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SPLICE VARIANTS IN APOPTOTIC PATHWAY

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Elimination of superfluous or mutated somatic cells is provided by various mechanisms including apoptosis, and deregulation of apoptotic signaling pathways contributes to oncogenesis. 40 years have passed since the term "apoptosis" was introduced by Kerr *et al.* in 1972; among the programmed cell death, a variety of therapeutic strategies especially targeting apoptotic pathways have been investigated. Alternative precursor messenger RNA splicing, by which the process the exons of pre-mRNA are spliced in different arrangements to produce structurally and functionally distinct mRNA and proteins, is another field in progress, and it has been recognized as one of the most important mechanisms that maintains genomic and functional diversity. A variety of apoptotic genes are regulated through alternative pre-mRNA splicing as well, some of which have important functions as pro-apoptotic and anti-apoptotic factors. In this article we summarized splice variants of some of the apoptotic genes including *BCL2L1, BIRC5, CFLAR, and MADD*, as well as the regulatory mechanisms of alternative splicing of these genes. If the information of the apoptosis and aberrant splicing in each of malignancies is integrated, it will become possible to target proper variants for apoptosis, and the trans-elements themselves can become specific targets of cancer therapy as well. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

***Key Words*: apoptosis, alternative pre-mRNA splicing, splice variants, malignancy, therapeutics.**

INTRODUCTION

Elimination of superfluous or mutated somatic cells is provided by various mechanisms including apoptosis [1], and deregulation of apoptotic signaling pathways contributes to oncogenesis. It was 40 years ago when Kerr, Wyllie, and Currie coined the term "apoptosis" to describe a special form of programmed cell death and published their original article on apoptosis [2]. Since that time the concept of apoptosis has contributed much to the various fields of biology and medicine especially in cancer research, and a variety of therapeutic strategies especially targeting apoptotic pathways have been investigated. On the other hand, alternative precursor messenger RNA (pre-mRNA) splicing, by which the process the exons of pre-mRNA are spliced in different arrangements to produce structurally and functionally distinct mRNA and proteins, is another field in progress [3]. After the completion of the Human Genome Project in 2004, alternative pre-mRNA splicing has been recognized as one of the most important mechanisms that maintains genomic and functional diversity. A variety of apoptotic genes are regulated through alternative pre-mRNA splicing as well, some of which have important functions as pro-apoptotic and anti-apoptotic factors. In this article, we summarized splice variants of some of the apoptotic genes as well as the regulatory mechanisms of alternative splicing of those genes.

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Abbreviations used: (Y)n – poly-pyrimidine tract; 3′SS – 3′ splice site; 5′SS – 5′ splice site; APAF1 – apoptosis-activating factor-1; APO-2L – apolipoprotein-2L; *BCL2L1 – BCL2-like 1*; BIR – baculovirus IAP repeat; *BIRC5 – baculoviral IAP repeat containing 5*; *CFLAR – CASP8- and FADD-like apoptosis regulator*; DD – death domain; DED – death effecter domain; DIABLO – direct IAP-binding protein with low PI; DISC – death-inducing signaling complex; ESE – exonic splice enhancer; ESS – exonic splice silencer; FADD – FAS-associated death domain; hnRNP – heterogeneous nuclear ribonucleoprotein; IAP – inhibitor of apoptosis protein; ISE – intronic splice enhancer; ISS – intronic splice silencer; MADD – MAP-kinase activating death domain; MAP – mitogen-activated protein; pre-mRNA – precursor messenger RNA; Smac – second mitochondria-derived activator of caspases; SR protein – serine/arginine-rich protein; TNF – tumor necrosis factor; TRADD – TNFR-associated death domain; TRAIL – TNF-related apoptosis-inducing ligand.

APOPTOTIC PATHWAY

There are two main pathways in apoptosis: the death receptor-mediated apoptotic pathway (extrinsic pathway) and the mitochondrial-mediated apoptotic pathway (intrinsic pathway) (Fig. 1).

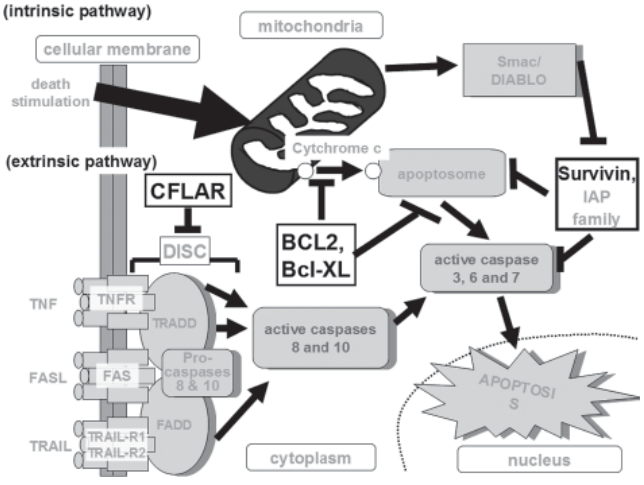

Fig. 1. A scheme of apoptotic pathway

The death receptor-mediated apoptotic pathway is initiated when tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL, also known as apolipoprotein-2L (APO-2L)) or FAS ligand (APO-1) bind to their receptors (TNFR, TRAIL-R and FAS, respectively), in association with adaptor molecules such as FAS-associated death domain (FADD) or TNFR-associated death domain (TRADD), and ini-

tiator caspases-8 and -10 are cleaved and activated, thus resulting in the activation of executor caspases-3, -6 and -7 and culminating in apoptosis. On the other hand, the mitochondrial-mediated apoptotic pathway is initiated when pro-apoptotic proteins release free cytosolic cytochrome *c*. Once released, cytochrome *c* promotes the assembly of a caspase-activating complex termed the apoptosome, which includes apoptosis-activating factor-1 (APAF1), dATP, and pro-caspase-9. The apoptosome activity functionally corresponds to the activity of caspase-9, and it activates executor caspases-3, -6, and -7, thus inducing apoptosis. The inhibitor of apoptosis protein (IAP) proteins such as cIAP1, cIAP2, and XIAP can prevent the proteolytic processing of pro-caspases-3, -6, and -7 by blocking the cytochrome *c*-induced activation of pro-caspase-9 in the intrinsic pathway; in contrast, these IAP proteins do not act in the extrinsic pathway [4]. APAF1/cytochrome *c*-independent and second mitochondria-derived activator of caspases (Smac)/direct IAP-binding protein with low PI (DIABLO)-dependent induction of apoptosis have also been reported. Alternative models of programmed cell death have been proposed, but still remain to be further clarified, including autophagy, paraptosis, mitotic catastrophe, and the descriptive model of apoptosis-like and necrosis-like programmed cell death [1, 5].

ALTERNATIVE PRE-mRNA SPLICING

Pre-mRNA splicing is a mechanism that removes introns from pre-mRNA and binds exons, eventually generating mature mRNA (Fig. 2). The process of pre-mRNA splicing is regulated by two elements: cis-elements and trans-elements, and the cis-elements are bound by the trans-elements in pre-mRNA splicing. Among the cis-elements, consensus splice sites are essential for pre-mRNA splicing and are comprised of a 5′splice site (5′SS), a branch point motif, a poly-pyrimidine tract ((Y)n), and a 3′splice site (3′SS) (Fig. 3). Among the consensus splice sites, the 5′SS is also known as a splice donor site and the 3′SS is also known as a splice acceptor site. Splice enhancers and silencers are known as cis-regulatory elements, and both of these sites are important for the recognition of the 5′SS and 3′SS regions, and depending on their localization within the genome, splice enhancers and silencers are subclassified into exonic splice enhancers (ESEs), intronic splice enhancers (ISEs), exonic splice silencers (ESSs) or intronic splice silencers (ISSs). Among the known trans-elements, spliceosomes are multicomponent complexes essential for pre-mRNA splicing. ESEs are predominantly bound and recognized by members of serine/arginine-rich proteins (SR proteins, symbolized by SRp) and SR-like proteins. However, heterogeneous nuclear ribonucleoproteins (hnRNPs) commonly bind to ESSs and ISSs. In many cases, hnRNPs block spliceosome assembly, thus resulting in exon skipping. Several of tissue-specific SR proteins and hnRNPs have recently been identified. Aberrant alternative pre-mRNA splicing in malignancies can be subclassified into two categories: (I) the generation of an aberrant splice variant as an individual transcript and (II) an aberrant expression profile of splice variants as an entire set of transcripts in malignant cells (Fig. 3).

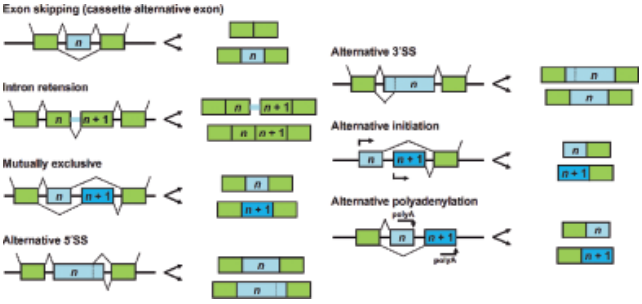

Fig. 2. Alternative pre-mRNA splicing. Seven types of alternative splicing were indicated

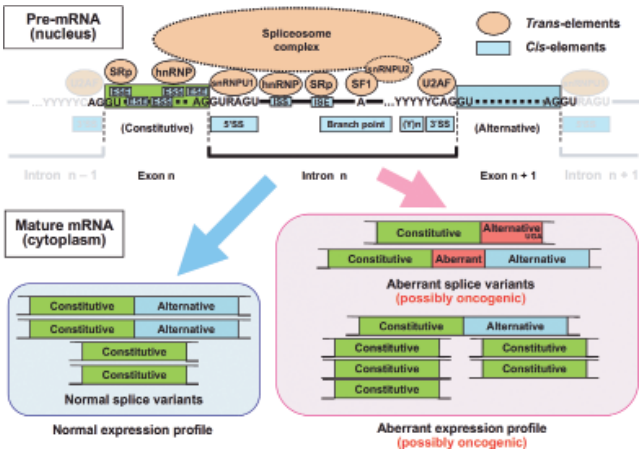

Fig. 3. Regulatory mechanism of alternative pre-mRNA splicing and its alteration in malignancies. Cis-elements are indicated with rectangles and trans-elements are indicated with ellipses. In the nucleotide sequences, Y denotes a pyrimidine (U or C) and R denotes a purine (G or A). ESE, exonic splice enhancer; ESS, exonic splice silencer; hnRNP, heterogeneous nuclear ribonucleoprotein; ISE, intronic splice enhancer; ISS, intronic splice silencer; snRNP, small nuclear ribonucleoprotein; SR, serine/arginine-rich protein; SS, splice site; U2AF, U2 small nuclear ribonucleoprotein auxiliary factor

SPLICE VARIANTS IN APOPTOTIC PATHWAY

Expression profiles of genes in the apoptotic pathway can also be regulated by alternative pre-mRNA splicing in both normal and malignant tissues, and some of those, *BCL2L1, BIRC5, CFLAR,* and *MADD*, will be highlighted in this session (Fig. 4).

BCL2-like 1 (BCL2L1)

Apoptotic factors of the BCL2 family are classified into two subgroups: anti-apoptotic (e.g., BCL2, MCL1, and Bcl-xL) and pro-apoptotic (e.g., BAX and Bcl-xS) [6], and the two subgroups differ in the number and variety of BH domains they contain [7]. In 1993 Boise *et al.* isolated *BCL2-like 1 (BCL2L1)* gene which functions as a *BCL2*-independent regulator of apoptosis (Fig. 1) [8], and alternative splicing resulted in two distinct *BCL2L1* mRNA isoforms: the larger variant (Bcl-xL) which is similar in size and predicted structure to BCL2 and the smaller variant (Bcl-xS) which inhibits the ability of BCL2 to enhance the survival (Fig. 4 *a*).

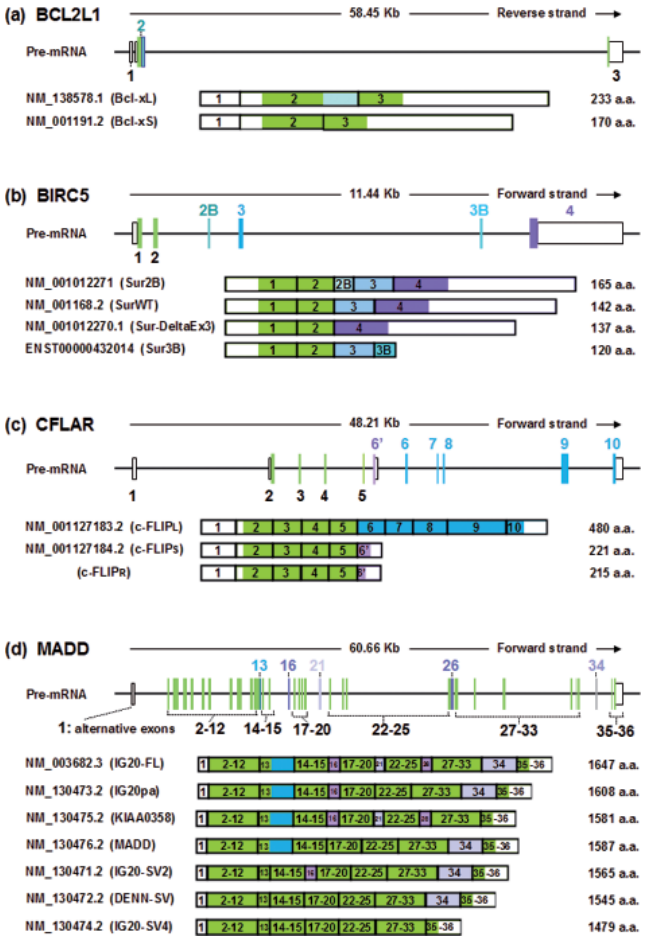

Fig. 4. Splice variants of genes in the apoptotic pathway. (*a*) *BCL2L1*, (*b*) *BIRC5*, (*c*) *CFLAR*, and (*d*) *MADD*. For each of the genes, pre-mRNA is indicated at the top, and mature mRNAs are indicated in the bottom. ID numbers of mRNAs and the numbers of amino acids were referred to the NCBI database (as of June 1, 2012)

The overexpression of Bcl-xL confers resistance to apoptosis (see Fig. 1); in contrast, Bcl-xS can induce apoptosis and alleviate multidrug resistance; hence the regulation of alternative splicing of the *BCL2L1* gene has been intensely explored since the 2000s [6, 9–21]. hnRNP F/H was reported to modulate the alternative splicing of *BCL2L1* gene [9], and SC35, a member of the SR proteins family, is required to switch the alternative splicing profile of various apoptotic genes such as *CFLAR, CASP8, CASP9*, and *BCL2L1*, towards the expression of pro-apoptotic splice variants [12]. Treatments with a chemical compound *emetine dihydrochloride* (CAS number: 316-42-7) down-regulated the level of Bcl-xL mRNA [11]. In 2012 Michelle *et al.* at Universite de Sherbrooke (Quebec, Canada) [6] identified four cis-elements in exon 2 of the *BCL2L1* gene which contribute to the splicing control: B2, B3, B1, and SB1 regions.

Baculoviral IAP repeat containing 5 (BIRC5, survivin)

In the process of apoptosis, the IAP family prevents apoptosis through direct caspase and pro-caspase inhibition (Fig. 1). The IAP family members are defined by the presence of a baculovirus IAP repeat (BIR) domain [22, 23]; currently seven genes have been isolated in this family: *XIAP, cIAP1, cIAP2, ILP2, BRUCE, BIRC5 (survivin)* and *livin*. The IAP proteins are abnormally regulated and expressed in the majority of human malignancies at elevated levels, and an increasing amount of evidence has been recently accumulated regarding the effects of survivin on the anti-apoptotic pathway; hence survivin has been investigated as a therapeutic target for malignancies.

Several of splice variants of survivin have been reported (Fig. 4 *b*). In 2007 Sampath and Pelus published a detailed review on alternative splice variants of survivin [24]. It seems that the splice variant Sur2B was regarded as pro-apoptotic until the middle of 2000s; the results of recent studies, however, indicate different outcome [25–27]. In 2011 Huang *et al.* reported that the wild-type survivin (SurWT), Sur-DeltaEx3, Sur2B, and Sur-2alpha splice variants were significantly elevated in astrocytoma and were associated with tumor grade and poorer prognosis [26], and Vivas-Mejia's study on taxane-resistant ovarian cancer cells showed that Sur-2B was more abundant in taxane-resistant cells than in taxane-sensitive cells [27]. These results should be comprehensively organized to develop therapeutic agents to target the variants. Currently novel drugs targeting sirvivin such as LY2181308 [28] and YM155 [29] are under development, and recently amiloride was also reported to regulate alternative splicing of *survivin* as well as *APAF1* and *CRK* [16].

CASP8- and FADD-like apoptosis regulator (CFLAR)

By searching EST databases for sequences of FADD, which forms a death-inducing signaling complex (DISC) with FAS-L, FAS, and pro-caspase-8 for the apoptotic pathway (Fig. 1), in 1997 Shu *et al.* reported cDNAs encoding a protein which they designated *CASPER* [30], and several groups simultaneously isolated genes such as *CASH, MRIT, CLARP* and others, all of which turned out to be a single gene. This gene is currently registered as *CASP8- and FADD-like apoptosis regulator (CFLAR)* with several types of splice variants in the NCBI database (Fig. 4 *c*). *CFLAR* has two death effecter domains (DEDs) only, in contrast to FADD, which have two death domains (DDs), and caspase-8 and -10, both of which have two DEDs along with caspase domain.

CFLAR is a major resistance factor and critical anti-apoptotic regulator that inhibits TNF-alpha, FAS-L, and TRAIL-induced apoptosis (Fig. 1) as well as chemotherapy-triggered apoptosis in malignant cells. CFLAR binds to FADD and/or caspase-8 or -10 in a ligand-dependent or ligand-independent fashion, which in turn prevents DISC formation and subsequent activation of the caspase cascade [31]. 13 distinct splice variants of CFLAR have been identified at the mRNA level [32–37], and CFLAR is expressed as long (c-FLIPL), short (c-FLIPS), and c-FLIPR splice variants in human cells (Fig. 4 *c*). Although CFLAR is a major resistance factor and critical anti-apoptotic regulator, the functional differences of the isoforms are still under exploration, and a cell-type specific pro-apoptotic role,

depending on caspase-8 to c-FLIPL ratio, has also been reported [38].

Mitogen-activated protein (MAP)-kinase activating death domain (MADD)

In 1996 Chow and Lee reported the cDNA sequence of *mitogen-activated protein (MAP)-kinase activating death domain (MADD)*, a novel human gene that is differentially expressed in normal and neoplastic cells [39]. MADD is implicated in cancer cell survival, proliferation, apoptosis, and other regulated functions [40]. Recently Prabhaker's laboratory at University of Illinois at Chicago has published a series of their studies on splice variants of *MADD* gene and their association with apoptosis [41–45]. The *MADD* gene encodes at least six different splice variants (Fig. 4 *d*), of which four variants, namely IG20pa, MADD, IG20-SV2, and DENN-SV, are expressed more ubiquitously [40]. Of these, MADD and DENN-SV are constitutively expressed, whereas the IG20pa and IG20-SV2 may or may not be expressed [40].

Other apoptotic genes with splice variants

In addition, splice variants which may have differential functions on apoptosis have been reported in some of other genes such as *RBM5* [46–48], *ING* family [49–51], *androgen receptor (AR)* [52–54], *BRCA1 associated RING domain 1 (BARD1)* [55, 56], *caspase 8 (CASP8)* [57, 58], *Kruppel-like factor 6 (KLF6)* [59, 60], *myeloid cell leukemia sequence 1 (MCL1)* [13, 61], and *spleen tyrosine kinase (SYK)* genes [62, 63].

THERAPEUTIC STRATEGIES TO TARGET SPLICE VARIANTS

In the present article we summarized splice variants of genes in the apoptotic pathway. Novel technologies such as microarray have enabled us to comprehensively screen alternative splicing, and the recent advance of sequencing technology will give us more powerful method to understand the whole picture of this field [64, 65]. The research on the mechanism of alternative pre-mRNA splicing [66, 67] and the trans-elements [68] are in progress, and the *in silico* computer predictions play another important role [69, 70] although they do not always correlate with *in vitro* and *in vivo* results. On the other hand, the experimental approaches of apoptosis and oncology are essential for the progress of research in this field.

The information of apoptosis will help us develop new strategies of cancer therapeutics. To target splice variants of apoptosis-associated genes, chemical compounds as small molecules are as important as ever, and RNA interference, antisense oligonucleotides, and splice-switching oligonucleotides [71] as well as monoclonal antibodies are new strategies to target specific sequences. If the information of the apoptosis and aberrant splicing in each of malignancies is integrated, it will become possible to target proper variants for apoptosis, and the trans-elements themselves can become specific targets of cancer therapy as well.

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