SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 14: 5114-5120, 2017

Alternative splicing regulation in tumor necrosis factor-mediated inflammation (Review)

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Received October 27, 2016; Accepted July 7, 2017

DOI: 10.3892/ol.2017.6905

Abstract. It is generally accepted that alternative splicing has an effect on disease when it leads to conspicuous changes in relevant proteins, but that the combinatorial effect of several small modifications can have marked outcomes as well. Inflammation is a complex process involving numerous signaling pathways, among which the tumor necrosis factor (TNF) pathway is one of the most studied. Signaling pathways are commonly represented as intricate cascades of molecular interactions that eventually lead to the activation of one or several genes. Alternative splicing is a common means of controlling protein expression in time and space; therefore, it can modulate the outcome of signaling pathways through small changes in their elements. Notably, the overall process is tightly regulated, which is easily overlooked when analyzing the pathway as a whole. The present review summarizes recent studies of the alternative splicing of key players of the TNF pathway leading to inflammation, and hypothesizes on the cumulative results of those modifications and the impact on cancer development.

Contents

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Key words: alternative splicing, inflammation, TNF signaling pathway, TNF isoforms

1. Introduction

The majority of eukaryotic genes undergo alternative splicing; once believed to be a peculiarity of a few genes, it is now closer to being the rule rather than the exception. Alternative splicing is a proteome-diversifying process through which several mature RNA messengers are obtained from a single gene, each of these transcripts potentially coding for a different protein isoform with a potentially different function (1).

The specialized molecular machinery that performs splicing is known as the spliceosome, a macromolecular complex composed of four small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4/U6 and U5) and >100 non-snRNP splicing factors. The spliceosome identifies individual splice sites along immature transcripts through the recognition of consensus sequences located in the exon/intron boundaries, which are complementary with the RNA present in the snRNPs. Next, through a series of spatial rearrangements, it facilitates two trans-esterification reactions that result in the excision of the intronic sequence; the process takes place at each intron to yield a mature mRNA (1).

On average, human genes have 8.8 introns (2) and ~90% of them produce more than one mature transcript (3). Alternative splicing can remove introns from a pre-mRNA in a number of different combinations, giving rise to different mRNAs. Known patterns of alternative splicing include exon skipping (removal of an exon along with the surrounding introns), usage of alternative intron donor (5') or acceptor (3') sites, intron retention and mutually exclusive exon splicing (Fig. 1). Alternative exons may possess splice sites with diverging sequences, thus are less likely to be recognized through RNA-RNA interaction; such sites are considered sub-optimal and their recognition is aided by the recruitment of *trans*-acting splicing factors that bind to *cis* regulatory elements in the vicinity of splice sites and recruit the main spliceosome components. These *cis* elements are classified in exonic/intronic splicing enhancers/silencers (ESE, ESS, ISE and ISS) according to their positions and functions, while the factors that bind to them mainly belong to two families, namely the SR and heterogeneous (hn)RNP proteins, which usually promote and prevent splice site recognition, respectively (Fig. 2). This way, the alternative splicing of a given precursor (pre)-mRNA is regulated by the abundance

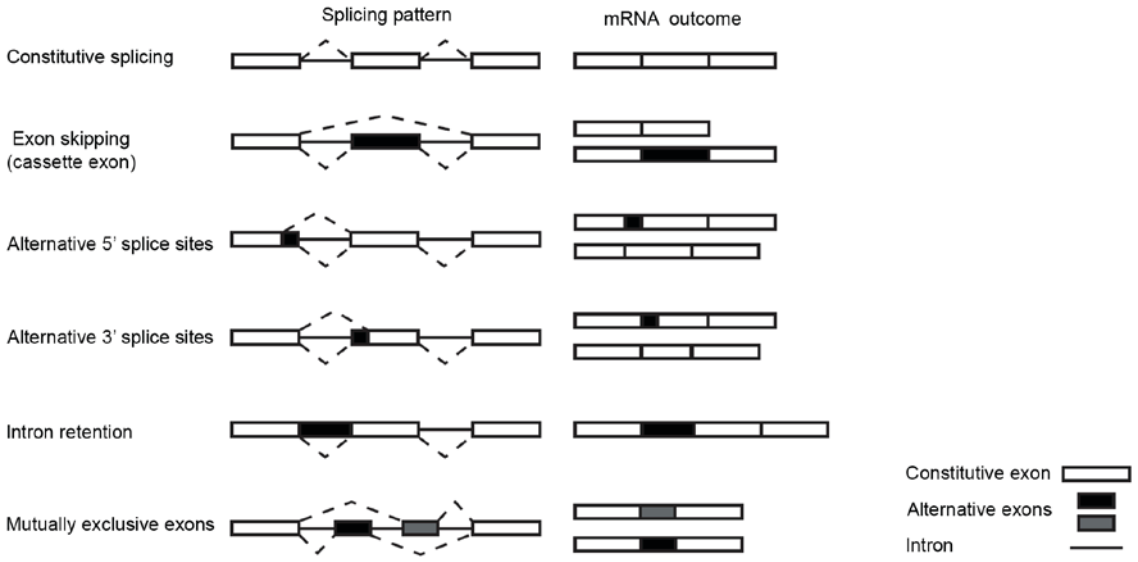

Figure 1. Diagrammatic representation of the known alternative splicing patterns in a pre-mRNA containing only three exons and two introns. Constitutive intron removal yields a single mRNA consisting only of exons (top). Conversely, alternatively spliced pre-mRNAs produce multiple mature messengers. Pre-mRNA, precursor-mRNA.

of the pre-mRNA itself, its sequence (presence/absence of splicing factor recognition sites) and the relative abundance of the splicing factors (4-6). So, it is now understood that the main components of the spliceosome remain largely unchanged and that the majority of the regulatory nature of alternative splicing relies on protein-protein, protein-nucleic acid interactions and their kinetics (7). Furthermore, RNA splicing is coupled to transcription physically and dynamically. Spliceosome factors are recruited to the carboxy-terminal domain of the RNA polymerase during RNA elongation, from where they are in turn recruited to the splice sites along the nascent transcript as they become available, thus making splicing dependent on the processivity of the RNA polymerase elongation complex (8).

Alternative splicing has been observed to have an effect on disease. There are a considerable number of hereditary diseases caused by point mutations, which in general, disrupt splice sites or ESE/ISEs and modify the aforementioned interactions preventing the expression of one particular isoform of a protein (9,10). The role that alternative splicing plays in the generation and/or maintenance of complex pathological conditions, such as inflammation, is not as straightforward, since these conditions are the result of several alterations in pathways that often comprise of dozens of proteins with diverse functions, each of which is prone to regulation through alternative splicing. High-throughput sequencing and bioinformatics have made it possible to assess alternative splicing modifications on a global scale (3,11); however, these methods are not yet without limitations and require complementing with particular, functional studies. The present review summarizes the impact of alternative splicing in the core components of the TNF signaling pathway.

2. Tumor necrosis factor (TNF) pathway

TNF-a (also known as cachectin) is a strong pro-inflammatory cytokine, which plays an important role in certain processes, including inflammation, cell proliferation, differentiation and apoptosis. Inflammation is an extremely important part of innate immunity and is regulated in a number of steps (12).

TNF-a binds two distinct receptors: TNFR1 and TNFR2. Activation of TNFR1 leads to the formation of signal complexes that activate pathways that lead to: i) Expression of pro-inflammatory genes through the recruitment of receptor-interacting protein 1 (RIP1) and TNF-receptor-associated factor 2 (TRAF2); and ii) apoptosis and cell death by recruiting Fas-associated death domain protein (FADD) and caspase 8. Binding to TNFR2 induces only inflammation through the direct recruitment of TRAF2, which in turn recruits TRAF1. The two pro-inflammatory complexes lead to IKK (inhibitor of nuclear factor κ-B kinase and mitogen-activated protein kinase (JNK and p38) activation (Fig. 3) (12).

The balance between TNF-activated inflammation and apoptosis is regulated on several levels, including signal strength, expression of signaling molecules and regulating proteins, and crosstalk with other cell signals (13), all of which can be heavily influenced by the alternative splicing of each element.

3. Known isoforms in TNF signaling

A search on the Ensmbl database (14) for the splice variants originated from the transcripts of the receptor-proximal elements of TNF signaling shows that all but FADD have at least 2 potential protein variants (Table I). Experimental evidence is not available for all of them; and thus far, it comprises mostly association data, rather than molecular evidence that offers an understanding of their regulation. In the following section, experimental evidence concerning alternative splicing of the key players in TNF signaling is reviewed.

TNFR. Two recent studies suggested that the TNF receptor superfamily member 1A (TNFRSF1A) transcript balance may depend on TNFRSF1A alleles. Gregory *et al* (15) investigated a single nucleotide polymorphism (rs1800693, c.625+10A>G) in the TNFRSF1A gene that was previously

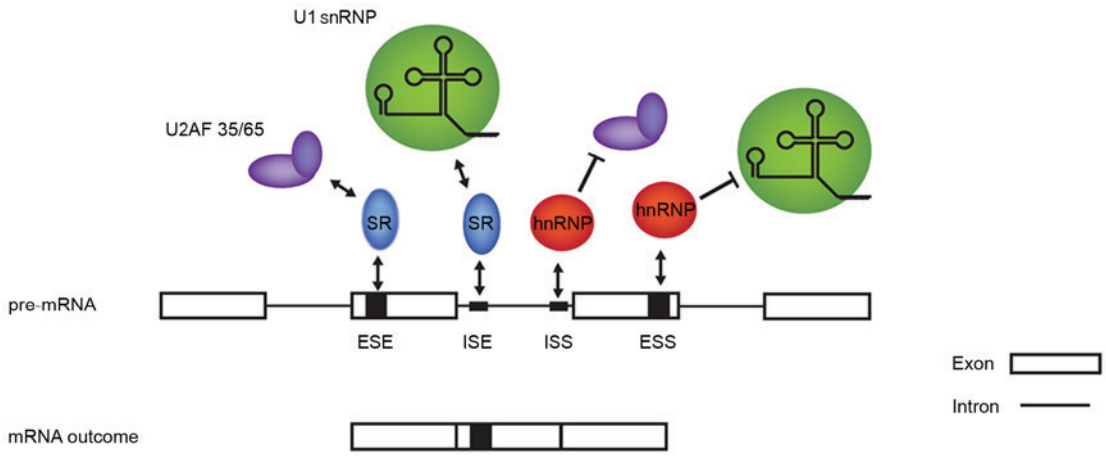

Figure 2. Splicing *cis* elements influence splice site recognition through recruitment of different spliceosome components. Generally, splicing enhancers recruit SR proteins, which in turn recruit the splicing machinery; splicing supressors recruit hnRNP proteins, which hinder these interactions. ESE, exonic splicing enhancer; ISE, intronic splicing enhancer; ISS, intronic splicing suppressor; ESS, exonic splicing suppressor; hnRNP, heterogeneous ribonucleoproteins; pre-mRNA, precursor-mRNA; snRNP, small nuclear RNP.

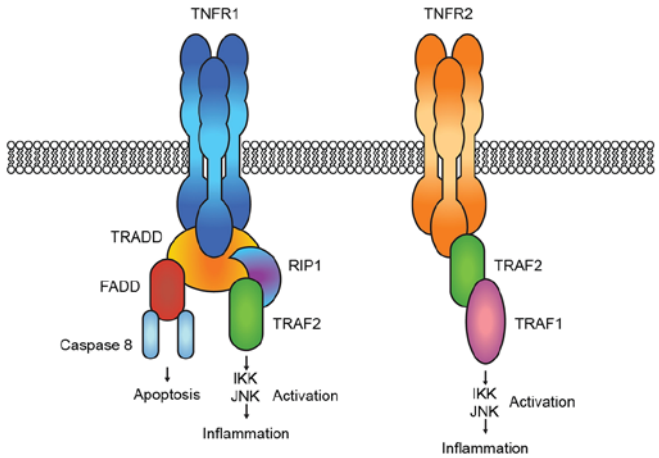

Figure 3. Proximal components of the TNF signaling pathway. TNFR1 transduces apoptotic signals through the recruitment of FADD and caspase 8, and inflammatory signals through the recruitment of RIP1 and TRAF2. TNFR2 binds TRAF1 and TRAF2 in order to transduce inflammation signals. IKK is an enzyme involved in the degradation of the inhibitor IKB, which binds NF-κB to inhibit its function. NF, nuclear factor; TNF, tumor necrosis factor; TNFR1, TNF receptor type 1; FADD, Fas-associated death domain protein; RIP1, receptor-interacting protein 1; TRAF1, TNF-receptor-associated factor 1; IKK, inhibitor of NF-κB kinase.

identified as a susceptibility marker for multiple sclerosis through genome-wide association studies. In *in vitro* splicing assays, only the G allele resulted in skipping of exon 6. TNFR1 exon 6 skipping results in a frameshift and a premature stop codon, which translates into a soluble form of TNFR [D6-TNFR, comprising only the amino-terminus of TNFR1, followed by a novel 45-amino acid (aa) sequence] for which a TNFR-antagonistic role was suggested. Another detailed study described a TNFR1 transcript lacking exon 2 (TNFR1-d2) and its association with a specific haplotype at 3 single nucleotide polymorphisms (SNPs) previously associated with TNF-receptor-associated periodic syndrome (TRAPS). These SNPs have distant locations along the TNFR1A gene: rs4149570 lies at the promoter region (c.610G>T), rs767455 at exon 1 (c.36A>G) and rs1800692 at exon 4 (c.473-33C>T), so it is plausible that an interplay of transcription and splicing dynamics determines transcript outcome (16). This evidence strongly suggests that these SNPs disrupt splicing enhancers/silencers along intronic or exonic sequences, an aspect yet to be studied for other reported SNPs associated with TRAPS (17) or other inflammatory conditions, including Crohn's disease (18).

TNFR2 isoforms have been described as well. Seitz *et al* (19) characterized hicpTNFR, a TNFR2 splice variant with an alternate exon 1 sequence that results from the usage of an alternate transcription start site and alternative splicing. This variant is mostly retained in the trans-Golgi network and in endosomes where it could function as a storage pool of preformed p75TNFR that is not affected by shedding. Upon emerging on the cell surface, hicp75TNFR is functionally no longer distinguishable from p75TNFR; furthermore, hicpTNFR colocalizes with endogenous TNF, hinting at intracellular activation of the hicp75TNFR by endogenous TNF (20). There are a number of studies on a soluble TNFR2 isoform lacking exons 7 and 8, known as differentially spliced (DS)-TNFR; as a consequence of splicing, it lacks the transmembrane and cytoplasmic domains. The data gathered suggests that it regulates TNFR function by antagonizing its biological activity. This soluble receptor was detected at increased levels in patients with sepsis and at higher concentrations in patients with rheumatoid arthritis, relative to the levels detected in the sera of healthy individuals (21). In another study, performed in insulin-resistant patients, this same isoform was found in 26% of samples from patients with type 2 diabetes and in 44% of non-diabetic subjects. An increase in waist size was associated with a progressive decrease in DS-TNFR2 concentration. Moreover, it was suggested that DS-TNFR2 exhibits anti-inflammatory activity, based on observations of the correlation between DS-TNFR2 and circulating adiponectin (22). This novel isoform and its potential anti-inflammatory properties have been associated with markers of liver injury (23) and with a favorable outcome in patients with rheumatoid arthritis (24), although no information is currently available on the dynamics of this splicing event.

Table I. Splicing isoforms of the main receptor-proximal elements of the TNF signaling pathway.

Gene	Ensembl ID	No. of exons	No. of transcripts	No. of protein-coding transcripts
TNFR1	ENSG00000067182	10	16	9
TNFR2	ENSG00000028137	10	4	2
TRADD	ENSG00000102871	5	5	2
FADD	ENSG00000168040	2	1	1
TRAF1	ENSG00000056558	8	3	3
TRAF2	ENSG00000127191	11	10	6
TRAF3	ENSG00000131323	11	9	7
RIP1	ENSG00000017797	10	5	5

TNF, tumor necrosis factor; TNFR1, TNF receptor type 1; TRADD, TNF receptor type 1-associated death domain protein; FADD, Fas-associated death domain protein; TRAF1, TNF-receptor-associated factor 1; RIP1, receptor-interacting protein 1.

Table II. Number of disease-associated SNPs of the main receptor-proximal elements of the TNF signaling pathway.

Gene	SNPs with association data available on SNPedia
TNFR1	Rs2234649, Rs4149570, Rs767455, Rs104895217, Rs104895218, Rs104895219, Rs104895220, Rs104895221, Rs104895222
TNFR2	Rs3397, Rs1061624, Rs652625
TRADD	Rs9939768, Rs6979, Rs9033, Rs868213
FADD	Rs387906839
TRAF1	Rs2476601, Rs3761847, Rs6457617, Rs7574865, Rs6920220, Rs10818488, Rs2416808, Rs10499194, Rs660895, Rs2416804, Rs7026551, Rs1930780, Rs1953126, Rs2900180, Rs2395148, Rs292001, Rs7021206
TRAF2	Rs7852970
TRAF3	Rs10133111, Rs11160706, Rs1131877

SNP, single nucleotide polymorphism; TNF, tumor necrosis factor; TNFR1, TNF receptor type 1; TRADD, TNF receptor type 1-associated death domain protein; FADD, Fas-associated death domain protein; TRAF1, TNF-receptor-associated factor 1.

RIP. Alternative splicing has been described in the RIP family only in the RIP3 gene; two splice variants (RIP3-β and RIP3-γ) with truncated N-terminal kinase domains and novel, shorter C termini have been found. These variants abrogate nucleocytoplasmic shuttling and are therefore not able to induce apoptosis. Additionally, they downregulate RIP3 pro-apoptotic activity (25). Suppression of RIP3-dependent apoptotic TNF signaling could potentially upregulate RIP1-dependent pro-inflammatory pathways.

Splice variants of the human RIP1 gene should not be ruled out, since it has been demonstrated that the associated *Xenopus* RIP1 gene produces at least one alternative splicing-derived isoform (26). According to the Ensembl database annotations, human RIP1 10 exon-pre-mRNA potentially produces 5 alternative transcripts, which are yet to be experimentally described.

TRAF. A splice variant of TRAF2 was described as early as 1998, this variant (TRAF2A) contains a 7-aa insertion within the RING finger domain, presumably produced by alternative usage of splice donor sites present at the 3' end of exon 1. TRAF2A is incapable of mediating the activation of nuclear factor (NF)-κB, thus it can act as a dominant inhibitor of TNFR2-mediated NF-κB activation; this way, cells can regulate NF-κB activation through TNF family receptors by modifying alternative splicing of primary transcripts from the TRAF2/TRAF2A gene to produce different ratios of TRAF2A and TRAF2 mRNAs (27). In a comparison of the structure of the human, murine and *Drosophila* TRAF genes, the TRAF2A transcript was only found to be expressed in mice, and not in humans or rats, although only Ramos (human B lymphoma) and HEK293 (human embryonic kidney) cells were assayed (28).

TRAF3 is another member of the TRAF family of proteins, (currently six genes have been identified, TRAF1 to 6). Three isoforms produced though alternative splicing of this gene were initially identified; they differ in the number of Zn fingers remaining from the five contained in the full-length TRAF3. The TRAF3b isoform (Δ25 aa) contains four Zn fingers with a C-terminal finger formed by the fusion of the N-terminal half of the 3rd and the C-terminal half of the 4th finger, the TRAF3c isoform (Δ52 aa) contains three Zn fingers with a C-terminal finger formed by the fusion of the 2nd and the 4th finger, while the TRAF3d isoform (Δ56 aa) contains three complete Zn fingers and the N-terminal portion of the 5th finger. Additionally, the study detected three variant

5'-untranslated regions (UTRs) and two variant 3' UTRs among the isolated clones, although they did not establish whether they corresponded to particular isoform-coding open reading frames (29).

A further study identified five additional TRAF-3 protein isoforms with alterations in the Zn finger domains (Δ27aa, Δ83aa, Δ103aa, Δ130aa and Δ221aa). TRAF3 splice-deletion variants, including Δ25 aa, Δ52 aa and Δ56 aa, were found to induce NF-κB activation in 293T cells, while full-length TRAF3 and TRAF3 Δ221 failed to do so. Unexpectedly, when full-length TRAF3 was co-expressed with each of the 7 TRAF3 splice variants capable of activating NF-κB alone, a 1.4- to 5-fold augmentation of their NF-κB activation was observed (30). All but the Δ130 aa were found to be expressed in four different lymphoma cell lines (Jurkat D1.1, BJAB, Daudi and Raji). Overexpression experiments of individual isoforms revealed that only the Δ27 aa, Δ103 aa or Δ130 aa isoforms are able to induce NF-κB activation in BJAB cells in contrast to full-length TRAF-3 or Δ221aa, which could not; notably, TRAF-3 Δ25 aa, Δ52 aa, Δ56 aa and Δ83 aa variants also failed to induce NF-κb activity, contrary to their activity in 293T cells (31). The difference in the TRAF-3 splice variants activation properties in different cell lines suggests an association between TRAF-3 isoforms and the cellular environment. The two examples provide evidence of the regulation of NF-κB activation in TRAF family genes through alternative splicing: The TRAF2 and TRAF3 shorter splice variants have this ability, while the full-length proteins lack them.

4. Splicing potential

The demonstration by Rittore *et al* (16) that SNPs can have an effect on the alternative splicing pattern of the TNFR1 gene may be the cornerstone that joins two groups of association studies, namely, transcript diversity and SNPs, and sheds light on their association. The Rittore group identified that SNPs rs4149570, rs767455 and rs1800692 have a combined effect on the TNFR1 transcript output through regulation of alternative splicing. When considering that the aforementioned TNFR1 SNPs have already been found to be associated with inflammation-related conditions, including susceptibility to develop invasive pulmonary aspergillosis (32), the prognosis of peripheral T-cell non-Hodgkin lymphoma (33), radiation-induced toxicity following treatment for non-small cell lung cancer (34), the risk of breast cancer (35), inflammatory demyelinating diseases (36), the response to Crohn's disease treatment (37) and adult onset Still's disease (38), it is plausible to consider whether it is the differential balance of TNFR1 isoforms produced by alternative splicing that causes these associations.

There is conclusive evidence that supports the fact that SNPs can modify the outcome of alternative splicing by disrupting canonical splice sites (39), or by modifying *cis* elements (enhancers or silencers) along both exonic and intronic sequences, as Pagani *et al* (40) found in a number of synonymous mutations that cause exon 12 skipping of the cystic fibrosis transmembrane conductance regulator. Curated repositories that gather SNP association data, such as SNPedia (41), pinpoint relevant SNPs worth analyzing for their potential effect on alternative splicing. A search in SNPedia reveals several disease-associated polymporphisms in virtually all of the receptor-proximal elements of TNF signaling (Table II).

Regarding human cancer, evidence points out that specific functions of certain genes regulating the TNF signaling pathway can be affected by alternative splicing, with an impact on tumor phenotype. In this sense, the TNF-apoptosis inhibitor c-FLIP (cellular FLICE inhibitory protein) has distinct alternative splicing variants (c-$FLIP_L$ and c-$FLIP_s$) that have distinct roles in the TNFα-induced signaling cascade. Park *et al* (42) demonstrated that c-FLIP alternative variants activate either Erk or NF-κB through the association with Raf and TRAF2, which contributes to TNF-induced cell cycle promotion. For instance, c-$FLIP_L$ showed stronger affinity to Raf in order to activate Erk and PI3K. Meanwhile, c-$FLIP_s$ showed strong affinity to TRAF2 to mediate activation of the TRAF-JNK pathway. Moreover, each splicing variant is regulated differentially at transcriptional level, that is, after TNF-α stimulation, a delayed c-$FLIP_L$ response is induced, whereas c-$FLIP_s$ shows a rapid response to the stimulation (42). Thus, alternative splicing enables protein diversity, generating structurally and functionally distinct proteins from the same gene. Later, Haag *et al* (43) reported the expression of these splicing variants in pancreatic cancer tissue. It was observed that c-FLIP is underexpressed in pancreatic intraepithelial neoplasm and pancreatic ductal adenocarcinomas compared with normal pancreatic tissues. Moreover, in pancreatic cancer cell line ULA-PaC, the downregulation of these isoforms by RNA interference enhances apoptosis, indicating that c-FLIP is an important regulator of death receptor-induced apoptosis (43).

In another study, in neoplastic and more notably, in non-neoplastic cells, two novel TNF-associated apoptosis-inducing ligand (TRAIL) splice variants were reported: TRAIL-β, lacking exon 3, corresponding to loss of 98 aa, and TRAIL-γ, lacking exons 2 and 3, corresponding to loss of 52 aa. These two variants result in a truncation of the extracellular domain, which is important for trimeric stability and ligand-receptor binding; consequently, these splice variants fail to trigger apoptosis signaling. The study suggested that these novel TRAIL variants may have implications for deepening our understanding of TRAIL-mediated apoptosis in neoplastic and non-neoplastic human cells (44). Recently, Krieg *et al* (45) reported the first study that describes the expression of TRAIL splice variants in 41 gastric carcinoma tissue samples by reverse transcription-quantitative polymerase chain reaction. Notably, all three TRAIL-splice variants could be detected in non-malignant and malignant tissues, but only TRAIL-γ had a prognostic value, since it was associated with a significantly higher survival rate (45).

5. Concluding remarks

TNF-mediated inflammation, as with numerous other signaling pathways, involves the concerted expression of a fairly well known number of genes; however, the human transcriptome has turned out to be far more complex than initially conceived, and the contribution of alternative splicing to the transcriptomic variability through the diversification of mature transcripts produced from the same gene, is becoming clearer and more

important (3). A considerable amount of research is currently being conducted into the search for transcriptomic signatures associated with inflammation-related diseases (among numerous other conditions), including osteoarthritis (46), atherosclerotic plaque progression (47), multiple sclerosis, systemic lupus erythematosus, juvenile rheumatoid arthritis, Crohn's disease, ulcerative colitis and type 1 diabetes (48), to name a few. Incorporation of alternative splicing data into this information can shed light on how these transcriptomic signatures come to be, and on the possible scenarios that they lead to (49).

The present study has reviewed the literature showing that protein isoforms derived from alternative transcripts of the genes involved in TNF signaling can have different, mainly antagonistic, functions. We therefore find it reasonable to hypothesize that, although TNF signaling ultimately leads to an inflammatory response, the pathway itself can be subject to regulation through alternative splicing of its elements, potentially modifying its end result.

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