Candidate Genes in Patients with Autoinflammatory Syndrome Resembling Tumor Necrosis Factor Receptor-associated Periodic Syndrome Without Mutations in the *TNFRSF1A* Gene

SILVIA BORGHINI, MICHELE FIORE, MARCO Di DUCA, FRANCESCO CAROLI, MARTINA FINETTI, GIUSEPPE SANTAMARIA, FRANCESCA FERLITO, FEDERICO BUA, PAOLO PICCO, LAURA OBICI, ALBERTO MARTINI, MARCO GATTORNO and ISABELLA CECCHERINI

ABSTRACT. ***Objective.*** Tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) is an autosomal-dominant multisystemic autoinflammatory condition. Patients display different mutations of the TNF receptor superfamily 1A gene (*TNFRSF1A*), coding for a nearly ubiquitous TNF receptor (TNFR1). No *TNFRSF1A* mutation has been identified in a proportion of patients with TRAPS-like phenotype.

Methods. We investigated mechanisms downregulating the TNF-induced inflammatory response such as (1) receptor shedding, producing a secreted form acting as a TNF inhibitor; (2) receptor internalization with subsequent induction of apoptosis; and (3) negative regulation of nuclear factor-κB (NF-κB) transcription. We analyzed the sequence of genes known to play a pivotal role in these pathways, in 5 patients with TRAPS symptoms and showing shedding and/or apoptosis defects, but without mutations of the *TNFRSF1A* gene.

Results. Sequence analysis of 3 genes involved in TNFR1 shedding (*ERAP1*, *NUCB2*, *RBMX*) and 3 genes involved in negative regulation of NF-κB signaling (*TNFAIP3*, *CARP-2*) or NF-κB transcription (*ZFP36*) revealed only a few unreported variants, apparently neutral.

Conclusion. Our study rules out any involvement in the pathogenesis of TRAPS of some of the genes known to regulate TNFR1 shedding and TNF-induced NF-κB signaling and transcription. Gene(s) responsible for TRAPS-like syndrome remain to be investigated among currently unidentified genes likely involved in these pathways, or by applying the genome-wide function-free sequencing approach. (J Rheumatol First Release April 1 2011; doi:10.3899/jrheum.101260)

Key Indexing Terms:

TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED PERIODIC SYNDROME TNFR1 SHEDDING *TNFAIP3* *CARP-2* *ZFP36* CANDIDATE GENE SCREENING

Tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) is an autosomal-dominant multisystemic autoinflammatory condition. Patients present with long-lasting recurrent fevers associated with abdominal pain, severe arthromyalgias, migratory rashes, fasciitis, periorbital edema, and frequent systemic AA amyloidosis as a longterm complication[1]. TRAPS has been associated with at least 64 different mutations of the TNF receptor superfamily 1A gene (*TNFRSF1A*; GenBank NM_001065.2) encoding for the transmembrane TNFR1 protein, also known as p55 TNFR (Institut de Genetique Humaine, CNRS-UPR1142, Montpellier, France; Website: http://fmf.igh.cnrs.fr/ISSAID/infevers). In particular, most TRAPS-associated *TNFRSF1A* mutations are missense substitutions mainly affecting the highly conserved cysteine residues in the extracellular cysteine-rich domains that are involved in the correct folding of the extracellular portion of the protein by forming disulfide bonds[2]. Indeed, *TNFRSF1A* mutations have been identified in a minority of patients and mainly in association with a positive family history[3,4].

The precise pathogenetic mechanisms underlying

From the Laboratorio di Genetica Molecolare, Istituto G. Gaslini, Genova; Dipartimento di Pediatria e CEBR, Università di Genova, Genova; UO Pediatria II–Reumatologia and Laboratorio di Immunologia delle Malattie Reumatiche, Istituto G. Gaslini, Genova; Laboratorio di Fisiopatologia dell'Uremia, Istituto G. Gaslini, Genova; and Laboratorio di Biotecnologie, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy.

Supported by Italian Telethon (grant GGP07236) and the European Community Seventh Framework Programme (the EUROTRAPS project).

S. Borghini, PhD; M. Fiore, MSc; F. Caroli, BSc; G. Santamaria, BSc; F. Bua, BSc; I. Ceccherini, PhD, Molecular Genetics, Gaslini Institute; M. Di Duca, BSc, Physiopathology of Uremia, Gaslini Institute; M. Finetti, MD; F. Ferlito, MSc; P. Picco, MD; A. Martini, MD, PhD; M. Gattorno, MD, Rheumatology Department, Gaslini Institute; L. Obici, MD, PhD, Biotechnology, IRCCS San Matteo.

Address correspondence to Dr. I. Ceccherini, Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini, Largo Gerolamo Gaslini 5, 16148 Genova, Italy. E-mail: isa.c@unige.it

Accepted for publication February 9, 2011.

TRAPS remain unresolved. TNFR1 is constitutively expressed as a trimeric protein on the cell membrane of most tissues. As well as TNFR1, TNF also binds another membrane receptor, TNFR2 (or p75 TNFR), that is typically found on cells of the immune system. The binding of TNF to TNFR1 results in immediate nuclear factor-κB (NF-κB) activation and subsequent apoptosis. Upon activation, cell-surface receptors are cleaved off by metalloproteinases, producing a secreted form that acts as a natural inhibitor of TNF activity. This process is known as "shedding" and this defect was originally proposed as a possible cause of the TRAPS phenotype[2]. Moreover, a constitutive release of full-length TNFR1 into the membranes of 20–50 nm exosome-like vesicles, capable of binding TNF, has been described in endothelial cells[5]. Finally, after binding TNF, the TNFR1 complex is also able to internalize into the cytoplasm, where the proapoptotic proteins FADD and caspase 8 are recruited. This pathway is induced when the NF-κB activity subsides, thus discontinuing the expression of antiapoptotic factors, and represents an additional means of downmodulating the TNF activity[6]. Recently, the generation of mouse models carrying *Tnfr1* missense mutations homologous to that described in patients with TRAPS suggests that mutant Tnfr1 protein accumulates intracellulary, where it activates JNK and p38 signaling in a ligand-independent fashion. An anomalous response was also reported upon stimulation with low doses of lipopolysaccharide and other immune stimuli, which induced enhanced production of inflammatory cytokines and chemokines[7].

On the basis of these considerations, we analyzed candidate genes possibly involved in the development of a TRAPS-like autoinflammatory phenotype in 5 patients with symptoms of this periodic fever, showing defects in the shedding and/or apoptosis, but without mutations, in the *TNFRSF1A* gene. In particular, we focused on (1) the 3 genes known to be involved in TNFR1 shedding, namely *ERAP1* (endoplasmic reticulum aminopeptidase 1) and its partners *nucleobindin 2* and *RBMX* (RNA-binding motif gene, X chromosome)[8,9,10]; (2) 2 genes responsible for negative regulation of NF-κB activation and TNF-induced apoptosis, namely *TNFAIP3* (tumor necrosis factor alpha-induced protein 3) and *CARP-2*[11,12]; (3) *ZFP36*, whose deficient mouse model develops overproduction of TNF-α[13].

MATERIALS AND METHODS

Patients. Five patients with clinical phenotype and cellular defects resembling TRAPS, and showing no mutation in the whole coding portion and flanking intronic sequences of the *TNFRSF1A* gene, were selected. No other affected family member was reported for any of these patients. Our study protocol was approved by the Ethics Committee of the Gaslini Institute and informed consent obtained from all patients or their parents before enrolment into the study.

Mutation screening. Coding sequences of the following genes were analyzed by polymerase chain reaction (PCR) and direct sequencing: *ERAP1*, *NUCB2*, *RMBX*, *TNFAIP3*, *CARP2*, and *ZFP36*. PCR conditions and primer sequences are reported in Table 1. Primers were designed to include exons and exon-intron boundaries in the respective amplimers. PCR products were enzymatically purified using ExoSAP-IT (GE Healthcare) and directly sequenced using Big Dye v1.1 and a 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA).

TNF-induced shedding and apoptosis on circulating monocytes. Monocytes from patients with TRAPS-like syndrome and healthy controls were obtained from blood samples, washed twice, resuspended in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with penicillin/streptomycin, L-glutamine, nonessential amino acids (Bio-Whittaker, Walkersville, MD, USA), and 10% fetal calf serum (FCS, complete medium; Invitrogen, Carlsbad, CA, USA) at 1×10^6 cells/ml, and seeded in 12-well plates. Apoptosis was induced by treatment with 30 ng/ml recombinant TNF-α (PeproTech EC, London, UK) and 1 μg/ml cycloheximide (Sigma) for 4 h at 37°C, and assessed by staining with fluorescein-2-2 isothiocyanate-conjugated human annexin V (Bender MedSystems, Vienna, Austria) and propidium iodide. Shedding was induced by PMA treatment, and assessed after incubation with anti-p55 TNFR1-PE monoclonal antibody (R&D Systems, Minneapolis, MN, USA) for 30 min at 4°C. In every case, treatments were followed by cell washing and cytofluorimetric analysis (FACScan; BD Biosciences, San Jose, CA, USA), as reported[14].

RESULTS

A total of 5 patients were selected among a large set of patients with autoinflammatory syndrome, with the following criteria: (1) presence of TRAPS-like clinical manifestations and/or positive diagnostic score indicative for a TRAPS phenotype[15]; (2) exclusion of severe, highly penetrant causative mutations of the coding sequence of the *TNFRSF1A* gene; and (3) evidence of a functional defect, assessed in patients' monocytes, of either shedding or TNF-induced apoptosis[14].

These patients displayed long-lasting episodes of fever associated with clinical manifestations typically observed in TRAPS (Table 2), as well as a functional defect (impaired shedding or TNF-induced apoptosis) associated with the TRAPS phenotype. In particular, in contrast to healthy controls, stimulation of patients' monocytes with either TNF and cycloheximide or PMA promoted neither apoptosis nor shedding, respectively, with values within the range for TRAPS patients (Table 3). Significant differences of induced apoptosis and shedding between control and TRAPS cells had been assessed in a previous study[14], and results of the current study clearly showed that patients had characteristics of TRAPS patients carrying *TNFRSF1A* mutations. Therefore all patients underwent molecular analysis for candidate genes possibly involved in the pathogenesis of the TRAPS syndrome phenotype.

DNA obtained from peripheral blood mononuclear cells of the 5 patients listed in Table 2 was analyzed under conditions reported in Table 1 for possible mutations of the coding sequence of the genes *ERAP1*, *NUCB2*, *RMBX*, *TNFAIP3*, *CARP2*, and *ZFP36*. As reported in Table 4, one of these patients was found to carry the missense variant p.R92Q, proposed to represent a low-penetrant hypomorphic mutation[16]. Nonetheless, his disease phenotype was quite severe, characterized by long-lasting fever episodes

Table 1. Polymerase chain reaction (PCR) conditions and primer sequences.

Gene	PCR Size (bp)	Forward Primer	Reverse Primer	PCR Conditions[1] Annealing Temp, Time, s	Cycles
ERAP1 NM_016442					
Exon 2	652	5'-gcttttgtacatttgtgccgc-3'	5'-gggaattttaaaaagacaatcaatct-3' *	59°C 30''	35
Exon 3	432	5'-gcaaacacctgttttagagttcctg-3' *	5'-gaatggcactgttactgaaatcttg-3' *	60°C 30''	35
Exon 4	334	5'-cctacctcggactccccaagtgctg-3' *	5'-tccagttttaaaaccagttccaagc-3' *	60°C 30''	35
Exon 5	559	5'-ggggattaatcaggaacagtcagta-3' *	5'-aataaaatcagaaggcaaaagagtc-3' *	59°C 30'' [2]	35
Exons 6-7	804	5'-atgattatgcacgggcagctg-3'	5'-ctgtcaaggaagttatcagtg-3'	56°C 45'' [2]	35
Exon 8	320	5'-agtatggacttcttgcc-3'	5'-tttaaccccacaaccttgcct-3'	56°C 45''	35
Exons 9-10	548	5'-taacagtgttcctgcagttgc-3'	5'-aagtttggcacagagctgcct-3'	58°C 45''	35
Exon 11	251	5'-taaatgggtgatgtgtctgcc-3'	5'-tcctacaaggcagatgttacc-3'	56°C 45''	32
Exon 12	335	5'-aaactaaccagtgcctggcac-3'	5'-ctatctggcaagatattggcc-3'	60°C 30''	35
Exon 13	333	5'-ccttgcccaatcttggatgag-3'	5'-gaacaataacagctgctttcag-3'	60°C 30''	35
Exon 14	482	5'-gggacatttatatctgattgcttgt-3' *	5'-catacacacacaatgattaacac-3'	60°C 30''	35
Exon 15	367	5'-gcccccatcatctgagaccataatg-3' *	5'-aacttttgtttttccctaatgttta-3' *	60°C 30''	35
Exon 16	411	5'-tcatggggaaagttcagcagttcat-3' *	5'-tttgcctttctcattagatgtgtgc-3' *	58°C 30''	35
Exon 17	404	5'-ttgtggttacccatttatcttttta-3' *	5'-tttctactgcctatcttcaatcaaa-3' *	58°C 30''	35
Exon 18	368	5'-catcatattttgtatttttcacacc-3' *	5'-tgatttatgctgctgtcaacaccat-3' *	60°C 30''	35
Exon 19	569	5'-atcctattcagacagctgggact-3' *	5'-gtgattagagataacaggaacc-3' *	60°C 30''	35
Exon 20		5'-gcacatgcacatgcacatac-3'	5'-catactagcagtagaagaacc-3'	58°C 45''	35
TNFAIP3 NM_006290					
Exon 1	498	5'-ctacagatcagggtaatgac-3'	5'-cttcatgaatggggatccag-3'	55°C 30''	30
Exon 2	351	5'-cttacatgcagataacttgac-3'	5'-catgtgacctagtccatcag-3'	52°C 30''	35
Exons 3-4	609	5'-gtagagtgatgttgtcagaatgac-3'	5'-cctgatgtttcagtgtctag-3'	52°C 30''	35
Exon 5	347	5'-gagatctacttacctatggc-3'	5'-cactctactgttgagcttc-3'	55°C 30''	30
Exon 6A	568	5'-gttctatgagctaatgatg-3'	5'-cttctggacagcagccgctg-3'	55°C 30''	30
Exon 6B	544	5'-cagtgagaccactgccatg-3'	5'-cttctggacagcagccgctg-3'	55°C 30'' [2]	30
Exon 7	401	5'-ctaatctgtatttggaaccc-3'	5'-atcatgaggagacagaacc-3'	55°C 30''	30
Exon 8	447	5'-gactccacactctccaatg-3'	5'-gcaccatgatgactgacagc-3'	56°C 30''	30
RBMX NM_02139					
Exons 2-3	647	5'-cttagttcaaattgcttcctac-3'	5'-cttggacaactacatgttattttatg-3'	56°C 30''	35
Exon 4	477	5'-gcagtgggttcaccttatgtttc-3'	5'-ccacttggtgtgagcttgcag-3'	66°C 30''	35
Exon 5	265	5'-ctttctgattcttgtgagtagtaata-3'	5'-caattctttgtgttacggtagtag-3'	56°C 30''	35
Exons 6-7-8	647	5'-catagcctgaatgtaagttcacg-3'	5'-cagcctaataaactggctcacg-3'	60°C 30''	30
Exon 9	438	5'-gtgttgccatattacctgacc-3'	5'-gaactggaattttggtccaaag-3'	60°C 30''	30

(> 15 days) with painful erythema of legs and arms and muscle-skeletal pain. An episode of fasciitis was also referred. For this reason he was included in our study.

In Table 4 we give all the possible variants detected in the coding regions of these candidate genes. No variant was found in *RMBX*, *TNFAIP3*, and *CARP2* genes. Among the variants identified in the *ERAP1* gene, p.H417H displayed a frequency of the T allele significantly different in our patients compared to that recorded in HapMap ($p = 0.0002$), while p.S453S, p.K528R, and p.L848L presented with frequencies slightly but not significantly higher in our patients. As the frequency comparison for p.S453S approached significance ($p = 0.08$), to identify potential additional splicing sites or altered exonic enhancers, we investigated variants

Table 1. Continued

Gene	PCR Size (bp)	Forward Primer	Reverse Primer	PCR Conditions[1] Annealing Temp, Time, s	Cycles
CARP-2 NM_057178					
Exon 2	353	5'-cactgctcacctgtgctctc-3'	5'-gcattcagaggggtcagtgg-3'	60°C 30''	30
Exon 3	532	5'-ctgaagctcccatcgcactc-3'	5'-gccctcaaactggcacagac-3'	60°C 30''	30
Exon 4	223	5'-gctatgcagatctctgggac-3'	5'-ctccactcgactctgctaag-3'	60°C 30''	30
Exon 5	295	5'-gagctgagcacactgatctc-3'	5'-gcaaacactcctgagctctg-3'	60°C 30''	30
Exon 6	243	5'-gatgtcagggcagtaagagac-3'	5'-cctgggtctcagtgagtttc-3'	60°C 30''	30
Exon 7	323	5'-cattctcctcactcctctgc-3'	5'-ctagcttgctcctctgcaag-3'	60°C 30''	30
NUCB2 NM_005013					
Exon 3	420	5'-cggaatgcatgtatatagagttaaaac-3'	5'gcatatgcttggccaatactc-3'	57°C 30''	30
Exon 4	369	5'-caaacaaggaaaaacctgcagaagca-3'	5'-caagcatggtggcacgcacc-3'	60°C 30''	30
Exon 5	411	5'-cagcctcccaaagtgctg-3'	5'-atcctacctttttgtttagcac-3'	60°C 30''	30
Exon 6	329	5'-ctagcttctagaaattgatcatttac-3'	5'-gggattttagacctgtatgtac-3'	57°C 30''	30
Exons 7-8	646	5'-ccacttctgttcaagagtatgc-3'	5'-agcagaccttggcagaaatc-3'	57°C 30''	30
Exons 9-10	449	5'-gatgcttcacatcagtactac-3'	5'-caaattctgtatattttccatcttaaag-3'	57°C 30''	30
Exon 11	281	5'-ctgtggaagttaagtgagtc-3'	5'-gtagaatagactgatgtagaatttg-3'	57°C 30''	30
Exon 12	425	5'-ccccttccttcttacctcca-3'	5'-ctatagtatagctatagcagtgtac-3'	57°C 30''	30
Exons 13-14	632	5'-gtcaaaggtcacacatatgatag-3'	5'-gatgaaaatagatgttgagttaacagc-3'	57°C 30''	30
ZFP36 NM_003407					
Exon 1	432	5'-ccaagctcaggcgcgtcc-3'	5'-ccaagctggtctgagcgg-3'	62°C 40''[2]	35
Exon 2A	512	5'-gcctggcaagctctagttccc-3'	5'-cggccctggaggtagaacttg-3'	56°C 40''	35
Exon 2B	469	5'-ggaccttctcagagagtgggc-3'	5'-gtggggtctcttcgagccag-3'	62°C 40''	35
Exon 2C	592	5'-ccttccctgtcctccagctc-3'	5'-cctcggaagacactccatccc-3'	62°C 40''	35
Exon 2D	408	5'-ggtctctgcatggaccccag-3	5'-gggtttggcaacggctttggc-3'	64°C 40''	35
Exon 2E	469	5'-gacctgaggttccagtgtctcc-3'	5'-cacttcctgccaggtaagcgc-3'	66°C 50''[2]	35

[1]Primers were designed to analyze exons and exon-intron boundaries. One hundred nanograms of DNA were amplified in 25 µl reactions containing 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl, 50 mM KCl, 200 µl dNTPs, and 1.25 U Taq polymerase (TaqGold, Applied Biosystems) with 1µM of each appropriate PCR primer. PCR of RBMX exon 5 was performed with 3 mM $MgCl^2$ and PCR of TTP exons 1 and 2F with 1 mM $MgCl^2$. An initial denaturation step at 95°C for 5 min and an extension cycle at 72°C for 7 min were performed. For each cycle, denaturation at 95°C and extension at 72°C were performed for the same time length of the annealing step, as reported. PCR products were enzymatically purified using Exo-SAP-IT and directly sequenced using Big Dye v1.1 and the 3130 automated sequencer (Applied Biosystems). [2]DMSO 5% was added as enhancer. *Primers as described[18].

p.H417H and p.S453S using the Spliceport software (Website: http://spliceport.cs.umd.edu/) and Rescue ESE (Website: http://genes.mit.edu/burgelab/rescue-ese/). No effect, however, was suggested by these *in silico* analyses.

The in-frame 3 nucleotide deletion found in the nucleobindin 2 gene has been recorded in the SNPs database: (http://www.NCBI.NLM.NIH.gov/projects/SNP/), although not reported yet in population studies. Therefore, we sequenced DNA samples from 64 control individuals and calculated a frequency of 0.33 for the variant allele, which was not significantly different from that estimated in our patients.

An unreported synonymous nucleotide variant was also identified in the *ZFP36* coding sequence in one of the 5 patients. *In silico* analysis using the software noted above revealed no formation of additive splice sites or exonic enhancers.

DISCUSSION

To determine the molecular basis sustaining pathogenesis in a small set of patients with TRAPS-like syndrome, we focused on a number of genes that, once mutated, may at some point dysregulate the TNFR1-mediated response to

Table 2. Characteristics of patients with TRAPS-like syndrome: clinical features and functional cellular defects and genotypes at the *TNFRSF1A* and other candidate gene loci.

Characteristic	Patient 1	2	3	4	5
Sex	M	F	F	M	M
Age, yrs	43	24	30	22	32
Disease onset, yrs	15.0	2.0	5.0	2	9
Duration of fever episodes, days	25.0	22.5	6.0	20	7
Frequency, episodes/yr	2	3	4	3	4
Abdominal pain	++	+	++	+	+
Arthritis/arthralgia	+	+	++	++	++
Rash	–	+	–	++	–
Ocular manifestations†	–	+	–	–	–
Treatment	CS, NSAID	CS, NSAID	CS, NSAID, anakinra	CS, NSAID	CS, NSAID

† Periorbital edema in Patient 2. CS: corticosteroids on demand; NSAID: nonsteroidal antiinflammatory drugs.

Table 3. Functional findings in patients with TRAPS-like syndrome. Data are percentages.

Group	Apoptosis*, median % (range)	Shedding** median % (range)
Healthy controls (n = 12)	+366 (+87/ +718)	–78 (–69/ –89)
TRAPS patients (n = 8)	0 (–25/ +50)	–48.6 (–31/ –53)
1	25	–49.2
2	ND	–36.1
3	31	–50.3
4	ND	–42.3
5	46.7	ND

* Expressed as percentage change of annexin V-positive cells after stimulation with tumor necrosis factor and cycloheximide compared to untreated cells; ** expressed as percentage change of p55 TNFR1 expression after PMA stimulation (see D'Osualdo, *et al*[15]). ND: not determined.

TNF, thus likely promoting a TRAPS-like autoinflammatory condition.

We started from analysis of genes coding for some membrane proteins that have recently been described in association with TNFR1, promoting TNFR1 release from human vascular endothelial cells. These are (1) ERAP1 (endoplasmic reticulum associated aminopeptidase 1), also known as ARTS-1 (aminopeptidase regulator of TNFR1 shedding), a type II integral membrane protein that binds full-length TNFR1 and regulates both the constitutive release of TNFR1 exosome-like vesicles and the proteolytic cleavage of soluble TNFR1 ectodomains[8]; (2) nucleobindin 2 (NUCB2), a calcium-dependent ERAP1 binding partner, which associates with cytoplasmic TNFR1 prior to its commitment to either release pathway[10]; and (3) RBMX (RNA-binding motif gene, X chromosome), which associates with ERAP1, regulating both constitutive and inducible pathways[9]. With the exception of the ERAP1 synonymous variant p.H417H, which was associated with patients reported here and which requires further investigation to exclude its role in the maturation of gene transcript, our study has ruled out that the 3 genes involved to date in TNFR1 shedding play a role in the TRAPS-like phenotype. This suggests that other genes are involved or that the observed shedding defect is only a secondary effect in this periodic fever.

However, shedding is not the only mechanism that down-modulates TNF-induced NF-κB activation. *TNFAIP3* (tumor necrosis factor alpha-induced protein 3), also known as A20, is an early NF-κB-responsive gene encoding for a dual ubiquitin-editing enzyme involved in the negative feedback regulation of NF-κB signaling. Although aspects of TNFAIP3 biology are unclear, its role in the regulation of TNFR1 signaling by ubiquitination has been demonstrated[11]. In particular, TNFAIP3 interacts with a multiprotein complex where it has been shown to remove the ubiquitin chains from lysine 63 of RIP1, a modification necessary for the translocation of NF-κB into the nucleus. Subsequently, TNFAIP3 catalyzes the addition of ubiquitin chains to lysine 48 of RIP1, promoting its degradation by proteasome and termination of NF-κB activation[11]. In addition, a recent report demonstrated that CARP-2, a protein with ubiquitin ligase activity, is recruited to early TNFR1 complexes inside the endocytic vesicles, where it targets internalized RIP for proteasome-mediated degradation, thus limiting TNF-induced NF-κB activation[12]. In light of this, we included *TNFAIP3* and *CARP-2* in our list of candidate genes. A further control mechanism relies on transcriptional regulation of NF-κB activity. Among the proteins involved in this process, ZFP36, also called TTP, a zinc finger-containing protein that destabilizes mRNA by binding AU-rich elements in their 3'UTR, has been described as a negative regulator of gene expression[17]. Since mice deficient in

Table 4. TNFRSF1A and other candidate genes and variants detected in their coding sequences.

Gene	GenBank ID	Exon	Nucleotide Variation	Amino Acid Variation	dbSNP Number	Public Minor Allele Frequencies (MAF)*	MAF in Present Patients**
TNFRSF1A	NM_001065	10	c.362 G>A	p.R121Q	Regarded as hypomorphic mutation***		1/10
ERAP1	NM_016442	2	c.171c>t	p.Y57Y	Unreported		1/10
		2	c.380g>c	p.R127P	rs26653	C = 0.28	2/10
		5	c.828a>g	p.1276M	rs26618	G = 0.25	2/10
		6	c.1045 a>g	p.M349V	rs2287987	G = 0.24	4/10
		6	c.1068 t>c	p.A356A	rs27434	T = 0.18	1/10
		8	c.1251c>t	p.H417H	rs3213809	T = 0.01	4/10†
		9	c.1359c>t	p.S453S	rs27529	T = 0.31	6/10
		11	c.1583a>g	p.K528R	rs30187	A = 0.30	5/10
		12	c.1723g>a	p.D575N	rs10050860	A = 0.24	3/10
		13	c.1911 g>a	p.A637A	rs469783	G = 0.39	5/10
		15	c.2174g>a	p.R725Q	rs17482078	A = 0.18	2/10
		15	c.2188c>g	p.Q729E	rs27044	C = 0.25	3/10
		17	c.2542 c>t	p.L848L	rs17481856	T = 0.12	3/10
NUCB2	NM_005013	12	c.1012c>g	p.Q338E	rs757081	C = 0.37	4/10
		13	c.1203delACA	ΔQ402	rs3842269	delACA = 0.33	1/10
RBMX	NM_02139	No variant of the coding portion of this gene has been detected					
CARP2	NM_057178	No variant of the coding portion of this gene has been detected					
TNFAIP3	NM_006290	No variant of the coding portion of this gene has been detected					
ZFP36	NM_003407	2	c.447 G>A	p.K149K	Unreported		1/10

* Reported in HapMap. ** Number of variant alleles out of total patients' chromosomes investigated. *** Infevers Website: http://fmf.igh.cnrs.fr/ISSAID/infevers/ † p = 0.0002.

ZFP36 develop a severe inflammatory syndrome characterized by overproduction of TNF-α, we included this gene in our study[13]. Our study has also ruled out causative mutations in the coding sequences of these genes, suggesting that other, unidentified genes are involved in these pathways, and novel candidate genes for TRAPS-like syndrome remain to be investigated.

We applied a candidate gene approach to a small set of patients, carefully selected for their clinical symptoms and for impairment of TNF-induced shedding and/or apoptosis, reflecting anomalous TNFR function. The inflammatory cell response is emerging as a broad and complex cellular mechanism, with many control steps and cross-talk pathways inducing opposite effects; we believe that identifying the causative gene(s) for TRAPS-like disorders will require a more straightforward and function-free approach than the strategies applied to date. In this respect, the emerging potential of the next generation of exome sequencing analysis provides a novel method for studies in this area of medical genetic research.

ACKNOWLEDGMENT

We are grateful to Loredana Velo for excellent secretarial assistance.

REFERENCES

1. McDermott M. Autosomal dominant recurrent fevers. Clinical and genetic aspects. Rev Rhum Engl Ed 1999;66:484-91.
2. McDermott M, Aksentijevich I, Galon J, McDermott E, Ogunkolade B, Centola M, et al. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell 1999;97:133-44.
3. Aksentijevich I, Galon J, Soares M, Mansfield E, Hull K, Oh H, et al. The tumor-necrosis-factor receptor-associated periodic syndrome: new mutations in TNFRSF1A, ancestral origins, genotype-phenotype studies, and evidence for further genetic heterogeneity of periodic fevers. Am J Hum Genet 2001;69:301-14.
4. Aganna E, Hammond L, Hawkins P, Aldea A, McKee S, van Amstel H, et al. Heterogeneity among patients with tumor necrosis factor receptor-associated periodic syndrome phenotypes. Arthritis Rheum 2003;48:2632-44.
5. Hawari F, Rouhani F, Cui X, Yu Z, Buckley C, Kaler M, et al. Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: a mechanism for generation of soluble cytokine receptors. Proc Natl Acad Sci USA 2004;101:1297-302.
6. Muppidi J, Tschopp J, Siegel R. Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity 2004;21:461-5.
7. Simon A, Park H, Maddipati R, Lobito AA, Bulua AC, Jackson AJ, et al. Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. Proc Natl Acad Sci USA 2010;107:9801-6.
8. Cui X, Hawari F, Alsaaty S, Lawrence M, Combs C, Geng W, et al. Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding. J Clin Invest 2002;110:515-26.
9. Adamik B, Islam A, Rouhani F, Hawari F, Zhang J, Levine S. An association between RBMX, a heterogeneous nuclear ribonucleoprotein, and ARTS-1 regulates extracellular TNFR1

release. Biochem Biophys Res Commun 2008;371:505-9.
10. Islam A, Adamik B, Hawari F, Ma G, Rouhani F, Zhang J, et al. Extracellular TNFR1 release requires the calcium-dependent formation of a nucleobindin 2-ARTS-1 complex. J Biol Chem 2006;281:6860-73.
11. Shembade N, Parvatiyar K, Harhaj N, Harhaj E. The ubiquitin-editing enzyme A20 requires RNF11 to downregulate NF-kappa B signalling. EMBO J 2009;28:513-22.
12. Liao W, Xiao Q, Tchikov V, Fujita K, Yang W, Wincovitch S, et al. CARP-2 is an endosome-associated ubiquitin ligase for RIP and regulates TNF-induced NF-kappa B activation. Curr Biol 2008;18:641-9.
13. Carballo E, Lai W, Blackshear P. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science 1998;281:1001-5.
14. D'Osualdo A, Ferlito F, Prigione I, Obici L, Meini A, Zulian F, et al. Neutrophils from patients with TNFRSF1A mutations display resistance to tumor necrosis factor-induced apoptosis: pathogenetic and clinical implications. Arthritis Rheum 2006;54:998-1008.
15. Gattorno M, Sormani M, D'Osualdo A, Pelagatti M, Caroli F, Federici S, et al. A diagnostic score for molecular analysis of hereditary autoinflammatory syndromes with periodic fever in children. Arthritis Rheum 2008;58:1823-32.
16. Ravet N, Rouaghe S, Dodé C, Bienvenu J, Stirnemann J, Lévy P, et al. Clinical significance of P46L and R92Q substitutions in the tumour necrosis factor superfamily 1A gene. Ann Rheum Dis 2006;65:1158-62.
17. Liang J, Lei T, Song Y, Yanes N, Qi Y, Fu M. RNA-destabilizing factor tristetraprolin negatively regulates NF-kappa B signaling. J Biol Chem 2009;284:29383-90.
18. Yamamoto N, Nakayama J, Yamakawa-Kobayashi K, Hamaguchi H, Miyazaki R, Arinami T. Identification of 33 polymorphisms in the adipocyte-derived leucine aminopeptidase (ALAP) gene and possible association with hypertension. Hum Mutat 2002;19:251-7.