

Targeted resequencing of 52 bone marrow failure genes in patients with aplastic anemia reveals an increased frequency of novel variants of unknown significance only in SLX4

The bone marrow failure (BMF) syndromes are a collection of heterogeneous disorders characterized by failure to produce blood cells of one or more lineage. Aplastic anemia (AA) is a rare, life threatening disorder defined by pancytopenia and a hypocellular bone marrow. Approximately 75% of cases are considered idiopathic (IAA), whereby no family history is observed and the cause remains unexplained.¹ In some cases, however, AA patients have been found to harbor mutations in genes encoding the RNA or reverse transcriptase component of telomerase (*TERC* and *TERT*, respectively).^{2,3} Here we performed targeted resequencing of 52 genes associated with 11 distinct BMF syndromes in 45 patients with AA (known to be normal for *TERC*) that was seemingly idiopathic in nature (patients had no extra-hematopoietic features, family history or stigmata of known inherited BMF syndromes and normal chromosomal breakage scores). Through this systematic approach we sought to verify whether a subset of patients had occult genetic variants causing or predisposing to dis-

ease. DNA samples were prepared using a TruSeq Custom Amplicon Kit and sequenced on a MiSeq Benchtop Sequencer (Illumina). The success of the method was confirmed by 3 positive control samples. A patient with AA, previously identified with the *TERC* variant r.95G>C, was included in the screen and this variant was successfully called by the on-system program MiSeq reporter. Furthermore, we identified disease-causing variants in 2 previously genetically uncharacterized BMF syndrome patients. In a Fanconi anemia (FA) patient, a homozygous frameshift deletion (c.1158delC, p.Ser387ProfsX16) was found in *FANCG*, which has not been described before. *FANCG* is a component of the FA nuclear complex involved in DNA repair.⁴ In a patient with Diamond-Blackfan anemia, we identified a heterozygous frameshift mutation in *RPL5* (c.172_173delAG, p.Asp59TyrfsX53) which has been reported previously.⁵ Both changes were confirmed by Sanger sequencing.

Screening of 45 IAA patients resulted in 8398 variant calls which were filtered using Ingenuity® Variant Analysis™ (Ingenuity Systems; www.ingenuity.com/variants) on the assumption that any disease causing or predisposing variant would not be seen at a high frequency in the general population. Variants were selected that were rare (minor allele frequency of 0.0001 or less), had passed the

Table 1. Rare variants identified in patients with IAA and their frequency per gene compared to a control population.

Gene	Transcript variant(s) ^a	Amino acid substitution	Ref depth ^b	Variant depth ^c	Polyphen score ^d	Patient frequency (rare variants per gene) ^e	Control frequency (rare variants per gene) ^f
<i>BRCA2</i>	c.1460C>A	Ala487Glu	509	506	0.815	1/90	1/93
<i>CDAN1</i>	c.1849G>A	Val617Ile	17	11	0.308	2/90	1/209
	c.2750T>G	Ile917Ser	28	25	0.000		
<i>FANCM</i>	c.4468A>G	Asn1490Asp	249	200	0.682	1/90	1/130
<i>GFII1</i>	c.65G>A	Gly22Glu	12	14	0.429	1/90	1/1270
<i>MPL</i>	c.932C>T	Ser311Phe	24	12	0.995	1/90	1/481
<i>RAD51C</i>	c.687C>G	Phe229Leu	35	20	0.118	1/90	1/813
<i>RPS10</i>	c.125A>G	Asn42Ser	37	36	0.940	1/90	1/3251
<i>RUNX1</i>	c.509T>C	Val170Ala	58	62	0.636	1/90	1/1652
<i>SBDS</i>	c.598G>A	Glu200Lys	80	56	0.963	1/90	1/1445
<i>SLX4</i>	c.929G>A	Arg310Gln	55	48	1.000	6/90	1/105
	c.1243G>A	Glu415Lys	29	15	0.897		
	c.1573C>A	Arg525Ser	44	33	0.026		
	c.1367-2A>G	Splice site loss	22	14	N/A		
	c.1799C>T	Pro600Leu	57	56	0.035		
	c.3424T>C	Ser1142Pro	8	19	0.000		
<i>TERT</i>	c.1570C>G	Pro524Ala	10	14	0.969	2/90	1/471
	c.2173C>T	Leu725Phe	27	32	0.996		
<i>WRAP53</i>	c.836C>T	Ala279Val	34	34	0.995	1/90	1/394

^a19 rare variants (with high quality scores) in 12 genes were validated by Sanger sequencing. Nine of the genes had a single rare variation in our patient cohort and it is not possible to differentiate this frequency from that of the control population. Two variations were identified in *CDAN1* (mutated in congenital dyserythropoietic anemia). Rare variants are reported in *CDAN1* in the control population at a frequency of 1 in 209 alleles, which is not significantly different from the 2 variations in 90 alleles identified in our IAA patients ($P=0.2$, Fisher's exact test); ^bRead depth of the reference base; ^cRead depth of variant call; ^dPolyphen-2 score as a prediction of possible impact of an amino acid substitution. Scores are between 0 and 1, with a higher score predicted to be more damaging. Calculated using <http://genetics.bwh.harvard.edu/pph2/index.shtml>. ^eThe allelic frequency of rare variants per gene identified in the IAA patients analyzed in this study. For each gene, the frequency of rare coding variants in a non-IAA control population were calculated using data on the EVS. First, all rare variants (minor allele frequency of 0.0001 or less) were totalled. The population size from which they came from was averaged. These values were then used to calculate the frequency stated. The 52 genes sequenced in the 45 idiopathic aplastic anemia patients belonged to the following syndromes: Fanconi anemia (15 genes), dyskeratosis congenita (10 genes), familial myelodysplasia/leukemia (4 genes), Shwachman-Diamond syndrome (1 gene), Diamond-Blackfan anemia (10 genes), severe congenital neutropenia (5 genes), congenital dyserythropoietic anemia (3 genes), Wiskot-Aldrich syndrome (1 gene), Barth syndrome (1 gene), WHIM syndrome (1 gene) and congenital amegakaryocytic thrombocytopenia (1 gene).

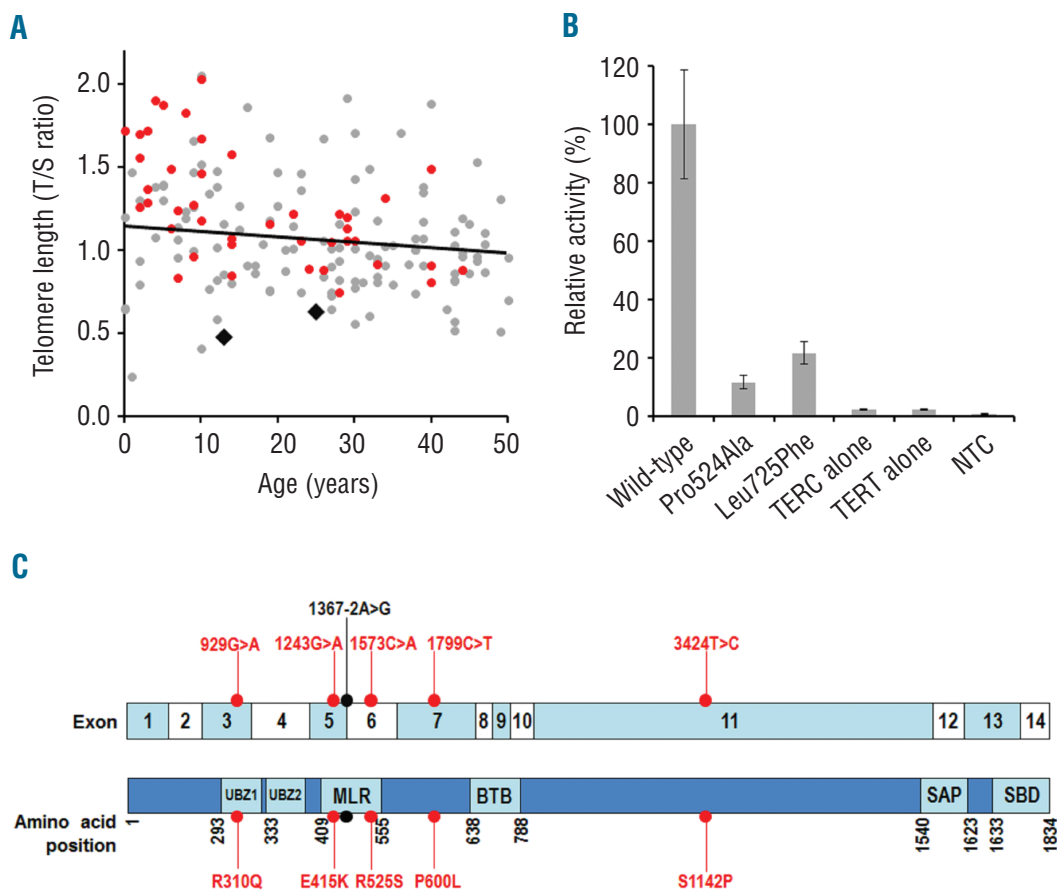


Figure 1. The impact of variants identified in *TERT* and the location of variants identified in *SLX4*. (A) Telomere lengths were measured in the 45 IAA patients studied and in 124 normal, age-matched controls by quantitative PCR and expressed as a T/S ratio. The 2 IAA patients with *TERT* variants had telomeres below the 5th percentile for their age group. Gray circles: controls; red circles: IAA patients; black diamonds: IAA patients with *TERT* variants. (B) The telomerase repeat amplification protocol showed that the two *TERT* variants reduced telomerase activity. Wild-type *TERC* and *TERT* were expressed in WI38 cells as a positive control to give 100% activity, and the variants were compared to this. NTC: no template control. Bars show standard error of mean of duplicate readings from 2 independent experiments. (C) Schematic illustration of *SLX4* with exon positions and functional domains highlighted. Variants in IAA patients are indicated. Red dots: missense variants; black dot: splice site variant; UBZ1/UBZ2: ubiquitin-binding zinc finger domains 1 and 2; MLR: MUS312-MEI9 interaction-like region; BTB: broad-complex, tramtrack, and bric à brac; SAP: SAF-A/B, Acinus and PIAS; SBD: SLX1 binding domain.

Illumina filter, had a quality score of Q80 or above and resulted in a missense, stop gain/loss, insertion/deletion or splice site change. All of these variant calls were then examined manually using the Integrated Genomics Viewer (Broad Institute) and variant calls that were clear sequence artefacts were ignored. Twenty rare variants remained and 19, from 12 different genes, were validated by Sanger sequencing (Table 1).

Two patients from our cohort presented with novel heterozygous *TERT* variants (Table 1). In one patient, the variant c.1570C>G, p.Pro524Ala was found in the RNA-interacting domain. In another patient, we identified c.2173C>T, p.Leu725Phe, which occurs in the reverse transcriptase domain necessary for catalytic activity. The telomere lengths of the 45 AA patients in the study were quantified by monochrome multiplex quantitative PCR⁵ and were compared to 124 healthy controls aged 0-49 years (Figure 1A). Although this group of IAA patients did not have shorter telomeres than the age-matched controls, the 2 patients with the *TERT* variants stand out, with telomere lengths below the 5th percentile. The effect of these variants on telomerase activity was measured using a TRAPeze RT Telomerase Detection Kit (Millipore). Both mutations had a

negative impact resulting in less than 22% of wild-type telomerase activity (Figure 1B). This finding demonstrates that pathogenic *TERT* variants can be successfully identified through a relatively large resequencing experiment.

Notably, 6 of the 45 IAA patients presented with rare heterozygous variants in the FA gene *SLX4*, 5 of which are novel and one (c.1367-2A>G) that has been reported in a homozygous state in a family with FA (Table 1).⁷ *SLX4* acts as a scaffold, binding proteins involved in DNA damage repair. These include the nuclease XPF, involved in DNA interstrand crosslink repair,⁸ and the DNA mismatch repair complex MSH2/MSH3.⁹ The integrity of *SLX4* is necessary for their binding and activity and loss of these interactions results in impaired DNA repair pathways.¹⁰ The *SLX4* ubiquitin-binding zinc finger domains 1 and 2 (UBZ1 and UBZ2) and the MUS312-MEI9 interaction-like region (MLR) act as the binding sites for MSH2/MSH3 and XPF, respectively.¹⁰ Five variants identified in our patients clustered between exons 3 to 7, which encode these domains (Figure 1C). Three impact on the MLR domain and another lies in the UBZ1 domain. When *SLX4* lacking a UBZ1 domain is ectopically expressed, cells exhibit an increased

sensitivity to the DNA cross-linking agent mitomycin C.¹¹ The importance of the UBZ1 and MLR domains in SLX4 is, therefore, well established, and it is possible that the variants we have identified here could impact on their function.

Data on the Exome Variant Server (EVS, <http://evs.gs.washington.edu/EVS>) were used to indicate the frequency of a rare SLX4 variant in a control population (likewise with a minor allele frequency of 0.0001 or less). When considering both European and African populations together, the frequency is one in 105 alleles. The increased frequency of 6 in 90 alleles that we observe in our IAA patient cohort is of borderline significance ($P=0.05$; Fisher's exact test). When considering only exons 3 to 7, the frequency of rare variants on the EVS is one in 461 alleles, which is significantly different to the 5 variants in 90 alleles seen in our cohort of AA patients ($P<0.01$; Fisher's exact test).

In summary, we have successfully utilized a method of targeted resequencing to analyze 52 genes responsible for inherited BMF syndromes in patients with IAA. This study is the first to investigate a gene panel of this scale using this technology and report on the frequency of rare variants in these genes in an IAA patient cohort. Intriguingly, we find an increased incidence of novel variants in the FA gene SLX4. These observations suggest heterozygous variations in SLX4 may be a predisposing factor for the development of AA, as proposed previously.¹² Functional studies may provide additional information on whether all or only some of these SLX4 variants contribute to the pathophysiology of IAA. It is also notable that in 50 out of 52 bone marrow failure genes, resequencing has not detected variants that can be presumed to predispose to IAA.

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