplified exonic sequences involved direct DNA sequencing (ABI3730 sequencer, Applied Biosystems, Warwick, UK).

Table 1 Primer sequence and PCR conditions for *SPRED2*, *SPRED3*, *SPRY1*, *SPRY2*, *SPRY3* and *SPRY4*

Gene	Exon	PCR fragments	Forward primer (5'-3')	Reverse primer (5'-3')	PCR temperature (°C)	PCR buffer
Spred 2	1	1a	aggaagcttttgactcca	gatagggttggggaagg	65	failsafe D
		1b	cgtcccttcccaaatct	acgccaaagtcaccaataa	61	failsafe D
	2		tgccctgtgcttcttct	cccacgagttgtatgtctgc	61	10x PCR buffer
	3		ttggtagttgccaccagtga	tactgacctggcccaaacc	61	10x PCR buffer
	4		tgcaaaaagaatggaatgtgg	acagcaacagcaaacactgc	64	10x PCR buffer
	5		ttttgggaagagcagcaaat	taaaggcaggagcaggagag	61	10x PCR buffer
	6	6a	ggagaaggtgggtgtttt	ctttgggtcctcgccta	61	10x PCR buffer
		6b	gaggtcccaagcatgacta	aaggcaataagagccatcca	61	failsafe D
		6c	ggaccccaggagactat	aagagacgagttccctgtg	61	10x PCR buffer
Spred 3	1		tgtgtgtccctgtcctcc	acctgacctgtctccacat	61	failsafe D
	2 + 3	Exon 2+3	atggacgagacgtcacagg	gcctgctacacagtaggtgct	68	failsafe E
	4		gattctgagaagcggaaga	acgtgggactggaggagatt	61	10x PCR buffer
Spry 1	1		ccctcctctaccacagaga	gaaaagcccatttcaagca	60	10x PCR buffer
	2	2a	ccccagcatcattgtaatcc	cgaggagcaggtctttcac	60	10x PCR buffer
		2b	tgctattttgtccttagaccaga	ggaaccctcaagtcacca	60	10x PCR buffer
		2c	ccagtccctggtcataggtc	ctaagcacatgcaggtcca	60	10x PCR buffer
		2d	accatcctgtttggcctgta	ggtgggaaacagggaataat	60	10x PCR buffer
Spry 2	1		acttggcgatctggcttag	caggagggaagagccaaac	60	10x PCR buffer
	2	2.1	attcaggtgggtggagagtg	agctgaccgtgcttatggat	60	10x PCR buffer
		2.2	ctccactcagcacaacacg	tggctggaaggtaacaccat	60	10x PCR buffer
		2.3	gacaacctatgttcttcag	gcctaggagtgtctgtgtgc	60	10x PCR buffer
		2.4	cggtgaaatgcttttgtcc	ttttgccttacagaaagccttt	60	10x PCR buffer
		2.5	tttgcaactgtgaaaaacaagt	gataaagaagacaaaacagctcagt	60	Fail safe C buffer
Spry 3	1	1a	atgtgccctgaaatccatgt	actgcgggggagagaagtag	60	10x PCR buffer
		1b	tgtaaacaggccctctccag	tcagcttctccctcagagc	60	10x PCR buffer

		1c	cttcaggccaatccatcatc	catcatcagtgaggcagtg	60	10x PCR buffer
		1d	gctgagagcctcctcgatta	ggggactcgaaggagaaaag	60	10x PCR buffer
Spry 4	1		tgtgccacccattcatag	tgaggaaggaacactgagg	60	10x PCR buffer
	2		taagtcaaggggaggggtg	caggatatcaaaatccccagaa	60	10x PCR buffer
	3	3a	ggctccaaggacctgat	ggtgacatcctggtcacagc	60	10x PCR buffer
		3b	tagcctggccctgaccac	agcacaggcactcctggtt	60	10x PCR buffer
		3c	aaatgcaaggagtgtgcatc	caggcaggtagcagagcag	60	10x PCR buffer
		3d	ggtgctctctccgtggtg	ttcttagatgtcagaagagaacca	60	10x PCR buffer

An (AT)_n microsatellite repeat **located** in intron 1 of the *SPRED1* gene was used to **assess gene heterozygosity or homozygosity in each** patient. The primer sequences for amplifying this polymorphic marker are: forward: 5'-CTTTGAAGTTTGTATGGCATGA -3' and reverse: 5'-ACAAATTATCCATTTTCAAGCTCT-3'. The PCR conditions used were 30 sec denaturation at 92⁰C, annealing at 60⁰C.

Bioinformatics analysis

Complexity analysis¹⁰ was applied to analyse the flanking sequences (±25 bp) that encompassed each of the six identified *SPRED1* mutations. Direct and inverted sequence repeats, as well as for symmetric sequence elements were sought. Mutational mechanism predicted to lead to micro-deletions, micro-insertions and indel mutations mediated by these types of repeats have been described.^{11,12} The possible mutational mechanism of single base-pair substitutions mediated by direct repeats identified by means of complexity analysis was explored in this study in the context of slipped non-B DNA structure formation.^{13,14}

Results

Germline *SPRED1* mutations were identified in 6 of the 85 unrelated patients screened. These included three nonsense mutations (R16X, E73X and R262X), two frameshift mutations (2bp del: c1048_1049 GG and 4bp del: c1149_1152), and a single missense mutation (V44D). The c.1048-1049 GG frame shift mutation has been reported by Brems et al (2007).⁶ The remaining 5 *SPRED1* mutations are all novel sequence changes. The mutation and clinical details on the 6 families is given in Table 2 and Figure 1. All the affected individuals had CAL and none exhibited obvious Noonan-like features. None of the individuals had Lisch nodules or cutaneous neurofibromas.

Table 2 Identification of *SPRED1* mutations in 6 unrelated families. The nature and location of mutations along with the clinical features are listed. **NA- not available**

Patients	Gender / Age	Germline Mutation	Clinical Features				
			Café-au-lait spots	Freckling	Lisch nodules	Macrocephaly	Other associated features
Family 1 : Possibly paternally inherited	Male / 30yrs	Exon: 8 c.1048-1049delGG	Present	Present	None	Normal range (50 th centile)	Excessive periorbital pigmentation
Family 2 : Sporadic	Male / 6yrs	Exon: 8 c.1149-1152del 4bp	Present	Absent	None	N/A	None
Family 3 : Father Son	Male / 28yrs Male / 6yrs	Exon: 8 c.784A>T: R262X	Present Present	Axillary Axillary & inguinal	None None	Head circumference normal range	Congenital soft tissue swelling on scalp Inguinal haemangioma
Family 4 : Son Father	Male / 3yrs Male / 26yrs	Exon: 4 c.217G>T: E73X	Present Present	Absent Absent	None None	Head circumf: 9 th Centile Head circumf: 90 th Centile	Protruding ears
Family 5 : Mother Daughter	Female / 35yrs Female / 3yrs	Exon: 3 c.46C>T: R16X	Present Present	Tiny dots in right groin Absent	None N/A	Head circumf: 50 th centile	Father said to have CAL

Family 6 :							
Grandfather	Male / 65yrs	Exon: 3 c.131T>A: V44D	Present	Present	N/A	Head circumf: 98 th centile Head circumf: normal range Head circumf: normal range	None
Father	Male / 33yrs		Present	Present	N/A		
Son	Male / 5yrs		Present	N/A	N/A		
Daughter	Female / 3ys		Present	present	N/A		

All but one proband (family 2) with *SPRED1* mutations satisfied the diagnostic criteria for NF1 (Table 2), no NF1-associated clinical complications were found in these patients. None had significant developmental or learning problems and no one had macrocephaly.

The proband in family one sought medical opinion in his twenties because of his marked peri-orbital pigmentation. At the age of 30 years, he had six CALS randomly distributed on his back. He had extensive freckling over his upper trunk, both axillae and groins. In addition he had freckles in his forearm creases. On the right of his trunk, at waist level, there was a right sided segment of freckles. In his neck there was a very distinct cut off to his freckles. These last two features raised the possibility of mosaicism and he was included in our laboratory studies of segmental NF1. .

In family 3, a 28-year old father and his 6-year old son, identified with a *SPRED1* nonsense mutation (R262X), both presented with CALS and axillary freckling, with a soft tissue swelling of the scalp noted in the father from birth.

In family 4, another father and son were found to carry a nonsense mutation (E73X). Father's head circumference was on the 90th centile, height was between 50th to 75th centile and he has protruding ears. His 3 year old son has similar features but the head circumference is at 9th centile.

In family 5, the mother had 6 CALs and freckling in the right groin. No Lisch nodules were detected and she was of normal intelligence. Her father was also reported to have CAL. Her daughter also had CAL and exhibited no other features of NF1.

SPRED1 mutation analysis in the four affected members from the 3-generation family 6 identified the same V44D *SPRED1* missense mutation in each individual. This valine residue is known to be highly conserved. The clinical features in each affected individual included CAL and axillary freckling but no cutaneous neurofibromas. Father had head circumference of 90th centile but the children's head circumference was found to be of normal range.

Several single nucleotide polymorphisms were also detected in the *SPRED1* gene, most previously reported in the Entrez SNP database. At least 10 patient DNA samples were homozygous for all *SPRED1* polymorphisms and 7 were also homozygous for an informative microsatellite marker. These samples will be tested with a MLPA kit when it becomes available.

We failed to identify disease causing mutations in *SPRED2*, 3 and *SPRY1-4* genes. Several polymorphic changes were identified in these genes. A potential *SPRY1* missense alteration was found in a similarly affected patient, who was *NF1* and *SPRED1* mutation-negative. However, in the absence of either parental DNA or a functional analysis, it is difficult to assess whether this is a true disease-causing mutation or only represents a rare neutral variant.

Complexity analysis of the flanking sequences surrounding each *SPRED1* mutation found inverted repeats adjacent to two of the single base-pair substitutions (Figure 2). Each inverted repeat, or palindrome, consists of a short sequence that is complementary to a second nearby sequence located upstream of the first one on the same DNA strand. Such inverted repeats can form hairpin loop structures and thus, any base mismatch in such a structure may promote aberrant templating that can lead to the formation of a more stable hairpin loop structure. A schematic explanation of the *SPRED1* mutation T>A [V44D] in exon 3 of the gene is shown in Figure 3a.

Short direct sequence repeats were found adjacent to the two frameshift deletions (Figure 2), and such repeats are reported to be involved in microdeletion formation in several human genetic diseases.¹² Direct repeats were also found in the vicinity of two nonsense mutations (E73X, R262X), with such direct repeats capable of forming non-B DNA slipped structures formation as shown in Figure 3b. The misaligned slipped structures illustrated may template a nucleotide change creating a perfect Watson-Crick base pairing.

Discussion

Although no evidence for locus heterogeneity in classical NF1 families has been found, some families with the 'café-au-lait only' phenotype were reported to be unlinked to the *NF1* gene region at 17q11.2,^{1,5} None of these families were large enough to permit a genome-wide mapping study. The recent identification of mutations of the 15q14-located *SPRED1* gene in patients with this phenotype⁶ is therefore the first indication of an alternative disease locus. The present study confirms these findings by identifying six *SPRED1* mutations, five novel, in 85 unrelated minimally affected patients, most of whom still satisfy the diagnostic criteria for neurofibromatosis type 1. **A *SPRY1* missense alteration was also found in a similarly affected patient who was both *NF1* and *SPRED1* mutation negative, whether this is a true disease-causing mutation however, or only represents a rare neutral variant, has still to be established.** Overall *SPRED1* mutations apparently account for ~10% of patients with a mild NF1 phenotype in the two cohorts reported (Brems et al, 2007 and the current study) to date. This has major implications for counselling NF1 families- the mild pigmentary phenotype is clinically indistinguishable from NF1 in childhood and the absence of major physical complications and neurofibromas greatly reduces any NF1 disease-related morbidity, especially in adults

The main feature exhibited by all these patients was the presence of multiple CAL spots, with skinfold freckling also evident in most cases, but with no other NF1 features. The current NIH diagnostic criteria for NF1 were met in five of the six probands. In children, the main clinical distinguishing feature between NF1 and *SPRED1* cases appears to be the lack of Lisch nodules and major physical complications. In contrast to the patients reported by Brems et al,⁶ none of the patients in this series had macrocephaly, learning problems or significant facial dysmorphism. Although one of our patients had the peri-orbital pigmentation seen in many NF1 adults. **In family 3, the underlying pathogenesis of the associated large haemangioma observed in the patient is difficult to explain. Such vascular abnormalities are more of a cardinal feature found in another RAS-MAPK syndrome resulting from mutations of the *RASA1* gene. The *RASA1* protein is another GTP-ase activating protein that also down-regulates the activated RAS signalling pathway, a function similar to that of neurofibromin. However, constitutional heterozygous *RASA1* mutations are associated with an autosomal dominant phenotype presenting with capillary malformations/AV malformations.**¹⁵

At a recent European NF group meeting it was agreed that the clinical phenotype resulting from *SPRED1* mutations should be referred to as Legius Syndrome in recognition that the group led by Professor Eric Legius defined this phenotype at the clinical and molecular level.⁶ It will be important to pool clinical data from different centres to document the variability in phenotype and natural history of Legius syndrome.

The confidence of clinical diagnosis in the majority of NF1 cases, coupled with the cost and complexity of current *NF1* mutation analysis, means that application of molecular testing is often not part of routine NF1 care. There are now however, 3 genotype/phenotype correlations of major clinical significance. These are the large *NF1*

microdeletion phenotype, that is often associated with a much more severe disease format,¹ the specific 3bp deletion of exon 17 of the *NF1* gene⁵ and now mutations of the 15q14-located *SPRED1* gene, both associated with NF1 patients exhibiting a generally milder clinical phenotype.⁶ Such findings strongly suggest that routine *NF1* genetic testing should be implemented, especially for sporadic cases. From a research perspective, the majority of patients with a mild phenotype, in both this and the previous study, apparently had neither an *NF1* nor a *SPRED1* gene mutation, emphasizing the need to expand the search for causative gene mutations to other related genes in the Ras/MAPK pathway. **The possibility that some of the *NF1*/*SPRED1* mutation negative cases might be mosaics for a *NF1* mutation also needs exploration.**¹⁶ However, we can not exclude the possibility of mutations in those regions of both the *NF1* and *SPRED1* genes not currently screened in our genetic analyses. Indeed, the mutation detection rate for the *NF1* gene, even in classical NF1 patients, is reported to vary from 50 to 95%¹⁷⁻²³

The Spred proteins contain several specific functional domains, including an N-terminal EVH1 domain, a central c-Kit binding domain (KBD) and a C-terminal Sprouty-like cysteine-rich domain (SPR domain) that is also present in the Sprouty proteins, although the N-terminal domain is more variable but does contain a conserved SH2 domain binding motif with a central tyrosine residue.⁶⁻⁹ All these proteins do however function as Ras/MAPK signalling pathway inhibitors. Three of the identified mutations in this study (G350FsX9 and S383FsX21 and R262X) are located within the C-terminal SPR domain known to interact with Raf proteins to make them inaccessible to Raf kinase phosphorylation when associated in this Spred-Raf complex.⁷

The frequency and types of mutations not only depend on the nature of the damage, but also on the sequence context. A number of sequence repeats in the adjacent *SPRED1* gene coding region were predicted to have mediated some of the observed mutational changes. *In-vitro* studies of various alternative conformations^{13,14} have shown that non-B DNA structures are mutation prone. Here we propose a potential mutational mechanism underlying single base pair substitutions and involving two direct repeats capable of a temporary misaligned non-B slipped structure formation which may lead to a nucleotide change.

In summary, a cohort of NF1-like disease patients, consisting of individuals with just CAL and axillary freckling, but no cutaneous neurofibromas or Lisch nodules, and without any detectable *NF1* mutations, have been identified. Amongst these patients, several individuals with different *SPRED1* mutations have been identified. As no other gene mutations were discovered, despite extensively screening for coding-region mutations of the *NF1*, *SPRED1*-3 and *SPRY1*-4 genes, it remains a challenge to define the molecular basis of the disease in these patients. It is possible that extending such comprehensive mutation analyses to many more of the genes involved in the RAS/MAPK signalling pathway^{24, 25} may provide further information in this group of mildly affected patients.

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Figure Legends

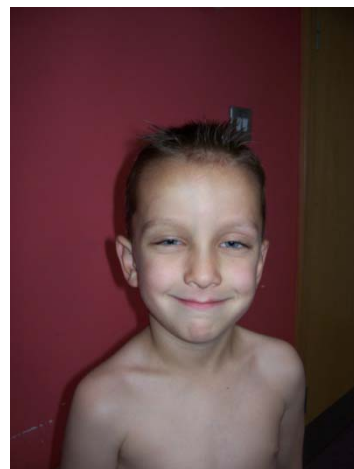
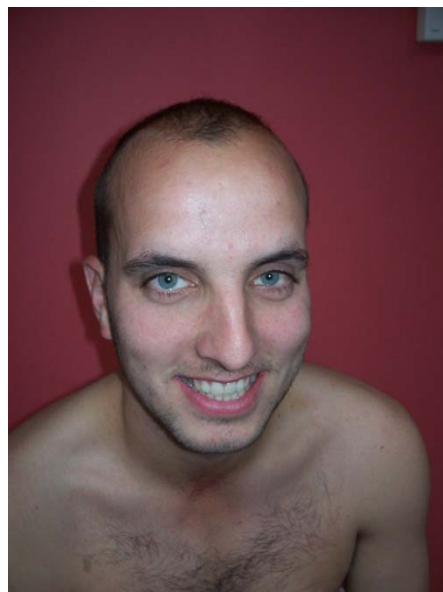
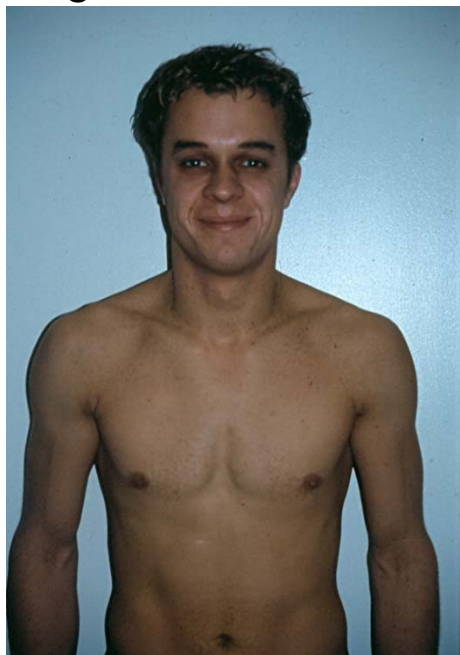
Figure 1. Photographs of patients with *SPRED1* mutation. Consent from all the patients included in this figure has been obtained.

Figure 2. Different types of repeat that could have mediated missense & nonsense mutations and short deletions in the *SPRED1* gene. Direct repeats are highlighted in yellow or underlined, inverted repeats are highlighted in blue; mutated nucleotides are shown in bold upper-case letters.

Figure 3. Schematic representation of potential mutational mechanism underlying (a) nonsense mutation G>T:E73X [GAA>TAA, c217] and (b) missense mutation T>A : V44D.

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Figure 1



Family1

Family3
Father and son

Family 4
Father and son

Figure2. Different types of repeat that could have mediated missense & nonsense mutations and short deletions in the *SPRED1* gene. Direct repeats are highlighted in yellow or underlined, inverted repeats are highlighted in blue; mutated nucleotides are shown in bold upper-case letters.

Nonsense mutation c217G>T:E73X [GAA>TAA]

at tt tgg c **tt t t g** t c a g G T G G **TT T T G** **G** A A T G T A T G C T T A A A A A A G T C A C T
↓
at tt tgg c **tt t t g** t c a g G T G G **TT T T G** **T** A A T G T A T G C T T A A A A A A G T C A C T

Missense mutation c784 A>T R262X **direct repeats**

T C T T A A T A C G T C G C T A T G C A G A C T A C **A** G A C A T C C T **G A C A T** G T G G A A A A A T
↓
T C T T A A T A C G T C G C T A T G C A G A C T A C **T** G A C A T C C T **T G A C A T** G T G G A A A A A T

G350fsX9 2 bp deletion c.1048-1049 **direct repeats**

A G G T T T A A T C A T G A A **G A A A A T G T** T A **G G** **G A A A A T G T** C A G G A T G C T C C
↓ deletion
A G G T T T A A T C A T G A A **G A A A A T G T** T A **G A A A A T G T** C A G G A T G C T C C

S383fsX21 Mutation 4 bp deletion c.1149-1152

T G T T G T A T C A T T G T A T G **T C A G A C T C** **A G A G** G G A G A T T T T T C T G A T C C C T G T T C G T G
↓ deletion
T G T T G T A T C A T T G T A T G T C A G A C T C G G A G A T T T T T C T G A T C C C T G T T C G T G

Missense mutation c46 C>T :ex3F **inverted repeats**

A T T T T A G T A A T A G T T A **T G C A** **C** G A G T G C G A G C T G T G G T G A T G
↓
A T T T T A G T A A T A G T T A **A T G C A T** **T** G A G T G C G A G C T G T G G T G A T G
↔↔↔

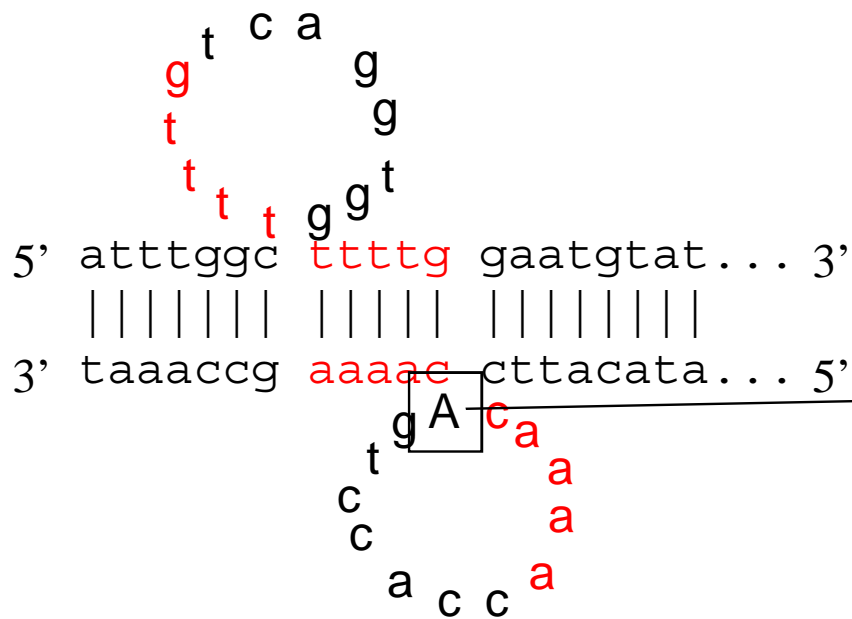
Missense mutation Exon 3 T>A : Val44Asp **inverted repeats**

T G G A C T A A G C A G C G T C A C T **G** **C T T** **C A A G T C** C C T C A T C A G G
↓
T G G A C T A A G C A G C G T C A C T **G A C T T** **C A A G T C** C C T C A T C A G G
↔↔↔

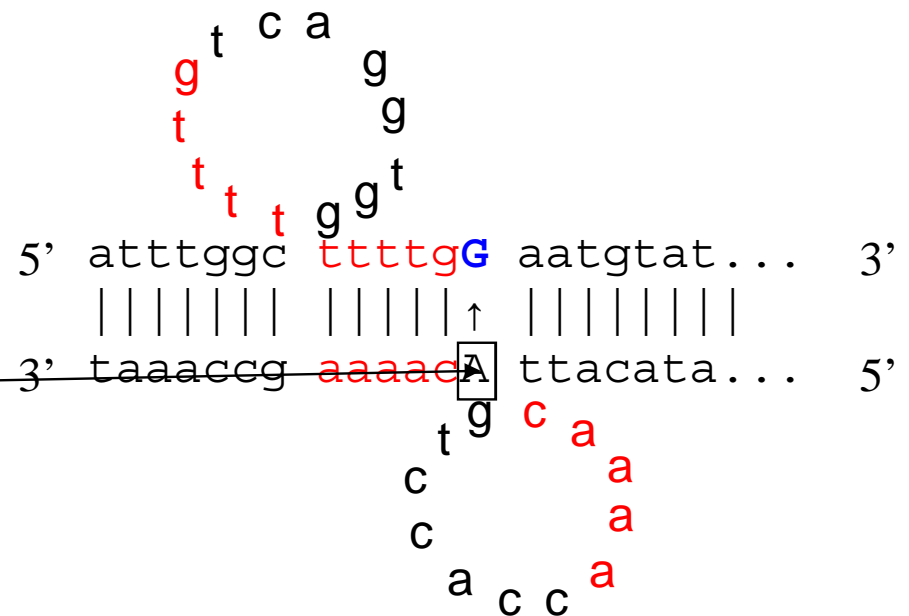
(a)

Normal sequence before mutation : **atttggc**ttttgtcag**GTGGTTTTG****G**AATGTAT...

Normal slipped structure



Misaligned slipped structure with nucleotide **A** from 3'-5' strand templating a **G**→**T** change



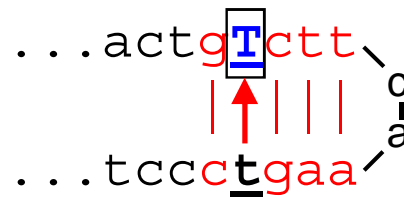
A slipped structure intermediate is formed by the direct repeats **ttttg** and this templates the **G**→**T** nucleotide change.

Mutated sequence: **atttggc**ttttgtcag**GTGGTTTTG****T**AATGTAT...

(b)

Normal sequence before mutation : **tggactaagcagcgtcactgTcttcaaagtcct...**

Predicted that a 'hairpin loop' structure formed by interaction between the imperfect inverted repeats **gTctt** and **aagtc** may have templated the t→A nucleotide change that resulted in this missense mutation



Missense mutated sequence : **tggactaagcagcgtcactgActtcaaagtcct...**