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Identification of a novel exon 2-skipped TNFR1 transcript: regulation by three functional polymorphisms of the TNFR-Associated Periodic Syndrome (TRAPS) gene

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ABSTRACT

Objectives: Mutations in the TNFRSF1A gene encoding the TNF cell surface receptor, TNFR1, cause TNFR-associated periodic syndrome (TRAPS) and polymorphisms in TNFRSF1A, including rs4149570, rs767455 and rs1800692, are associated with inflammatory diseases. We describe a novel exon 2-spliced transcript, named TNFR1-d2, and the impact of these 3 SNPs on exon 2 splicing, transcriptional activity of TNFRSF1A and TRAPS phenotype.

Methods: Expression of TNFRSF1A transcripts was performed by RT-PCR in a range of human cells and tissues. Exon 2 splicing and transcriptional activity were analysed in HEK293T and SW480 cells by in vitro alternative splicing and luciferase assays, respectively. We constructed haplotypes containing rs4149570, rs767455 and rs1800692 in controls (n=70), TRAPS (n=111) and TRAPS-like patients (n=450) to compare their distribution and association with clinical features of TRAPS.

Results: TNFR1-d2 was expressed in a tissue-specific manner, whereas TNFR1 expression was ubiquitous. Alternative splicing assays revealed that the T-A-T haplotype at rs4149570-rs767455-rs1800692 showed the highest expression of exon 2-skipping product ($p=0.02$). Transcriptional activity from the T-T haplotype at rs4149570-rs1800692 was increased compared to the G-C haplotype ($p=0.03$). In TRAPS patients, rs1800692 T/T homozygotes were excessively rare ($p<10^{-4}$) and TRAPS-like patients with this genotype experienced less fever.

Conclusions: Our study provides a novel mechanism of TNFRSF1A regulation whereby three polymorphisms in the promoter, exon 1 and intron 4 have a functional and combined effect on exon 2 splicing, via a coupling mechanism between transcription and splicing. These polymorphisms may impact the phenotype of TRAPS and TRAPS-like patients.

Keywords

TNFR1 transcript; alternative splicing; TRAPS; autoinflammatory disease; recurrent fever

INTRODUCTION

The biological response to tumor necrosis factor-α (TNF) is mediated by specific cell surface receptors. Among these, TNFR1 (TNF receptor 1, also known as p55 or CD120a) plays a pivotal role by inducing various cellular responses including cell death, differentiation or inflammation[1-2]. Inappropriate activation of TNF signalling is implicated in the pathogenesis of a wide spectrum of inflammatory disorders with a major role in autoinflammatory and autoimmune diseases[3].

Mutations in the TNFR1 gene, TNFRSF1A (TNFR superfamily, member 1A, NC_000012.11), are responsible for the dominantly inherited autoinflammatory disease, TNFR-Associated Periodic Syndrome (TRAPS[4-5]). TRAPS, belongs to the group of hereditary recurrent fevers (HRF)[6] which are characterized by recurring inflammatory bouts of fever, abdominal pain, arthritis, and cutaneous manifestations. Patients with symptoms compatible with TRAPS but no demonstrable TNFRSF1A mutations (named TRAPS-like patients)[7-9] remain difficult to manage and treat. The reported mechanisms underlying TRAPS pathophysiology, including defective TNFR1 receptor shedding, NF-κB activation, apoptosis, autophagy, mitochondrial reactive oxygen species generation and disordered protein folding may be cell- and/or mutation-dependent[10-13]. In addition to the TRAPS-causing mutations, a growing number of genetic studies have identified several single nucleotide polymorphisms (SNPs) in the TNFRSF1A gene as susceptibility or predictive markers of multifactorial inflammatory disorders[14-16]. One of the functional consequences of these variants could be linked to regulation of gene expression[17].

In the course of TNFRSF1A expression studies, we have identified a new TNFRSF1A spliced transcript that lacks exon 2, which we named TNFR1-d2. We describe the impact of rs4149570, rs767455 and rs1800692 SNPs in the promoter, exon 1 and intron 4, respectively of TNFRSF1A, on the regulation of expression of TNFR1-d2 and on the TRAPS phenotype.

MATERIALS AND METHODS

Healthy controls and patients

Genotyping analyses were conducted on a total of 72 healthy controls and 561 patients referred to either our laboratories (n=504) or to the EUROTRAPS consortium (n=57). All individuals were of European ancestry. The 450 patients in whom we found no mutation through our routine screening strategy were named "TRAPS-like" because their referring physician considered their symptoms sufficiently evocative to request a genetic diagnosis of TRAPS (see online supplementary table S1). Borghini et al addressed genetic heterogeneity in TRAPS-like patients, but this study did not validate the candidate genes[8].

The project was approved by the Comité de Protection des Personnes Sud Méditerranée IV, and all participating individuals signed an informed consent.

Expression analysis of TNFRSF1A transcripts

Blood samples were collected in heparin tubes. PBLs were purified in blood lysis buffer whereas peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated using lymphocyte separation medium (Eurobio), as described previously[18]. Cells were saved in TRIzol RNA isolation reagent (Gibco BRL) until RNA extraction. Total RNA from various tissues (human total RNA master panel II, Clontech) were also examined. One µg RNA was reverse-transcribed (RT) into complementary DNA (cDNA) using random primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV, Fisher).

The TNFR1 (NM_001065.3), TNFR1-d2 and the reference B2-microglobulin (B2M) cDNAs were co-amplified by multiplex PCR and were separated on an 8% acrylamide gel and stained in ethidium bromide (see also online supplementary text and table S2).

Plasmid construction

To generate the splicing reporter constructs, we inserted TNFRSF1A genomic fragments into the pSpliceExpress (pSE) reporter vector[19]. To test the transcriptional activity by luciferase assay, the TNFRSF1A promoter and intron 4 were cloned into the pGL3-basic vector (Promega Corporation). Plasmid constructions are described in online supplementary text and table S3.

Cell culture

Human embryonic kidney 293 (HEK293T) cells and colon adenocarcinoma (SW480) cells were grown as described in online supplementary text. Cells were seeded onto 12 (minigene experiments) or 24 (luciferase experiments) well plates, one day prior to transfection. Cells were transfected with Lipofectamine^{TM}2000 (Invitrogen) according to the manufacturers' instructions and were harvested with appropriate lysis buffer 24h after transfection.

Alternative splicing assay

HEK293T and SW480 cells were co-transfected with 1.6µg of minigene construct and 200ng of pRL-TK Vector (Promega) to correct for transfection efficiency. Total RNA was purified with the RNAeasy Plus Mini Kit using the automated QIAcube system (Qiagen). After reverse transcription using random primers and M-MuLV Reverse Transcriptase, the cDNAs obtained were subjected to quantitative PCR. All results were obtained from four independent transfections. Quantification of the exon 2 skipping and inclusion was performed by Real-Time quantitative PCR in SYBR-Green I mixture (Roche Diagnostics). We moreover quantified the expression of both the endogenous GAPDH and transfected Renilla luciferase cDNA as an additional reference transcript to correct for transfection efficiency (see online supplementary text).

Luciferase assay

HEK293T and SW480 cells were transfected with 700ng pGL3-construct. One hundred ng of vector containing the β-galactosidase gene (pCMVβ, Clontech Laboratories) was co-transfected to normalize the transfection efficiency of all luciferase reporter constructs. Cells were lysed in 200µl 1×lysis buffer (Promega) per well. Protein concentrations were determined using the Bradford protein assay kit (Sigma-Aldrich). Firefly luciferase activity was measured using Dual-Luciferase reporter Assay System, (Promega), according to the manufacturer's instructions. β-galactosidase activity was measured on 20µl cleared lysate after addition of 140µl 0.34mg/ml Chlorophenol Red β-D-galactopyranoside, 2mM $MgCl_2$ containing buffer. Relative luciferase activities were normalized according to protein concentration and β-galactosidase activity. Data were expressed as a mean of seven independent experiments performed in duplicate and were represented as a percentage of the relative luciferase activity obtained with the pGL3-promoter vector.

Genotyping analyses

The genotyping analyses performed in controls and patients are detailed in online supplementary text and table S4.

Statistical analysis

Data and statistical analyses of minigene-splice products, and luciferase assays were processed using the GraphPad Prism 4 software (GraphPad Software). Statistical significance was calculated using the non-parametric Mann-Whitney U test.

The SNP genotype distributions and their association with the clinical signs of TRAPS were evaluated using a Chi square test (χ^2) with 2 degrees of freedom or the Fisher's exact test. Statistical significance was defined as $p<0.05$ for all calculations.

RESULTS

Identification of TNFR1-d2, a novel TNFRSF1A spliced transcript

In the course of TNFRSF1A expression studies by multiplex RT-PCR, we identified a novel alternatively spliced transcript that skipped exon 2 which we named TNFR1-d2 (GenBank accession number JN172914) (figure 1A). Whereas TNFR1 was ubiquitously expressed, TNFR1-d2 was predominantly expressed in human peripheral blood leukocytes (PBLs), brain, heart, kidney, skeletal muscle, small intestine and spinal cord, but not in liver or lung, for example (figure 1B). These results suggest that expression of these two TNFRSF1A transcripts is differentially regulated, with a tissue-specific expression of TNFR1-d2.

Identification of potential cis-regulatory elements of TNFRSF1A expression

In order to gain insight into the regulation of TNFRSF1A alternative splicing, we selected potential cis-regulatory elements of TNFRSF1A expression located in regions surrounding SNPs previously identified as functional polymorphisms and/or susceptibility markers of inflammatory disorders.

The SNP closest to exon 2, rs1800692 (c.473-33C>T) located in intron 4, belongs to a chromosomal segment known to contain a linkage block[16], which includes rs767455 (c.36A>G, p.Pro12Pro) in exon 1. Stanke et al. showed that the A-T haplotype at rs767455-rs1800692 was associated with disease severity in cystic fibrosis patients[16]. Moreover, rs767455 associated with Crohn's disease[20], is likely to be located in a splicing regulatory sequence in the 3'end of exon 1 (see online supplementary text).

rs4149570 (c.-610G>T) in the TNFRSF1A promoter was previously correlated with TNFRSF1A expression[21]. Moreover, linkage disequilibrium (LD) analyses of our controls and patients disclosed that rs4149570 was in complete LD with rs1800692 in intron 4 (D'=1, see online supplementary table S5). We therefore studied the effects of the genomic regions including rs1800692, rs767455 and rs4149570 on exon 2 splicing and transcriptional activity.

The haplotype T-A-T at rs4149570-rs767455-rs1800692 promotes exon 2 skipping

To address whether sequences in the promoter, exon 1 and intron 4 have an effect on the exon 2 splicing, we performed an in vitro alternative splicing assay, using minigene vectors containing the 5' end of TNFRSF1A, extending from the promoter to intron 1 and a genomic region extending from the 3' end of intron 1 to intron 4 (figure 2A). Minigene constructs containing the four haplotypes observed in LD analyses (see online supplementary table S5): G-G-C, G-A-C, T-G-T and T-A-T at rs4149570-rs767455-rs1800692 were transfected into HEK293T and SW480 cell lines.

Quantitative experiments, using specific primers designed to amplify the exon 2-skipping (figure 2B), revealed that the highest expression of exon 2-skipping product in HEK293T cells was obtained with the major haplotype T-A-T, and lowest with the G-G-C haplotype (p=0.02), whereas the two minor G-A-C and T-G-T haplotypes resulted in intermediate levels. This haplotype-dependent differential exon 2 splicing was not seen in SW480 cells (figure 2B). In contrast, the level of exon 2-inclusion product was not influenced by the genotype whatever the cell line (figure 2C). These results were confirmed by considering exon 2 skipping/inclusion ratio (see online supplementary figure S1).

To better tackle the impact of these three regions on exon 2 splicing, we investigated two additional series of minigene constructs. The results obtained with constructs containing the TNFRSF1A promoter and exon 1 regions revealed that the level of exon 2 skipping/inclusion ratio was higher with the A-allele at rs767455 in exon 1 regardless of the allele at rs4149570 in the promoter and the cell line (see online supplementary text and figure

S2). In addition, the results obtained with constructs containing only the genomic region extending from the 3' end of intron 1 to intron 4 demonstrated that rs1800692 in intron 4 was sufficient to modulate TNFRSF1A exon 2 splicing, in HEK293T cells (see online supplementary text and figure S3).

Taken together, our results show that these three TNFRSF1A regions may have a combined functional effect on exon 2 splicing in a cell-specific manner.

Combinatorial effect of the intron 4 sequence and the TNFRSF1A promoter on transcriptional activity

Since it is well documented that regulation of transcription can also modulate splicing, as transcription and splicing processes are coupled[22], we evaluated the impact of intron 4 on the transcriptional activity of the TNFRSF1A promoter. We generated luciferase vectors containing the promoter together with the full intron 4 sequence, or with intron 4 deleted from c.473-72 to c.473-29 including rs1800692. As the G allele at rs4149570 was always found with the C allele at rs1800692 (see online supplementary table S5), only the two G-C and T-T haplotypes were assessed. In HEK293T cells, the presence of the intron 4 sequence carrying the C allele significantly decreased the relative luciferase activity compared to that with the TNFRSF1A promoter alone ($p=0.009$, figure 3) or the deleted intron 4, whereas the T-allele in intron 4 did not significantly modify the transcriptional level. The transcriptional activity obtained with the T-T haplotype was increased as compared to the G-C haplotype ($p=0.03$, figure 3). In contrast, no differences were observed in SW480 cells. These results suggest that the intron 4 sequence could contain transcriptional regulatory element(s). In addition, the effect of intron 4 on transcriptional activity was only rs1800692-dependent in HEK293T. This hypothesis was confirmed by investigating the effect of intron 4 on the transcriptional activity of constitutively active SV40 promoter (see online supplementary text and figure S4).

We conclude that intron 4 and the promoter of the TNFRSF1A gene have a combinatorial effect on its transcription.

rs1800692 in intron 4 of TNFRSF1A as a possible TRAPS phenotype modifier

To evaluate whether the rs1800692 and rs767455 polymorphisms (in intron 4 and exon 1, respectively) could influence the patients' phenotype, we compared the genotype distribution in controls, in patients suffering from TRAPS, and in a TRAPS-like group (patients in whom no TNFRSF1A mutation was found). rs4149570 (in promoter) was not

investigated since it was found in complete LD with rs1800692 (see online supplementary table S5) and therefore association studies would be expected to give similar results.

While the rs1800692 genotypic distribution was comparable in our controls and in the TRAPS-like group, only one T/T homozygote carrying a p.Thr79Lys (usual name T50K) mutation (c.236C>A)[5] was present in 111 available genetically confirmed TRAPS patients ($p<10^{-4}$) (table 1). The skewed distribution of T/T homozygotes was observed in both patients with mild p.Arg121Gln (usual name R92Q) variant (c.362G>A; n=53, p=0.003)[23-24] or other mutations (n=58, p=0.007) ruling out that the rarity of T/T homozygotes was attributable to strong LD between p.Arg121Gln and the C allele at rs1800692. We observed similar genotype distributions in two other control Caucasian populations (table 1). Using allele-specific PCR or RFLP strategies, we determined phase in our TRAPS patients heterozygous at rs1800692 (in intron 4) and observed that the T-allele was always located in cis with the wild type allele, apart from 3 patients, carrying a cysteine mutation (data not shown). This result, together with the fact that one T/T TRAPS patient was identified, suggests that the combination of two T-alleles plus one TRAPS mutation is not lethal. Homozygotes for A/A were also very rare at rs767455 (in exon 1, n=9) in TRAPS patients compared to controls ($p<10^{-7}$, data not shown).

We then compared the 3 genotypes at rs1800692 for the major clinical signs of TRAPS and detected a significant association of this SNP with fever in TRAPS-like patients (chi-square with two degrees of freedom, p=0.01, table 2). We observed a protective effect of the T/T genotype as compared to the other genotypes (CC+CT vs TT); p=0.007, OR=0.44 [0.25-0.78]). rs767455 (in exon 1) alone was not associated with fever in TRAPS-like patients, but the A-T haplotype at rs767455-rs1800692 was protective, as determined by haplotype simulation studies (p=0.02, OR=0.66 [0.46-0.94]).

DISCUSSION

We have identified and characterized TNFR1-d2, a novel spliced transcript of the TNFRSF1A, the gene responsible for TRAPS. Whilst the existence of two functional alternative transcripts expressed by the second TNFR gene (TNFSFR1B encoding TNFR2 or p75) was identified in 2001 and 2004[25-26], data about TNFR1-d2 are absent in the literature. Interestingly, a TNFRSF1A alternative transcript lacking exon 6 (Δ6-TNFR1) has recently been identified[27].

In vitro alternative splicing and transcriptional activity assays revealed that exon 2-skipping increased with the T-A-T haplotype at rs4149570-rs767455-rs1800692 in the promoter, exon 1 and intron 4, respectively, as compared to the G-G-C haplotype (figure 2 and online supplementary figure S1); furthermore transcriptional activity increased with the T-T haplotype compared to the G-C haplotype at rs4149570-rs1800692 in the promoter and intron 4 (figure 3). A similar effect was observed when rs1800692 in intron 4 was investigated alone, i.e. increased exon 2-spliced ratio and transcriptional activity with the C-allele as compared to the T-allele (see online supplementary figures S3 and S4, respectively). These results suggest that regulation of TNFR1-d2 expression may occur via a coupling mechanism between TNFRSF1A transcription and exon 2 splicing with a combined functional effect of the promoter, exon 1 and intron 4.

Several studies support the existence of such coupling[21, 28-29]. Both processes are extremely complex, involving DNA sequences, RNA molecules and thousands of protein factors which can establish physical interactions among components of both machineries[22, 30-32]. Among other mechanisms, transcription can modulate splicing via regulation of the RNA polymerase II elongation rate by the promoter itself and/or by other sequences in the gene that could influence the recruitment of transcriptional factors[28, 33]. As summarized in figure 4, this coupled mechanism could explain our experimental results whereby a low RNA polymerase II (RNAPII) elongation rate, or a weaker transcriptional activity would favor exon 2 inclusion, whereas a fast elongation rate RNAPII, or a stronger transcriptional activity would favor exon 2 skipping in the TNFR1-d2 transcript. In this TNFRSF1A coupling model, the rs767455 genotype in exon 1 would modulate the exon 2 splicing process, since this SNP is in an alternative splice site selection location, and we observed a higher expression of exon-2 skipping with the A-allele of rs767455 regardless of the TNFRSF1A promoter sequence and intron 4 (figure 2, and online supplementary text and figure S2). In contrast, rs4149570 and rs1800692 in the TNFRSF1A promoter and intron 4 respectively would predominantly act on the transcriptional machinery to influence TNFR1-d2 expression in a cell-dependent manner, since we observed a decreased luciferase activity (figure 3) when the G-allele in the promoter was associated with the C-allele in intron 4. In agreement with our hypothesis, co-amplification of the two transcripts was significantly lower in G carriers at rs4149570 in the promoter (G/G or G/T) than in T/T patients suffering from haematological malignancies with pulmonary aspergillosis[15].

To further assess the association between genotype and TNFRSF1A expression, we carried out a preliminary quantitative study of both transcripts, TNFR1 and TNFR1-d2, in frozen PBLs from controls and patients. The relative TNFR1-d2/TNFR1 transcript ratio did not achieve significant associations between TNFRSF1A expression levels and the genotypes at rs4149570-rs767455-rs1800692, regardless of the phenotypic group investigated (see online supplementary figure S5). The small size of each genotype group, especially the homozygotes (no T-T at rs1800692 in the TRAPS group), the genetic background of the individuals apart from this gene, time conservation of sample, and/or the unknown inflammatory status of the individuals at the time of the blood test, most likely influence TNFRSF1A expression independently of the genotype at rs4149570-rs767455-rs1800692. A prospective experiment would be warranted.

Our results raise interesting questions: why does the T-A-T haplotype at rs4149570-rs767455-rs1800692 increase exon 2-skipping, whereas in TRAPS patients, rs767455 A/A and rs1800692 T/T homozygotes are excessively rare and, in TRAPS-like patients, the A-T haplotype carriers have less fever. It would be interesting to extend these SNPs association studies with the clinical signs to other HRF syndromes. Recently, Gregory et al. demonstrated that the G allele at rs1800693 in intron 6, a susceptibility marker for multiple sclerosis[34], was associated with exon 6 skipping in the Δ6-TNFR1 transcript which, in turn, directs expression of a soluble form of TNFR1[27]. This study and ours suggest that the TNFRSF1A transcript balance may depend on TNFRSF1A alleles. It is tempting to speculate that regulation of exon 2 splicing by TNFRSF1A polymorphisms may partly account for heterogeneity in TRAPS phenotype and may also participate in the molecular mechanisms that determine susceptibility to multifactorial diseases, as these SNPs have been consistently associated with several autoinflammatory or autoimmune diseases e.g. Behcet's[35], Crohn's disease[20], rheumatoid arthritis[36], and multiple sclerosis[37].

Our study provides a novel mechanism of TNFRSF1A regulation that could account for some of the known complexity of the TNFR1 signalling pathway.

Tables

Table 1. Comparative distribution of genotypes at rs1800692 in intron 4 (c.473-33C>T)

	Controls n (%)		TRAPS n (%)			TRAPS-like n (%)
	Montpellier	NCBI[a]	Montpellier	EUROTRAPS[b]	Montpellier + EUROTRAPS[b]	Montpellier
CC	28 (38.9)	19 (26.9)	27 (50)	28 (49.1)	55 (49.5)	186 (41.3)
CT	30 (41.7)	26 (50)	27 (50)	28 (49.1)	55 (49.5)	191 (42.5)
TT	14 (19.4)	7 (13.5)	0	1 (1.8)	1 (1)	73 (16.2)
Total	72 (100)	52 (100)	54 (100)	57(100)	111 (100)	450 (100)

[a] Distribution of rs1800692 in two Caucasian populations (CAUC1 and PGA-European-Panel) from the SNP database National Center for Biotechnology Information (NCBI). TRAPS : TNFR-associated periodic syndrome.
[b] Patients from the EUROTRAPS consortium

Table 2 The rs1800692 (c.473-33C>T) genotype distribution and its association with fever in TRAPS-like patients

	Fever, n (%)	No fever, n (%)
CC	153 (41.5)	33 (40)
CT	164 (44.5)	27 (33)
TT	51 (14)	22 (27)
Total	368 (100)	82 (100)

rs1800692 (c.473-33C>T) association with fever was evaluated using a Chi square test (χ^2) with 2 degrees of freedom (p=0.01)

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Contributors

IT, SG designed the experiments. CR, ES, SS, MA, SG, performed the experiments. CR, MB, IT, SG contributed to the interpretation of the data. LO, MM, IT participated to patient recruitment with clinical data. CR, ES, SG processed the controls and patients samples. IT, SG wrote the paper. LO, MM made revisions to the paper.

Competing interests

None declared

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Legends to Figures

Figure 1. Identification and tissue-specific expression of the novel TNFR1-d2 transcript.

A. Schematic representation of the TNFRSF1A gene and its transcripts, TNFR1 and TNFR1-d2. Introns are shown as horizontal lines in the gene, and the splicing events as broken lines in the transcripts. The dark grey box represents the promoter. The other boxes symbolise exons and the translated regions are in grey. The TNFR1-d2 transcript coding sequence represented here is that predicted from the same start methionine codon as in the full-length TNFR1 form. The arrowheads depict the location of primers used to co-amplify TNFR1 cDNAs by RT-PCR.

B. Expression analysis of TNFRSF1A transcripts. A multiplex RT-PCR was performed using exonic primers spanning TNFR1 exon 1 to exon 4 and primers specific to the β-2-Microglobulin (B2M) internal reference transcript. cDNAs were amplified from human leukocytes (top panel) or human tissues (bottom panel). PBLs: peripheral blood leukocytes. PBMCs: peripheral blood mononuclear cells. Control sample cDNA free (H_2O) was run in all experiments. PCR products were separated on an 8% acrylamide gel and stained with ethidium bromide. The size in base pairs (bp) of each amplicon is given on the right. MW: molecular weight.

Figure 2. Combinatorial effects of rs4149570, rs767455 and rs1800692 in the promoter, exon 1 and intron 4 on exon 2 splicing

A. Constructs used in the minigene experiments. On the top is shown the TNFRSF1A gene structure as represented in figure 1 and the SNP location. Below are depicted the four pSpliceExpress (pSE) minigene constructs including the TNFRFS1A promoter, exon 1, the genomic fragment spanning the 3'end of intron 1 to the full-length intron 4 and rat insulin exon 3. The TNFRFS1A promoter is in dark grey and the exons 2-4 are in grey, whereas the rat insulin exon 3 is in white. A comprehensive description of all constructs is available in online supplementary text. Vectors containing each of the four haplotypes at rs4149570-

rs767455-rs1800692 observed in our genotyping analysis (see online supplementary table S5) were co-transfected with a vector constitutively expressing Renilla luciferase into HEK293T and SW480 cells.

B and C. Quantitative RT-PCR experiments of the level of exon 2-skipping (B) and exon 2-inclusion (C). The forward primer spanned the TNFRSF1A exon 1 and exon 3 junction or was located in exon 2 to specifically amplify the exon 2-skipping or exon 2-inclusion products, respectively, whereas the reverse primer was in rat exon 3. Quantification using a SYBR Green technique of both spliced products were normalized to those of GAPDH and Renilla luciferase as an internal control of transfection efficiency: Data are means from four independent transfection experiments each followed by duplicated qPCR, with errors bars indicating standard error of the mean (SEM). Data were compared by using the nonparametric Mann-Whitney U test (GraphPad Prism 4 software). Only significant differences ($p<0.05$) are shown.

Figure 3 rs1800692 in intron 4 modulates the transcriptional activity of the TNFRSF1A promoter

On the top is depicted the SNP location in the TNFRSF1A gene structure, as represented in figure 1. Below are represented the 7 luciferase expression vectors used in this experiment.

The luciferase vectors contained the TNFRSF1A promoter in dark grey carrying the G (in PrG-pGL3) or T (in PrT-pGL3) allele at rs4149570. The intron 4 sequence, denoted by a thick grey line carrying either the rs1800692 C or T alleles, were inserted in PrG-pGL3 and PrT-pGL3, respectively. Two constructs containing the intron 4 deletion from c.473-72 to c.473-29 represented by a thick grey line broken by square brackets, were investigated. The luciferase activities obtained after co-transfection of HEK293T or SW480 cells with either of the pGL3 constructs, and the β-galactosidase vector are represented as horizontal histograms. All luciferase activity values were normalized to the β-galactosidase activity and protein concentration to correct for transfection and lysis efficiencies and the basal luciferase activity obtained with the pGL3-basic construct was subtracted. Relative firefly luciferase activity of each construct was represented as a percentage of that of the pGL3-promoter construct (pGL3) with errors bars indicating standard error of the mean (SEM). Data are means from seven independent and duplicate transfections and were compared by using the nonparametric Mann-Whitney U test (GraphPad Prism 4 software). Only significant differences ($p<0.05$) are shown.

Figure 4. Schematic model representing coupled transcriptional and splicing regulation of TNRF1-d2

This putative model illustrates that the carboxyl terminal domain (CTD) of the large subunit of RNA polymerase II (RNAPII) recruits components of the pre-mRNA splicing machinery and that the transcription elongation rate contributes to splice site recognition.

On the top, the TNFRSF1A gene is represented. The dark and light grey box boxes represent the promoter and constitutive exons, respectively. The alternative exon 2 is depicted by a white box. Introns are shown as horizontal lines in the gene. Vertical arrows locate the three polymorphisms analysed in this study, and horizontal grey and black arrows schematize the speed and amount of the transcriptional activity, respectively. The RNAPII and its CTD, involved in transcription, the bound splicing factors, and the nascent pre-mRNA, are shown. In the pre-mRNA molecules, exons are represented by circles.

The two alternative splice selection sites, leading to either exon 2-inclusion (left, TNFR1 transcript) or exon 2-skipping (right, TNFR1-d2 transcript) are depicted. The mRNA maturation process is represented on the right as horizontal black arrows of variable thickness. Below are shown the transcript levels according to the two major haplotypes at rs4149570-rs767455-rs1800692, and their associated transcriptional activity: a low transcriptional elongation with G-G-C haplotype favors exon 2 inclusion, whereas a higher transcriptional activity with T-A-T favors exon 2 skipping.

This figure was drawn using the Servier medical art software available at http://www.servier.fr/servier-medical-art.