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TITLE Mosaic variants in *TNFRSF1A*: An emerging cause of tumor necrosis factor receptor-associated periodic syndrome

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KEY WORDS Autoinflammatory disease, TRAPS, *TNFRSF1A*, mosaic, post-zygotic, variant, NGS, genetic counseling.

ABBREVIATIONS

CRD: cysteine-rich domain

CRP: C-reactive protein

DD: intracellular death domain

gnomAD: genome aggregation database

HGVS: human genome variation society

NGS: next generation sequencing (i.e. deep targeted sequencing)

NSAIDs: non-steroidal anti-inflammatory drugs

PBMC: peripheral blood mononuclear cell

S-S: disulfide bridge

SAA: serum amyloid A

SAIDs: systemic autoinflammatory disorders

SP: signal peptide

TM: transmembrane region

TNFR: tumor necrosis factor receptor

TNFRSF1A: tumor necrosis factor receptor superfamily 1A

TRAPS: tumor necrosis factor receptor-associated periodic syndrome

VAF: variant allele fraction

WBC: white blood cells

ABSTRACT

***Objective*:** To identify the molecular basis of a systemic autoinflammatory disorder (SAID) evocative of tumor necrosis factor receptor-associated periodic syndrome (TRAPS).

***Methods*:** (i) Deep next generation sequencing (NGS) through a SAID gene panel; (ii) variant allele distribution in peripheral blood subpopulations; (iii) *in silico* analyses of mosaic variants using TNFRSF1A crystal structure; (iv) review of the very rare *TNFRSF1A* mosaic variants reported previously.

***Results*:** In a 36-year-old man suffering from recurrent fever for 12 years, high-depth NGS revealed a *TNFRSF1A* mosaic variant c.176G>A p.(Cys59Tyr) that Sanger sequencing failed to detect. This mosaic variant displayed a variant allele fraction of 14% in whole blood; it affects both myeloid and lymphoid lineages. The p.(Cys59Tyr), a recurrent germline pathogenic variant, affects a crucial cysteine located in the first cysteine-rich domain (CRD1) and involved in a disulfide bridge. Introduction of a tyrosine at this position is expected to disrupt the CRD1 structure. Review of the three previously reported *TNFRSF1A* mosaic variants revealed that they are all located in a small region of CRD2 and that germinal cells can be affected.

***Conclusion*:** This study expands the localization of *TNFRSF1A* mosaic variants to the CRD1 domain. Noticeably, residues involved in germline *TNFRSF1A* mutational hot spots can also be involved in post-zygotic mutational events. Including our study, only four patients have been so far reported with *TNFRSF1A* mosaicism highlighting the need for a high-depth NGS-based approach to avoid the misdiagnosis of TRAPS. Genetic counseling has to consider the potential occurrence of *TNFRSF1A* mosaic variants in germinal cells.

KEY MESSAGES

- In sporadic cases with clinical suspicion of TRAPS, search for *TNFRSF1A* mosaic variants is recommended.
- High-depth NGS is required for detection of low-level mosaicism.
- The risk to transmit a *TNFRSF1A* variant to the offspring also exists for mosaic variants.

INTRODUCTION

Tumor necrosis factor receptor (TNFR)-associated periodic syndrome (TRAPS) is a rare systemic autoinflammatory disorder (SAID) caused by heterozygous germline variants in the tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*) gene (1). TRAPS is characterized by recurrent episodes of high-grade fever lasting from 1 to 3 weeks accompanied by abdominal pain, arthralgia, migratory erythematous plaques and patches involving the limbs and often associated with underlying myalgia and ocular manifestations (2–4). During febrile episodes, patients have elevated serum levels of acute phase reactants such as C-reactive protein (CRP) and serum amyloid A (SAA). AA amyloidosis is a severe complication of chronic untreated inflammation of TRAPS patients (5). In most patients, disease-onset occurs in the first decade of life, although around 10% of patients are diagnosed beyond the age of 30 (6,7).

TRAPS is caused mainly by missense variants in *TNFRSF1A*, a gene that encodes the 55-kD TNF receptor, TNFRSF1A (also known as TNFR1, TNFR, CD120a, p55), a transmembrane protein belonging to the large TNF-receptor superfamily. TNFRSF1A comprises a 29-residue N-terminal signal peptide (SP), an extracellular domain (ECD), a transmembrane region (TM), and a C-terminal cytoplasmic region with a characteristic intracellular death domain (DD) **(Figure 1A and 1B)**. The extracellular domain of TNFRSF1A consists of four cysteine-rich domains (CRDs) **(Figure 1A and 1B)**. The ligand-binding pocket for TNF is mainly formed by CRD2 and CRD3, while CRD1, which mediates receptor self-assembly (homotrimerization) in the absence of ligand, is also called “the pre-ligand-binding assembly domain” (PLAD). CRD1 is an essential domain for efficient TNF binding and receptor function (8,9).

TRAPS was initially described in familial forms of SAID transmitted as an autosomal dominant disease (1,3,10). It also has been reported in sporadic cases, due to *de novo* variants either in germline (12) or more recently, in mosaic state (13–15). Here, we report a 36-year-old patient presenting recurrent febrile attacks for more than 12 years in whom high-depth next generation sequencing (NGS) revealed a *TNFRSF1A* mosaic variant that Sanger sequencing failed to identify. We discuss, in light of the previous reports, the diagnostic challenges and consequences of somatic mosaic *TNFRSF1A* variants.

PATIENT AND METHODS

Patient's clinical features were collected using a standardized form. Written informed consent was obtained from the patient participating in the study for genetic tests according to French legislation and the principles of the Declaration of Helsinki.

Molecular analyses

Genomic DNA was extracted from the patient's peripheral whole blood as well as from patient's monocytes, neutrophils, T and B cells isolated from peripheral blood mononuclear cells (PBMCs) by positive selection using immunomagnetic microbeads (MACS, Miltenyi Biotec) as previously described (16).

Sanger sequencing: *TNFRSF1A* exons 2-4 and their intronic junctions were amplified by polymerase chain reaction (PCR) and sequenced using the Big Dye Terminator v3.1 sequencing kit on an ABI 3730XL automated capillary DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed using the SeqScape software (Applied Biosystems). The current nomenclature of *TNFRSF1A* variants, which differs by 29 amino acids from the former nomenclature in which the variant numbering began after the N-terminal signal peptide of the protein, follows the Human Genome Variation Society (HGVS) recommendations (https://www.hgvs.org/) and is based on the cDNA sequence (NM_001065).

Deep-targeted next generation sequencing (NGS): The DNA samples were extracted and sequenced following a deep targeted sequencing approach (sequencing depth: 500 to 1000X) using a NextSeq500 (Illumina) platform as previously described (16). We used a custom targeted capture that contains the coding regions and flanking intronic sequences of the main genes so far implicated in SAIDs. All the results obtained through this targeted sequencing were confirmed by Sanger sequencing.

TNFRSF1A crystal structure analysis

The protein 3D structure was rebuilt using human crystals of the extracellular domain (ECD), the

transmembrane region (TM), and the intracellular death domain (DD) of the human TNFRSF1A (Protein Data Bank accession numbers, 1EXT (ref: 8939750), 7K7A (ref: 33163490), 1ICH (ref: 11453696), respectively. The 3D structures were visualized with the Pymol software.

In order to study *in silico* the structural changes involved by the p.(Cys59Tyr) *TNFRSF1A* variant in the extracellular domain of TNFRSF1A, we used the corresponding human crystal structure 1EXT (A chain) available on Protein Data Bank (17) and PremPS (https://lilab.jysw.suda.edu.cn/research/PremPS/) bioinformatics tool.

RESULTS

Disease phenotype

The patient was a 36-year-old male, born to healthy non-consanguineous French parents, with no family history of autoinflammatory or autoimmune disorders. He presented since the age of 24 years with recurrent episodes of fever (38-40C°) lasting for 1-2 weeks with spontaneous resolution. The febrile attacks were accompanied by severe lower back pain and myalgia. During his clinical course, the patient complained of recurrent thoracic pain and reported an acute episode of pericarditis that lasted for several weeks and which was efficiently treated with aspirin. He also developed migratory erythematous plaque eruptions localized at elbows and left lower limb. From the age of 30, he also complained from recurrent attacks of abdominal pain. The patient never displayed ophthalmological or neurological symptoms, nor lymphadenopathy or organomegaly.

Laboratory tests performed during the febrile episodes showed elevated inflammatory biological markers such as increased serum levels of CRP (171 mg/l) and leukocytosis (15,000 WBCs/µl). Serum ferritin levels and kidney function tests were normal and extensive analyses of the immunological and infectious profile were found to be negative. Between attacks, CRP levels ranged from 6 to 36 mg/l. During the attacks, he was efficiently treated with analgesics and non-steroidal anti-inflammatory drugs (NSAIDs).

Molecular diagnosis and 3D modelling of the TNFRSF1A regions surrounding the identified sequence variant

With the aim of identifying the molecular basis of the patient's disease that was reminiscent of TRAPS, Sanger sequencing of *TNFRSF1A* exons 2 to 4 (in which the great majority of the variants are localized) was performed on DNA extracted from whole blood. No pathogenic heterozygous *TNFRSF1A* variant was identified. To deepen the molecular investigations, the DNA sample was re-analyzed by NGS using a panel containing the main genes so far implicated in SAIDs. This high-depth sequencing approach enabled us to identify a somatic mosaic variant in exon 2 of *TNFRSF1A* c.176G>A p.(Cys59Tyr) with a variant allele fraction (VAF) of 14% in whole blood DNA. The p.(Cys59Tyr) variant is not described in the genome aggregation database (gnomAD). Noticeably, the c.176G>A variant has already been reported as a recurrent germline mutational event in TRAPS patients (7,18,19). Cys59 (also referred to as C30), has also been involved in other germline missense pathogenic variants (C30R, C30S, and C30F) (1,19,20). Located in the first cysteine-rich domain (CRD1) of TNFRSF1A, Cys59 is involved in the second disulfide bridge (S-S2; Cys59-Cys72, also referred to as C30-C43) of CRD1 as shown in the 3D structure of the protein **(Figure 1B** and **C-right panel)**. Introduction at this position of a tyrosine, an aromatic amino-acid, is expected to disrupt the CRD1 structure, by loss of the disulfide bridge and by the establishment of several aromatic, polar and hydrophobic interactions **(Figure 1C-left panel).**

Cellular distribution of TNFRSF1A mosaicism

To study the cellular distribution of the *TNFRSF1A* mosaicism, different cell types were subsequently isolated from the patient's PBMCs. The analyzed cells included monocytes, neutrophils, B and T cells. Deep next generation sequencing showed a wide distribution of the variant allele in the studied cells with average VAF of ~15% in myeloid cells (i.e. 15.8% in monocytes and 14.1% in neutrophils) and ~9% in lymphoid cells (i.e. 8.9% in B cells and 9.6% in T cells) **(Figure 2, lower panel)**. Hindsight search for the *TNFRSF1A* variant c.176G>A p.(Cys59Tyr) by Sanger sequencing revealed the presence of a superimposed sequence, mimicking a background signal, but indeed corresponding to this variant **(Figure 2, upper Panel)**.

Review of the previously reported TNFRSF1A mosaic variants

To better assess the impact of *TNFRSF1A* mosaic variants in TRAPS, we reviewed the three previously reported patients with a mosaic pathogenic variant in this gene **(Table 1)**. The first reported patient carried a *de novo TNFRSF1A* gonosomal mosaic variant (c.255_278del, p.Ser86_Glu93del) with a VAF of ~21% in whole blood. He presented from early adolescence recurrent febrile episodes that were lasting for 2 weeks and accompanied with both clinical and biological inflammation **(Table 1)**. The variant was detected at different fractions in the studied tissues which included myeloid cells (19%), lymphocytes (20-30%), hair (4%), nails (11%) and sperms (16%). The patient was efficiently treated with Anakinra (13).

The second reported patient carried a *de novo TNFRSF1A* missense somatic mosaic variant (c.265T>C, p.Phe89Leu) with a VAF of ~30% in whole blood. He presented since the age of 54 with an intermittent daily fever over a period of 3-4 weeks and accompanied by systemic inflammation and highly elevated CRP levels with leukocytosis (18,500 WBC/µl) **(Table 1).** Kidney function tests were normal. The variant was detected in the patient's buccal swab, natural killer (NK) cells, neutrophils, and B lymphocytes with an estimated VAF ranging from 25 to 45%, but with no variant detected in monocytes, T cells and hair roots. The administration of Canakinumab which followed the identification of the molecular defect showed complete clinical and biological remission (14).

In addition, a *TNFRSF1A* postzygotic missense variant (c.269C>A, p.Thr90Asn) with a very low VAF of ~1.3% in whole blood was identified in a patient for whom phenotypic data are not available (15). All these three mosaic variants were found to be clustered in a small region of the CRD2 domain and involved residues Phe89-Thr90 (15). Noticeably, the Phe89 and Thr90 residues have already been involved in recurrent germline missense pathogenic events in TRAPS patients (7,21,22). Modelling of TNFRSF1A 3D structure showed that these variants are localized within the first loop of the CRD2 domain, a 16-residue-long loop formed by the first disulfide bridge of CRD2 between Cys84 and Cys99 residues (S-S1 of CRD2, also referred to as C55-C70) **(Figure 1B).**

DISCUSSION

Post-zygotic mosaicism, defined by the presence of two or more cell populations with different genotypes in the same individual, is an emerging field in systemic autoinflammatory diseases adding to the complexity of monogenic disorders and raising new challenges from both clinical and molecular viewpoints.

Here we illustrate the diagnostic challenges in relation with a *TNFRSF1A* mosaic variant in a 36-year-old patient with no family history of autoinflammatory disease, presenting with recurrent febrile episodes accompanied by systemic inflammatory manifestations. Despite broad biological and radiological investigations carried out for more than 10 years, the underlying etiology remained unknown. Although the clinical presentation of the patient was suggestive of TRAPS, Sanger sequencing of *TNFRSF1A* failed to identify the causative variant. A NGS-based high-depth sequencing analysis revealed a somatic mosaic pathogenic variant in *TNFRSF1A* c.176G>A p. (Cys59Tyr) with a VAF of ~14% in whole blood, enabling us to establish the diagnosis of TRAPS. The c.176G>A variant occurred on a CpG dimer in keeping with the fact that a significant excess of variants occurs in CpG dinucleotides (23) leading to *de novo* pre-zygotic (germline) or post-zygotic (mosaic) variants. Already known as a *TNFRSF1A* recurrent germline variant, c.176G>A could also be a hot spot for mosaic variants. Moreover, despite the advances in NGS-based sequencing technologies (including high-depth targeted NGS) that allows identification of mosaic variants, only three patients with *TNFRSF1A* mosaic variants have been reported so far suggesting that this diagnosis may be underreported.

It should be noted that the low-depth sequencing (about 20X to 100X) used in whole genome or exome sequencing does not allow identification of low-level mosaicism. High-depth NGS (500-1000X) is necessary to detect low-level mosaicism. However, detection of very low VAF (below 1-2%) remains challenging. To do so, specific deep sequencing method using single-molecule molecular inversion probes (smMIP) was requested in a previously reported patient carrying a *TNFSRF1A* mosaic variant (15).

The tissue distribution of the variant *TNFRSF1A* allele raises important questions on the mechanism leading to mosaicism and the impact of such mosaicism on genetic counseling. Based on the body parts and tissues that harbor the variant cells and the potential for transmission to

offspring, mosaicism can be divided into three types: the germline mosaicism (also known as gonadal mosaicism); the somatic mosaicism; and the gonosomal mosaicism which is a combination of both germline and somatic mosaicism (24). Notably, different tissue distributions of the variant alleles were observed in two of the three previously studied patients. In the first patient, who developed the disease at early adolescence, an in-frame mosaic *TNFRSF1A* deletion (c.255_278del, p.Ser86_Glu93del) was detected with a VAF of ~21% in whole blood but also in all tissues tested (VAF ranging from 4 to 30%) (13). In the second patient, who had an adult-onset TRAPS, a *TNFRSF1A* mosaic missense variant (c.265T>C, p.Phe89Leu) was detected in whole blood (VAF of 30%) and more precisely in neutrophils, NK cells, B lymphocytes and buccal cells, but not in monocytes, T cells or hair samples (14). In our patient, in whom the VAF was of 14% in whole blood, the variant allele was detected in two cell types of myeloid origin (i.e. 15.8% in monocytes and 14.1% in neutrophils) as well as in lymphoid cells (i.e. 9.6% in T cells and 8.9% in B cells). Taken together, these distributions are in favor of an early embryonic *TNFSRF1A* mutational event and underline that genetic counseling should take into account the fact that mosaic variants are not restricted to the disease-target cells (i.e. leukocytes in TRAPS) and may be present in other cell lineages including germinal lineage. Interestingly, the phenotypic severity of TRAPS does not appear to be correlated with the *TNFRSF1A* variant, the germinal or mosaic status or the VAF of mosaic variants (1,7,13,14,18–20).

Our study extends the region targeted by mosaic variants from a small region (Phe89-Thr90) of the CRD2 domain (15) to Cys59 located in the CRD1 domain. Among the six highly conserved cysteine residues found in CRD1, Cys59 is believed to play a crucial role in the structure and function of the receptor (1,20) and, according to our study, the p.(Cys59Tyr) pathogenic variant is expected to disrupt the protein structure.

In conclusion, in sporadic cases of autoinflammatory disorders with long lasting episodes of fever and no germline *TNFRSF1A* variant, search for a somatic mosaic variant in this gene should be done by high-depth NGS. *TNFRSF1A* mosaic pathogenic variants can be located not only in the CRD2 domain but also in CRD1 and the affected residues belong to the germline mutational hotspots. As for genetic counseling, the stage of variant occurrence is of prime importance: while germline variants have a one-in-two risk of being passed on to the next generation, this risk is lower in case of post-zygotic variants and exists only if the variant affects the gonadal cells.

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Authors' contribution: Study design: IG and SA. Sample collection and clinical description: GG. Data analysis: FDM, WP, BC. Tables and figures: EA, CL, EEK, JD. Data interpretation: IG, ML, EA, CL. Writing of the manuscript: EA, IG and SA. Critical proofreading of the manuscript and final approval of the submitted version: IG, SA, GG, SAK, ML, EA, CL, EEK, JD, BC, FDM, WP.

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Data availability statement: The data are available upon request from the corresponding author.

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365 Table (1): Clinical and molecular characteristics of TRAPS patients with somatic mosaic
366 *TNFRSF1A* variants.

	Current study	Rowczenio et al. (13)	Kontzias et al. (14)	Kant et al. (15)
***TNFRSF1A* genotype**				
HGVS sequence name	c.176G>A	c.255_278del	c.265T>C	c.269C>A
HGVS protein name	p.(Cys59Tyr)	p.(Ser83_Glu93del)	p.(Phe89Leu)	p.(Thr90Asn)
Former nomenclature	C30Y	S54_E64del	F60L	T61N
VAF (% in whole blood)	14%	21%	30%	1.1-1.4%
Reported in germline state	Yes (7, 18, 19)	No	Yes (7, 21, 22)	Yes (7)
gnomAD (allele frequency)	0	0	0	0
Variant classification*	Pathogenic	Likely pathogenic	Pathogenic	Likely Pathogenic
Age at disease onset (Y)	24	Early childhood	54	NA
Age at diagnosis (Y)	36	41	60	NA
Gender	M	M	M	NA
Origin	France	United Kingdom	Caucasian	NA
Recurrent episodes	Yes (6 episodes)	10-12/Y	Every 7-8 weeks	NA
Duration (Weeks)	1-2	2	3-4	NA
Main symptom(s)	Recurrent fever and severe lower back pain	Recurrent fever and severe abdominal pain	Intermittent daily fever	NA
Thoracic symptoms	Acute pericarditis, recurrent thoracic pain	Pleuritic chest pain	No	NA
Neurological symptoms	No	Headache	Headache	NA
Cutaneous lesions	Migratory erythematous plaque eruptions at elbows and left lower limb	Generalized erythema	Erythematous non pruritic rash at trunk	NA
Lymphadenopathy	No	Cervical (painless)	Cervical (painless)	NA
Ocular symptoms	No	Red eye	Bilateral episcleritis	NA
Other symptoms	No	Night sweats and late-onset puberty	No	NA
CRP levels (mg/l) (during one of the attacks)	171	87	210	NA
Family history	No	No	No	NA
Treatment	NSAIDs	Anakinra	Canakinumab	NA

HGVS: Human Genome Variation Society, VAF: variant allele fraction**,** gnomAD: genome aggregation database, Y: year, NSAIDs: non-steroidal anti-inflammatory drugs, CRP: C-reactive protein, NA: not available, *according to ***Infevers*** database (25) (https://infevers.umai-montpellier.fr/web/search.php?n=2).

Figure Legends:

Figure 1. *TNFRSF1A* variants and their impact at the protein level

(a) TNFRSF1A protein domain-organization diagram. Location in the extracellular domain of the protein of the c.176G>A p.(Cys59Tyr) pathogenic variant identified in this study (in red) and the previously reported *TNFRSF1A* mosaic variants (Ser86_Glu93del, Phe89Leu and Thr90Asn) (in black). SP: Signal peptide, CRD: Cysteine-rich domain, TM: transmembrane region, and DD: intracellular death domain.

(b) Upper part: The TNFRSF1A 3D structure includes the four cysteine-rich domains (CRD); CRD1 (blue), CRD2 (purple), CRD3 (green) and CRD4 (yellow) belonging to the extracellular domain, the transmembrane region (TM) (dark red) and the intracellular death domain (DD) (grey). The Cys59 residue (in red) involved in the sequence variant identified in this study is located in CRD1. The previously reported mosaic variants are localized within the first loop of CRD2 (in black). **Lower part:** An illustrative scheme of the amino acid sequence encompassing the three disulfide bridges of CRD1 (S-S1, S-S2 and S-S3, in cyan) and the first disulfide bridge of CRD2 (S-S1, in purple).

(c) *In silico* prediction of structural changes involved by the p.(Cys59Tyr) variant using PremPS tool. **Right Panel**: Cys59 is involved in the second disulfide bridge (S-S2) of CRD1 domain. **Left panel:** Cysteine-to-tyrosine substitution results in disruption of the disulfide bridge and in establishment of a bulky cluster formed by the dense interaction between the tyrosine residue and the surrounding amino acids.
Red balls indicate oxygen molecule; nitrogen atoms are in blue, sulfur atoms are in yellow and carbon atoms are in grey. Dashed lines stand for the interacting bonds between the amino acids; Polar interactions are in cyan, hydrophobic interactions are in blue and carbonyl interactions are in orange.

Figure 2. *TNFRSF1A* DNA sequencing in different cell types

Upper panel: Sanger electrophoregrams showing the identified mosaic *TNFRSF1A* variant c.176G>A p.(Cys59Tyr) (red). The red arrow shows the point mosaic variant (c.176G>A) which

is revealed by overlapping peaks of the WT nucleotide (G in black) and the variant one (A in green). **Lower panel:** Fraction of the variant allele and ratio of the variant reads to the total number of reads identified by a deep-targeted sequencing approach.

Figure 1

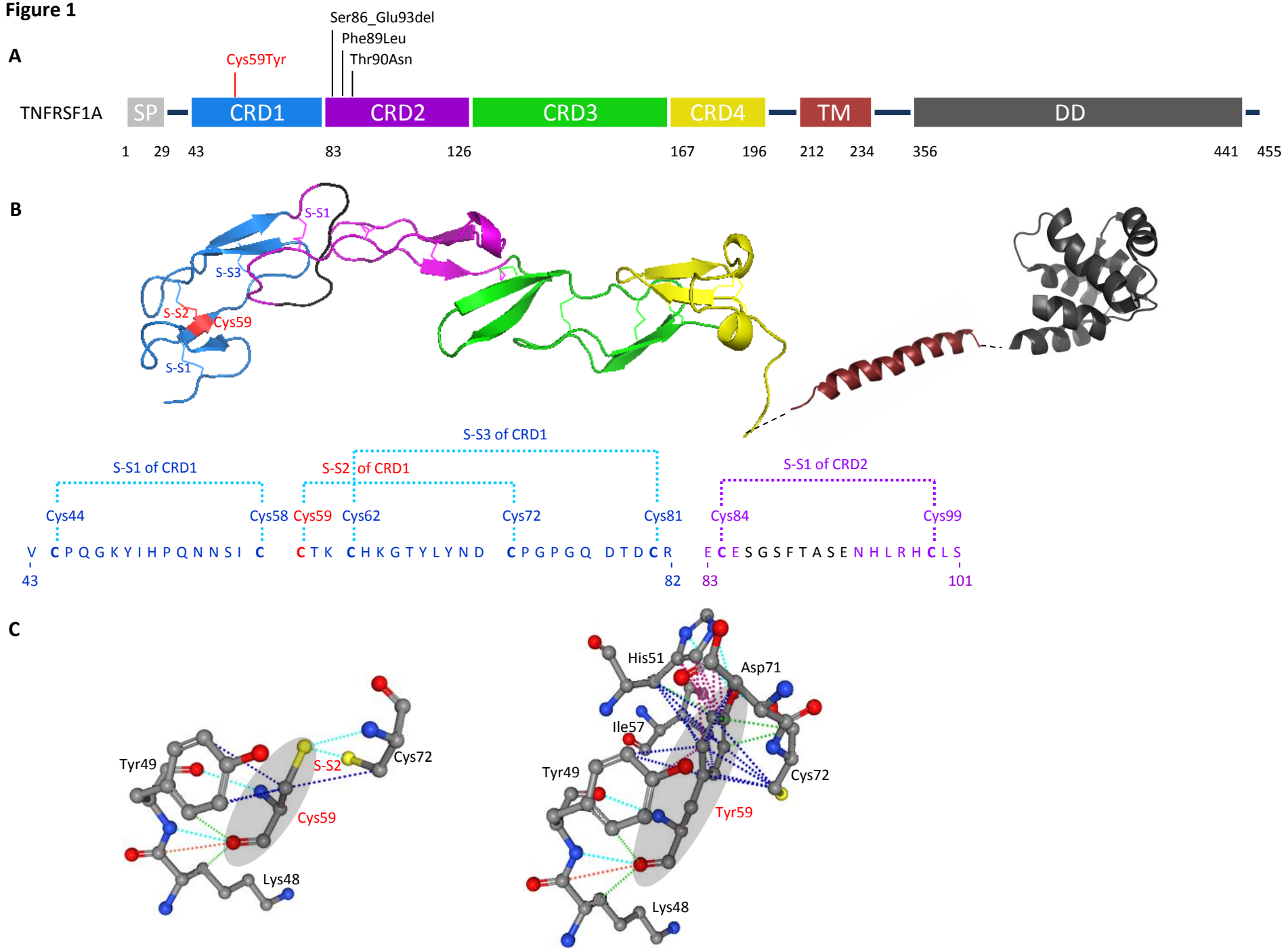
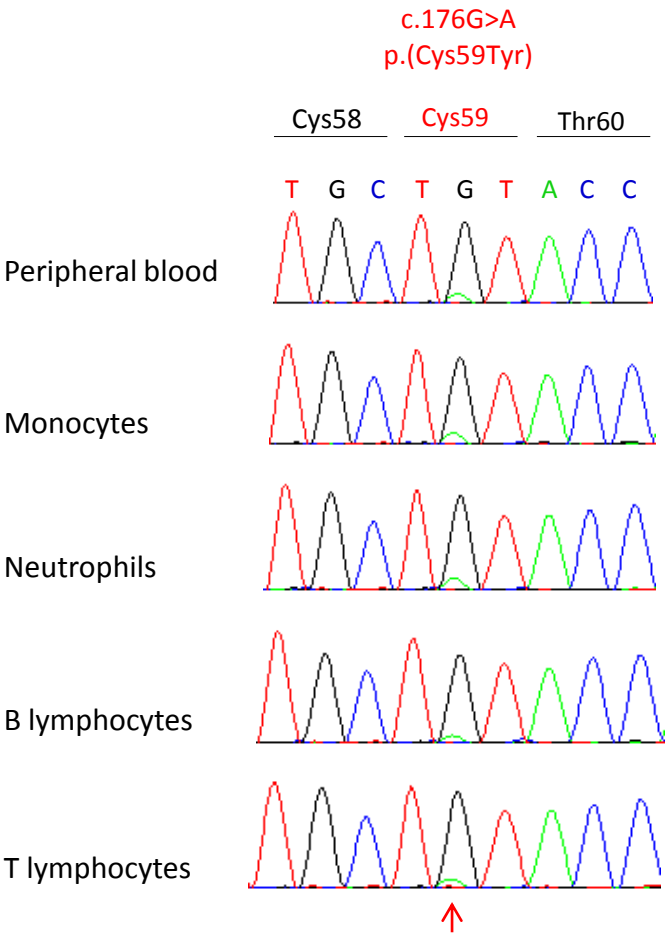

A
Ser86_Glu93del
Phe89Leu
Thr90Asn
Cys59Tyr
TNFRSF1A
SP
CRD1
CRD2
CRD3
CRD4
TM
DD
1
29
43
83
126
167
196
212
234
356
441
455
B
S-S1
S-S3
S-S2
Cys59
S-S1
S-S3 of CRD1
S-S1 of CRD1
S-S2 of CRD1
S-S1 of CRD2
Cys44
Cys58
Cys59
Cys62
Cys72
Cys81
Cys84
Cys99
V C P Q G K Y I H P Q N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S F T A S E N H L R H C L S
43
82
83
101
C
Tyr49
S-S2
Cys72
Cys59
Lys48
His51
Asp71
Ile57
Tyr49
Cys72
Tyr59
Lys48

Figure 2



	Variant allele fraction (variant reads / total reads)
Peripheral blood	14% (125/811)
Monocytes	15.8% (80/864)
Neutrophils	14.1% (59/368)
B lymphocytes	8.9% (130/889)
T lymphocytes	9.6% (85/899)