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EDITORIAL

Impairment of pre-mRNA splicing in liver disease: Mechanisms and consequences

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Abstract

Pre-mRNA splicing is an essential step in the process of gene expression in eukaryotes and consists of the removal of introns and the linking of exons to generate mature mRNAs. This is a highly regulated mechanism that allows the alternative usage of exons, the retention of intronic sequences and the generation of exonic sequences of variable length. Most human genes undergo splicing events, and disruptions of this process have been associated with a variety of diseases, including cancer. Hepatocellular carcinoma (HCC) is a molecularly heterogeneous type of tumor that usually develops in a cirrhotic liver. Alterations in pre-mRNA splicing of some genes have been observed in liver cancer, and although still scarce, the available data suggest that splicing defects may have a role in hepatocarcinogenesis. Here we briefly review the general mechanisms that regulate pre-mRNA splicing, and discuss some examples that illustrate how this process is impaired in liver tumorigenesis, and may contribute to HCC development. We believe that a more thorough examination of pre-mRNA splicing is still needed to accurately draw the molecular portrait of liver cancer. This will surely contribute to a better understanding of the disease and to the development of new effective therapies.

Key words: Cell signalling; Hepatocarcinogenesis; Pre-mRNA splicing; Splicing factors; Targeted anticancer therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a deadly disease, and is currently ranked as the fifth most common cancer worldwide[1]. Most cases of HCC emerge on a background of chronic liver injury and inflammation induced by viral infection (hepatitis B and C viruses), chronic alcohol abuse, exposure to hepatotoxins (such as aflatoxin B), and genetic or metabolic conditions (such as haemochromatosis, 1-antitrypsin deficiency, obesity or diabetes)[2]. The end-stage of chronic liver disease is liver cirrhosis, characterised at the histological level by the massive deposition of extracellular matrix in fibrous septa that surround regenerative nodules. In these regenerative

nodules, partially dedifferentiated hepatocytes are driven by a pro-inflammatory milieu to divide in an attempt to restore the lost functional mass, frequently giving rise to foci of dysplastic cells[3,4]. Accumulating evidence indicates that this microenvironment favours the development of mutations and genetic alterations that are at the origin of the malignant transformation of the liver[5-7]. In view of this, chronic hepatitis and cirrhosis are considered as pre-neoplastic conditions. Nevertheless, a small but sizeable proportion of HCCs develop in normal liver tissues, in the absence of any known risk factor for liver cancer. The mechanisms leading to cancer in this small but sizeable minority of HCC cases are not known[1].

HCCs are complex tumors from the molecular point of view[8,9]. Over the past decade many efforts have been made to identify the molecular alterations that take place during the development of HCC. High throughput array-based techniques evaluating gene expression profiles have provided valuable clues for the identification of key molecular pathways driving the neoplastic conversion of the liver[10-12]. Such studies have significantly contributed to the definition of accurate prognostic genetic signatures, and to the identification of relevant therapeutic targets, some of which are currently being validated in the clinical setting[13-15]. However, conventional microarray approaches are not robust enough to detect the subtle differences in the transcriptome that arise through alternative splicing mechanisms, and therefore potentially relevant alterations involved in the carcinogenic process can be missed[16,17]. Indeed, a large proportion of the diversity within the transcriptome is generated by alternative splicing, a mechanism through which multiple mRNAs and structurally different proteins can be produced from a single gene and that may affect more than 70% of human genes[18,19]. The protein products generated by alternative splicing can have different or even antagonistic biological roles, and therefore their relative levels may impact significantly on cell function. Alterations in mRNA splicing are important cause of disease, as illustrated by the fact that single-point mutations affecting splicing represent at least 15% of all disease-causing point mutations[20]. The relationship between alternative splicing and cancer has also been clearly established, cancer-specific splice variants and cancer-associated changes in the relative levels of spliced isoforms of genes with an established role in carcinogenesis have been observed[21-25]. Moreover, in some cases the pro-tumorigenic effects of these changes have been directly demonstrated, and the mechanisms leading to the appearance of these tumor-associated alterations in normal mRNA splicing are now being elucidated[24,25]. Together these findings attest to the importance of dysregulated pre-mRNA splicing in cancer. However, compared to other types of tumors, less information is available on the disruption of normal splicing in liver cancer and its biological significance. Here we discuss representative cases that illustrate the importance that perturbations in this process may have from the early stages of hepatocarcinogenesis.

OVERVIEW OF THE BASIC MECHANISMS OF PRE-MRNA SPLICING AND ITS REGULATION

Most eukaryotic pre-mRNAs contain noncoding sequences (introns) that need to be removed to generate the correct concatenation of exonic sequences[18,19]. Introns represent more than 90% of the length of pre-mRNAs, and the spliceosome, a complex nuclear machine, needs to accurately identify specific nucleotide sequences at intron-exon boundaries to carry out intron excision and exon joining. The spliceosome is composed of five types of small nuclear ribonucleoproteins (snRNPs), plus a large number of ancillary proteins. This complex is able to recognize a 5' donor splice site beginning with a GU dinucleotide, and a 3' acceptor site ending with an AG dinucleotide at the boundaries of introns, as well as the so-called branching sequence that precedes the 3' acceptor site. The 5' splice site is recognized by the U1 snRNP, while the branching sequence and the 3' acceptor site are recognized and bound by the U2 snRNP and the auxiliary factor U2AF, respectively (Figure 1A)[21]. Subsequent interaction with the U4, U6 and U5 snRNPs leads to the formation of the catalytically active complex that carries out the trans-esterification reactions, resulting in the cleavage and religation of the mRNA chain.

The accurate excision of introns requires the recognition of the above-mentioned consensus sequences that identify the splice sites[26]. Deviations from these consensus sequences generate weak sites that have less affinity for their respective snRNPs, resulting in less efficient exon recognition and therefore allowing the alternative selection of exons[21,24]. In these cases an additional class of sequence elements present both in introns and exons may come into play and modulate the selection of splice sites by the spliceosome machinery. These sequences, known as splicing enhancers and silencers, are short conserved elements of about 10 nucleotides that can enhance or repress exon recognition. The specific binding of a wide range of splicing regulatory proteins, such as SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNP), to these elements in pre-mRNAs influences (positively or negatively) the placement of the spliceosome on the appropriate splice sites (Figure 1B). This mechanism allows the facultative use of weak splice sites and mediates the generation of alternatively spliced mRNAs[19,24]. Nevertheless, many if not all exons contain exonic splicing enhancers that tend to be clustered around splice sites, and these elements are of particular high density within constitutive exons. As discussed by Jensen *et al*[26], this suggests that enhancers are important for the control of constitutive exon splicing, even in the presence of consensus splice sites. Exonic splicing silencers also help to define exon boundaries, and strongly affect splicing when there are cryptic or multiple splice sites of similar strength. These elements are thought to play a more prominent role in the control of alternative splicing[26,27]. Figure 2 summarizes

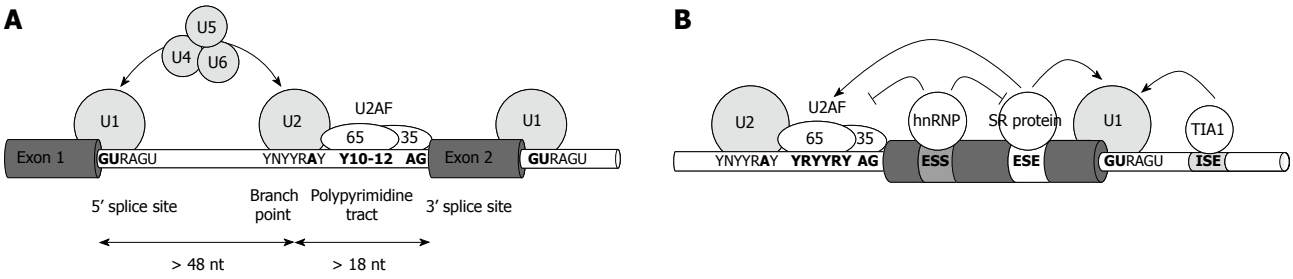

Figure 1 Splicing consensus sequences and interactions with small nuclear ribonucleoproteins (snRNPs), SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). A: Arrangement of donor and acceptor sites in a eukaryotic gene and interaction with snRNPs during splicing. Core elements necessary for pre-mRNA splicing include the 5' and 3' splice sites and a branch point sequence and a polypyrimidine-rich tract located upstream of the 3' splice site. The splicing factor U2AF (U2 auxiliary factor) consists of two subunits which bind to the 3' splice site and the polypyrimidine tract. U2AF promotes the binding of U2 snRNA in the U2 snRNP complex to the branch site. The U1 snRNP particle binds to the upstream and downstream 5' splice sites through base pairing of the U1 snRNA. The additional assembly of the snRNPs U4, U5 and U6 is required for the constitution of the spliceosome and the removal of introns; B: Interaction of splicing regulatory proteins with exonic and intronic target sequences. SR (Ser-Arg) proteins bind to exonic splicing enhancers (ESEs) to stimulate the binding of U2AF to a weak 3' splice site, which here is interrupted by purines (R). They also stimulate the binding of the U1 snRNP to the downstream 5' splice site. SR proteins antagonise the negative effect on splicing of hnRNPs bound to exonic splicing silencers (ESSs). In many cases, U-rich sequences situated immediately downstream of 5' splice sites known as intronic splicing enhancers (ISEs) are bound by factors such as T cell-restricted intracellular antigen 1 (TIA1) to facilitate U1 binding.

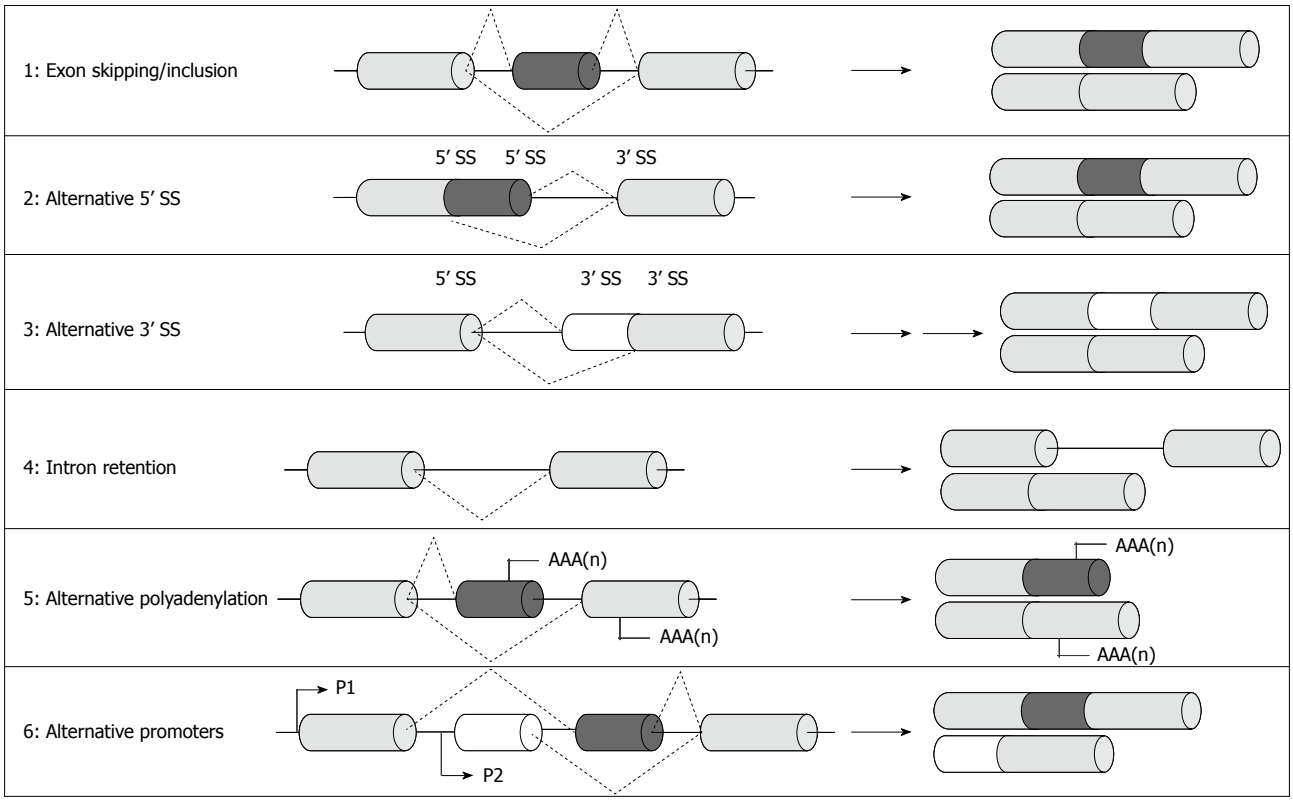

Figure 2 Different modes of alternative splicing. After pre-mRNA processing a single gene can encode multiple mRNA isoforms that possess distinct coding and regulatory sequences. Alternative splicing isoforms can result from: 1: The skipping or inclusion of alternative exons; 2: The selection of alternative 5'; or 3: 3' splice sites (SS); 4: The inclusion of introns; 5: The selection of different polyadenylation sites [AAA(n)]; and 6: The transcriptional initiation at an alternative promoter associated with the inclusion of specific exons.

the different alternative splicing events observed, these include: (1) exon skipping (cassette exons), in which the exon can be spliced from the transcript together with its flanking introns; (2) alternative 5' and (3) 3' splice site selection, where two or more splice sites can be recognised in an exon; (4) intron retention, in which an intron can remain in the mature mRNA; (5) alternative polyadenylation signals, which allows the generation of isoforms with 3' untranslated regions (UTRs) of different length; and (6) the alternative usage of promoters, with the generation of isoforms with promoter associated exons[18,19].

SR proteins are highly conserved RNA-binding proteins that facilitate splice site recognition and promote the inclusion of exons in the mature mRNA. The SR protein family members mostly interact with exonic splicing enhancers (ESE), stabilising the interaction of snRNPs and other factors at splice sites. Among other factors this family includes the ASF/SF2, 9G8, SC35, SRp30c, SRp20,

SRp40 and SRp46 proteins[27]. In contrast to the SR proteins the hnRNPs, such as hnRNPA1 and hnRNP I, have been mainly implicated in exon skipping. hnRNPA1 binds to exonic splicing silencers (ESS) and is able to prevent the binding of SR proteins to their specific sites in the exonic region, while hnRNPI competes with the U2AF auxiliary factor for its binding sequence close to the 3' splice site[24,28]. Variations in the expression levels of these splicing factors are known to affect the selection of splice sites, and therefore are thought to significantly influence the relative levels of splice variants among different tissues and also between normal and transformed cells[23,24].

Interestingly, accumulating evidence points also to the influence of extracellular stimuli on the regulation of alternative splicing[28,29]. In this respect, the activity of splicing factors is known to be affected by their phosphorylation status[30]. For instance SR proteins can be extensively phosphorylated on Ser residues, and their phosphorylation status affects protein-protein and protein-RNA interactions[28,30]. Moreover, extracellular signals can also impinge on the subcellular localisation of splicing factors, and consequently modulate their ability to interact with pre-mRNAs. This has been shown for different members of the SR protein family[29], and also for the splicing factor Slu7[31]. Several kinases and phosphatases responsible for the post-translational modification of splicing factors have been identified, and these include for instance the SR protein kinases (SRPKs), Clk/Sty, CDC-2 related protein kinase-7, and more recently, Akt and Fas-activated Ser/Thr kinase (FASTK)[28,29,32,33]. Among the phosphatases able to dephosphorylate splicing factors of the SR family we can cite PP1 and PP2A[29]. Less is known regarding the extracellular signalling pathways that control the activity of splicing factors, however, recent experimental efforts are beginning to pay off and relevant pathways are being delineated[29]. For example, insulin treatment has been shown to increase SRp40 phosphorylation and to stimulate protein kinase CβII alternative splicing *via* Akt2 activation[34]. Similarly, growth factors like the epidermal growth factor (EGF) have been demonstrated to modulate the splicing of the surface antigen CD44 in HeLa cells through the activation of Ras/mitogen-activated protein kinase (MAPK) cascade[35]. More recently, the activation of the EGF receptor (EGFR) was also shown to regulate the alternative splicing of the tumor suppressor genes *Krüppel-like zinc finger transcription factor 6* (*KLF6*) and *p73* in HCC cells. Although this will be discussed later in more detail, alternative splicing of *KLF6* pre-mRNA triggered by EGFR stimulation involved the Ras/phosphatidylinositol 3-kinase (PI3K)/Akt cascade, while that of *p73* specifically depended on c-Jun N-terminal kinase (JNK) activation[36,37]. Complex interactions between intracellular signalling pathways in the control of alternative splicing are also emerging. Such as the case of the antagonistic effect of JNK signalling on PI3K-mediated splicing regulation of the fibronectin gene in response to extracellular stimuli[38]. Taken together, these observations underscore the dynamic nature of alternative splicing, and also the high sensitivity to environmental signals displayed by this fundamental mechanism of gene expression regulation.

MECHANISMS AND SIGNIFICANCE OF ALTERATIONS IN PRE-MRNA SPLICING IN HEPATOCARCINOGENESIS

As previously mentioned, alterations in pre-mRNA splicing patterns, including changes in the normal tissular patterns of alternative splicing and the appearance of tumor-specific aberrantly spliced mRNAs, are increasingly being reported and implicated in cancer[39,40]. The mechanisms responsible for these alterations observed in cancer cells are still not well known. Nevertheless, some of these mechanisms are currently being elucidated and include: (1) mutations that create or disrupt splice sites or splicing enhancers or silencers[21]; (2) the abnormal expression of splicing factors[22,23,41,42]; and (3) the activation of cell signalling pathways that affect the activity of the splicing machinery[21,29]. In spite of recent advancements a major challenge in the field is still to discriminate whether these changes in alternative splicing can be the cause or are the consequence of human diseases. This is especially difficult in neoplastic diseases like HCC, if we take into account the multiple and heterogeneous genetic alterations that occur along the multi-step process of hepatocarcinogenesis, and the profound effects that the tumor microenvironment may have on transformed cells. Nevertheless, in some cases direct evidence has been provided on the oncogenic potential of liver cancer-associated splice variants and on the mechanisms involved in their generation. Interestingly, some cancer-associated isoforms have already been detected at pre-neoplastic stages, suggesting their potential early contribution to liver malignisation. Below we discuss some examples that are summarised in Table 1.

One interesting case of dysregulated alternative splicing occurring early during human hepatocarcinogenesis was reported by Saito *et al*[43]. These authors described the overexpression of a splice variant of DNA methyltransferase 3b (*DNMT3b*), namely *DNMT3b4*, in liver tissues showing chronic hepatitis and cirrhosis, as well as in HCC tissue samples. In contrast to *DNMT3b3*, the major variant in normal liver tissues, the *DNMT3b4* splice variant lacks the conserved methyltransferase motifs IX and X in exon 21, and probably also lacks this enzymatic activity. An elevation of the ratio of *DNMT3b4* to *DNMT3b3* mRNA was significantly correlated with the degree of DNA hypomethylation on pericentromeric satellite regions in precancerous conditions and HCC[43]. Hypomethylation of pericentromeric satellite regions may induce chromosomal instability, and is considered an early event during hepatocarcinogenesis. The authors hypothesised that the protein product of *DNMT3b4* transcript might compete with the active variant DNMT3b3, behaving as a dominant negative isoform. They confirmed this possibility by transfecting HEK293 epithelial cells, which express the DNMT3b3 active variant, with a DNMT3b4 expres-

Table 1 Examples of genes with altered splicing in liver carcinogenesis

Gene	Function	Selected ref.
DNMT3b4	DNA methyltransferase	[43,44]
MDM2	E3 ubiquitin ligase	[47]
Aurora kinase B Sv2	Serine/Threonine kinase	[45,46]
Hugl-1	Tumor suppressor	[49]
Tensin 2var3	Focal adhesion	[48]
Cadherin 17	Cell surface adhesion	[55]
MAD1 (β)	Spindle-assembly checkpoint	[50]
KLF6	Tumor suppressor	[36]
SVH	Unknown	[52]
p73	Tumor suppressor	[37,72]

sion vector and observed satellite DNA demethylation[43]. Furthermore, subsequent observations demonstrated that DNMT3b4 transfection resulted in enhanced growth rate and increased expression of transformation-related genes, even before chromosomal alterations appeared, underscoring the profound biological consequences of this altered splicing event[44].

Other examples of aberrantly-spliced genes detected in the preneoplastic cirrhotic liver include the serine/threonine kinase aurora kinase B (*AURKB*), and the E3 ubiquitin ligase and p53-antagonistic protein *MDM2*. AURKB, which is expressed in normal liver tissue, had been shown to be overexpressed in HCC correlating with tumor recurrence and a poor prognosis[45]. It was subsequently observed that over 60% of metastatic liver cancer tissues expressed the *AURKB* splice variant 2 (*AURKB Sv2*), which was also present in about 16% of the cirrhotic tissues tested regardless of their aetiology, but was absent in the normal liver parenchyma[46]. Interestingly, *AURKB Sv2* variant-positive HCC samples were mostly obtained from younger patients, and correlated with a poor outcome and short disease-free period[46]. From a mechanistic point of view the contribution of AURKB Sv2 to hepatocarcinogenesis is not known. This splice variant lacks parts of the kinase domain, and it could compete with the full length AURKB in a dominant negative manner, but experimental data on this aspect are not available yet. Regarding *MDM2*, two splice variants with tumorigenic potential, which are absent in the normal liver tissue, were found in alcoholic and autoimmune liver cirrhosis. However, for still unknown reasons these variants were not detected in hepatitis C virus (HCV)-infected cirrhotic samples[47].

As previously mentioned, the experimental demonstration of the pro-oncogenic effects of the splice variants found in transformed tissues is important to establish a cause and effect relationship. This is commonly done by expressing the splice variant under evaluation in cultured cell lines, as presented above for DNMT3b4, and more recently also by the generation of transgenic animals with targeted expression of the spliceoform in question. Although these approaches can be limited by the generally unphysiological expression levels obtained, relevant mechanistic information may be drawn from these studies. For instance, Yam *et al*[48] found that the Tensin 2 splice variant 3 (*TENSIN 2sv3*) was overexpressed in 46% of HCC tissues and most HCC cell lines examined, and that the expression of this variant correlated with a more aggressive tumor phenotype and other pathological features of poor prognosis. Tensins constitute a new family of focal adhesion proteins that link the extracellular matrix to the actin cytoskeleton and to intracellular signalling pathways. To directly assess the consequences of *TENSIN 2sv3* overexpression, the authors generated stable clones in a HCC cell line with low *TENSIN 2sv3* expression. These clones displayed increased growth, invasive properties, and tumorigenicity in an orthotopic model of HCC[48]. Together these findings identified a novel determinant in the metastatic behaviour of HCC which is generated by alternative splicing. Another cytoskeletal protein recently shown to be aberrantly spliced in HCC is the product of the *Hugl-1* gene, a tumor suppressor mainly expressed in the cytoplasm and involved in the regulation of cell polarity. Lu *et al*[49] found that 32.5% of HCCs displayed aberrant spliced variants of this gene that lacked a conserved repeat motif involved in protein-protein interactions. The presence of these Hugl-1 variants correlated with poor differentiation and large tumor size, and their overexpression in HCC cells resulted in enhanced invasion and tumorigenicity when injected into nude mice. Similar findings were recently reported for the *mitotic arrest deficient 1* (*MAD1*) gene. Sze *et al*[50] identified a novel *MAD1* splice variant in human HCC samples which they named *MAD1β*, while the original wild-type isoform was renamed *MAD1α*. MAD1α is a key protein in the mitotic checkpoint complex that monitors the status of kinetochore-microtubule attachment and the formation of the connections to the mitotic spindle. Defective mitotic checkpoint leads to DNA aneuploidy and chromosomal instability, which are features of HCCs[51]. MAD1β was overexpressed in 24% of the HCC tissue samples compared to the surrounding parenchyma, and interestingly more than 50% of the cases expressed MAD1β both in the tumoral and nontumoral tissue. MAD1β lacks the exon 4 of MAD1α, and is localised to the cytoplasm instead of the cell nucleus. MAD1β overexpression also affected the subcellular location and protein levels of other key components of the mitotic checkpoint such as MAD2. Functional studies overexpressing MAD1β in HCC cell lines demonstrated that the presence of this splice variant resulted in severe chromosome aberrations, thus demonstrating that MAD1β induces mitotic checkpoint incompetence[50]. Finally, there are also examples in which the specific targeting and downregulation of an aberrant splice variant found in HCC cells and tissues results in reduced tumorigenesis. This is the case of the "specific Splicing Variant involved in Hepatocarcinogenesis" (*SVH*) gene, an armadillo repeat domain containing gene of still unknown biological function that can produce four splice variants (*SVH-A, -B, -C* and *-D*). Only the splice variant *SVH-B* was upregulated in HCC tissues and hepatoma cell lines, and only the overexpression of this variant in non-transformed liver cell lines resulted in accelerated cell growth and tumorigenicity in nude mice[52]. Interestingly, the specific inhibition of *SVH-B* with antisense oligode-

oxynucleotides reduced HCC cell growth and survival[52]. This observation also suggests the possibility of specifically targeting aberrantly spliced mRNAs to quell liver cancer, as will be discussed later.

The observations described above illustrate the oncogenic potential of aberrantly spliced isoforms in HCC tissues. However, for all these cases no information is so far available regarding the molecular events involved in their generation[43,48-50,52]. In fact, the mechanisms that lead to the appearance of aberrantly-spliced genes in hepatocarcinogenesis have been so far elucidated for a limited number of genes. Among them we find *Cadherin 17* (*CDH17*), also known as liver-intestine cadherin (LI-Cadherin), a non-classic member of the cadherin family of cell-cell adhesion proteins overexpressed in about 90% of HCCs[53,54]. CDH17 expression was shown to confer tumorigenic potential to premalignant liver progenitor cells[53]. Interestingly, it was later found that 50% of HCC samples, and 30% of peritumoral tissues, also expressed a CDH17 splice variant lacking exon 7[55]. The expression of this splice variant was strongly associated with decreased overall survival of the patients[55]. While the reason why overexpression of this splice variant may have a pathogenic role remains speculative, the mechanisms behind its generation have been characterised. Wang *et al*[55] detected a base change at position 651 in exon 6, and another single nucleotide polymorphism (SNP) in a putative branch point at intron 6 position 35 (IVS6+35) was also found. The mutation at position 651 in exon 6 could affect exon 7 inclusion by generating an ESS, or by disrupting an ESE located in exon 6[21]. GG and AG polymorphisms were identified in 73% of the patients at IVS6+35, and in the same patients TT and CT polymorphisms were also observed at 651 (exon 6), in the control group normal livers showed the wild-type phenotype 651 CC, IVS6+35 AA. The functional role of these two SNPs was confirmed in a minigene assay, in which the presence of 651 T and IVS6+35 G SNPs resulted in exon 7 skipping[55]. It was later observed that the 651 T IVS+35 G haplotype, specially 651 TT and IVS6+35 GG homozygotes, seemed to be strongly associated with HCC, and therefore could be considered as a genetic susceptibility factor for HCC development in a Chinese population[56].

While point mutations in splice or regulatory sites are perhaps the most common alterations leading to aberrant splicing in cancer, the role of signalling pathways is gaining recognition. This is very well illustrated by the study of fibronectin alternative splicing regulation. Fibronectin is a glycoprotein found in blood, body fluids and tissues that plays a key role in cell adhesion and migration. Fibronectin has a domain structure consisting of three internally homologous repeats, termed type Ⅰ, Ⅱ and Ⅲ domains. There are two type Ⅲ domains that can be alternatively spliced, and incorporated or not in the mature transcript, the extra domains A and B, denominated EDA and EDB[57]. In addition, the so-called type Ⅲ connecting segment (ⅢCS) can also be alternatively spliced, generating in total up to 20 fibronectin isoforms in humans[57]. Fibronectin is biologically classified in two forms, namely plasma and cellular fibronectin, and only cellular fibronectin contains the EDA and EDB sequences. Plasma fibronectin is secreted by the hepatocytes, but these cells, as other normal adult cells, express limited amounts of EDA-containing fibronectin, which is expressed by foetal hepatocytes[57]. Several functions have been described for the EDA domain, including cell adhesion, wound healing, matrix assembly, matrix metalloproteinase expression, cell differentiation and tissue injury and inflammation[57]. Alternative splicing of the EDA exon is controlled by a bipartite element comprising an ESE and an ESS[29,57]. Srebrow and co-workers[38,58,59] have characterised the effect of extracellular matrix components and growth factors like EGF, hepatocyte growth factor and fibroblast growth factors, on fibronectin splicing. These authors observed that extracellular matrix components such as laminin and collagen Ⅳ downregulated EDA inclusion, while growth factors promoted the inclusion of EDA in fibronectin mRNA[38,59]. The signalling pathways connecting the cell surface with the nuclear splicing machinery controlling fibronectin splicing were also investigated. Exposure to a laminin-rich basement membrane resulted in JNK activation and sustained extracellular signal-regulated kinase (ERK) dephosphorylation, and these events were mechanistically linked to the described downregulation of EDA inclusion[38]. On the other hand, growth factor-activated Ras-PI3K-mediated signalling was shown to alter the phosphorylation levels of the SR proteins SF2/ASF and 9G8, and direct evidence was also provided on the importance of these events in mitogen-stimulated EDA inclusion[32,38].

All the mechanistic observations on the modulation of fibronectin splicing described above were carried out in mammary epithelial cells, however, they can be highly relevant to the alterations of fibronectin splicing found in liver disease. Indeed, EDA and EDB containing variants have been detected in the rodent liver during acute and chronic injury, and during liver regeneration after partial hepatectomy[60-63]. In models of liver injury, EDA-containing fibronectin was mainly produced by sinusoidal endothelial cells, while extracellular matrix-producing cells expressed EDB fibronectin[60,63]. In contrast, in human chronic hepatitis and in liver fibrosis EDA-containing fibronectin was detected not only in non-parenchymal cells, but also in hepatocytes[63,64]. Moreover, both EDA- and EDB-containing fibronectin variants were detected in human HCC tissues, and were localised to hepatoma cells[62,65]. The molecular mechanisms involved in fibronectin alternative splicing in liver injury and transformation are currently unknown. However, the pro-inflammatory milieu characteristic of chronic liver injury and HCC activates the same intracellular pathways that promote fibronectin splicing in mammary epithelial cells. Therefore, it is likely that cytokines and growth factors upregulated during hepatocarcinogenesis promote the alternative splicing of fibronectin in the liver. In support of this is the positive effect of transforming growth factor-β (TGFβ) on EDA inclusion observed in isolated mouse liver endothelial cells, or that of TGF and EGF found in human HCC cells in culture[58,59,62]. The expression of EDA-containing fibronectin during liver injury has been shown to contribute to the activation of ECM-

producing cells[60]. This effect, together with its ability to stimulate cell cycle progression and cellular migration observed in other cell types[57], suggests its likely involvement in the neoplastic conversion of the liver.

Together with fibronectin, other relevant examples of genes for which extracellular signals are known to affect their normal alternative splicing are the tumor suppressor genes *KLF6* and *p73*. As previously mentioned KLF6 is a ubiquitously expressed Krüppel-like zinc finger transcription factor, and tumor suppressor gene that is functionally inactivated in a number of cancers including HCC[66]. The *KLF6* pre-mRNA can give rise to three alternative splice variants, and at least one of them acts as a dominant-negative protein that antagonises the full length KLF6. This variant, named KLF6 SV1, lacks the three zinc finger DNA binding domains but retains the activation domain that mediates protein-protein interactions essential for the biological activity of this family of transcription factors. KLF6 SV1 is overexpressed in several human cancers, correlating with poorer outcome and reduced survival[67]. Increased *KLF6* alternative splicing, expressed as the ratio of *KLF6 SV1* to full length *KLF6*, was observed in 76% of the HCC samples examined[36]. While full length KLF6 overexpression in HCC cells inhibited growth and promoted cell differentiation[68], the overexpression of KLF6 SV1 partially restored the decreased cell proliferation induced by inhibition of the Ras pathway[36]. Mechanistically, the first cause that was identified for the generation of KLF6 SV1 in cancer cells was the presence of a SNP in intron 1 of the *KLF6* gene. This SNP abrogates a binding site for the splicing factor ASF/SF2 and generates a binding site for the SR protein SRp40, provoking the use of two cryptic splice sites in exon 2[66]. The presence of this SNP in association with HCC development has not been tested so far. However, it was known that *KLF6* could still be alternatively spliced into *KLF6 SV1* in normal and cancerous tissues without this SNP[66]. One relevant mechanism for the generation of KLF6 SV1 in HCC cells was recently outlined by Yea *et al*[36]. It involved the oncogenic activation of the Ras/PI3K/Akt pathway and the splice regulatory protein ASF/SF2. Interestingly, upstream of Ras the authors identified the EGFR tyrosine kinase activity as a signal that could trigger KLF6 SV1 generation. These findings may be of special relevance for the biology of HCC, they link a signalling pathway hyperactive in liver cancer[69] to the functional inactivation of a tumor suppressor gene through dysregulated alternative splicing.

p73 is another tumor suppressor gene which is frequently altered in different cancers, including HCC[70]. This gene is structurally related to *p53*, sharing common transcriptional targets that promote cell cycle arrest, apoptosis and limit anchorage-independent cell growth[70]. However, at variance with *p53*, no inactivating mutations have been described for *p73* in cancer cells. Inhibition of p73 tumor suppressive activity derives from the coexpression of N-terminally truncated isoforms. Two of these isoforms are known as ΔEx2p73 and ΔEx2/3p73, and are splice variants of the full length *p73* pre-mRNA that lack the transactivation domain, but retain the DNA binding domain and a C-terminal oligomerisation domain. ΔEx2p73 and ΔEx2/3p73 behave as dominant negative inhibitors of both p53 and p73, and are not expressed in normal tissues, but are frequently up-regulated in HCC[37,71-73]. Forced expression of these variants induces malignant transformation in fibroblasts and chemotherapy resistance in cancer cells[74,75]. Moreover, transgenic mice overexpressing human ΔEx2/3p73 in hepatocytes spontaneously developed HCC, attesting to the *in vivo* oncogenicity of aberrantly spliced p73 variants for liver cells[76]. The mechanisms regulating the generation of protumorigenic p73 variants are not known, however, a recent study by Castillo *et al*[37] identified a signalling pathway that promoted the generation of ΔEx2p73 in liver cells. It was observed that the stimulation of the EGFR by its ligand amphiregulin (AR) in an autocrine manner triggered the splicing of p73 into its ΔEx2p73 variant[37]. AR expression is undetectable in the healthy liver, but it is upregulated during injury and inflammation, contributing to tissue repair but also to the progression of liver disease[77-79]. Overexpression of AR is detected in liver tumors, and significantly participates in the maintenance of the neoplastic phenotype of HCC cells[79]. Interestingly, the expression of AR significantly correlated with the presence of ΔEx2p73 transcripts not only in tumor tissues, but also in cirrhotic livers. Furthermore, this correlation was also observed in the non-neoplastic normal parenchymal tissue of livers that developed HCC in the absence of any risk factor for this neoplasia[37]. These findings suggest that AR-mediated dysregulation of *p73* pre-mRNA splicing can be an early event during hepatocarcinogenesis, including those cases in which cancer develops in a tissue not chronically injured. Mechanistically it was demonstrated that downstream of the EGFR the activation of JNK1 resulted in the downregulation of the splicing factor Slu7 in HCC cells[37]. Slu7 is involved in the correct selection of the 3' splice site during the second step of splicing, and it has been demonstrated that Slu7 knockdown results in exon skipping of endogenous genes[80]. Inhibition of Slu7 expression by AR/EGFR/JNK1 signalling involved the activation of the transcription factor Elk-1, a previously recognised repressor of *Slu7* transcription[80]. Of importance, the expression of Slu7 was also found to be significantly decreased in chronic liver disease and HCC. Together, these observations identify a cancer-related extracellularly triggered signalling pathway that culminates in the transcriptional knockdown of a splicing factor, and the consequent generation of an oncogenic splice variant.

As mentioned before, abnormally expressed splicing factors can induce the production of mRNA isoforms nonexistent or present in low amounts in normal cells. Increasing evidence points to the significant contribution of this phenomenon to malignant progression, as recently demonstrated by the oncogenic consequences of SF2/ASF overexpression[41,81], or the contribution to the cancerous phenotype of hnRNP proteins upregulation[42]. However, a decrease in the expression of splice factors is less frequently observed in cancer cells, although some cases have been reported[23]. For instance reduced expression of

U2AF was found in pancreatic cancer[82], and siRNA-mediated inhibition of its expression in HeLa cells increased the level of the oncogenic CDC25B phosphatase[83]. In view of this, the downregulation of Slu7 gene expression in the cirrhotic liver may have mechanistic implications for the progression towards HCC.

POTENTIAL THERAPEUTIC INTERVENTIONS TARGETING SPLICING DEFECTS

The appreciation that dysregulated splicing may influence the development of human disease makes this process a potential therapeutic target. Different strategies are currently being explored to correct or inhibit pathological splicing events. They may be grouped in two major categories: molecules that can change alternative splicing and antisense strategies[39,84-86].

High throughput screens and individual studies on specific molecules have identified a number of compounds that can change splice site selection. For instance two different natural products with broad anticancer activity, pladienolide and spliceostatin A, have been recently reported to bind and interfere with the essential splicing protein SF3b, a core component of the U2 snRNP, inhibiting the splicing of a number of transcripts resulting in growth inhibition[87,88].

Targeting the expression and the post-translational modifications of splice modulators have also been attempted with promising results. As mentioned before, there are several kinases that phosphorylate the SR proteins in their arginine/serine domains (RS), affecting their intracellular localisation and their interaction with other splice factors and the pre-mRNAs. These kinases include topoisomerase I (Topo I) and members of the Clk/Sty and SRPK families[84,85]. Several antineoplastic compounds that target Topo I, such as diospyrin and indole derivatives like NB-506, can modify splice site selection. NB-506 inhibited the phosphorylation of SF2/ASF and altered the splicing pattern of several target genes including the apoptosis regulator Bcl-X in tumor cells[89]. The Clk1 kinase inhibitor TG003 also interferes with SF2/ASF-dependent splicing and can suppress alternative splicing in reporter genes[90]. The specific targeting of SRPK1 may be of special relevance, given its prominent role in cancer cells. Downregulation of SRPK1 expression by siRNA was reported to inhibit cancer cell proliferation and increase the sensitivity to chemotherapeutics[91], therefore the inhibition of this kinase may be an effective pharmacological strategy. Another approach to modify the activity of splice modulators is to target protein phosphatases (PPs). PP1 can bind directly to a conserved motif in the RNA-recognition region of at least nine splicing-regulatory proteins[84]. Modulation of PP1 activity shows a strong influence on alternative splicing, as illustrated by the lipid ceramide which activates PP1 and promotes the alternative splicing of the apoptosis regulator Bcl-X into its pro-apoptotic variant Bcl-Xs[92]. Activation of PP1 expression and upregulation of Bcl-Xs levels have also been demonstrated to occur in response to S-adenosylmethionine (AdoMet) and 5'-methylthioadenosine (MTA) treatment in HCC cells[93]. Interestingly, these two naturally occurring compounds show antitumoral effects in HCC and can induce growth inhibition and apoptosis in liver cancer cells, while normal hepatocytes are spared from this effect[94,95]. The reasons why AdoMet and MTA promote PP1 expression and Bcl-Xs upregulation only in HCC cells are still unknown, but this fact represents an advantage over other experimental approaches in which splicing can be altered both in normal and cancerous cells.

The selective modulation of splicing reactions is a big challenge, and the specific target of one splicing event is difficult to achieve with the above-mentioned compounds. This can be addressed with antisense oligonucleotides that can complementarily bind to a target site in pre-mRNAs and regulate the splicing process. Antisense oligonucleotides can block cryptic splicing sites created by a mutation, and redirect splicing back to the correct splice site. They can be used to induce exon skipping, and can also be targeted to regulatory sequence elements within exons and introns, like ESEs and ESSs[86]. These antisense oligonucleotides must be stable in biological fluids, and also need to be efficiently delivered inside target cells. To achieve the desired stability, chemical modifications are introduced that prevent degradation. To increase cellular delivery, formulations coupling oligonucleotides to arginine-rich cell penetrating peptides have been devised[39]. Moreover, intracellular delivery of antisense oligonucleotides to correct splicing defects has also been attained using adenoviral vectors[96]. The new generation antisense oligonucleotides include: phosphoroamidate morpholino oligomers, peptide nucleic acids (PNA), and locked nucleic acids (LNA)[86]. Morpholino oligonucleotides have been used to inhibit splicing silencers and to activate otherwise repressed exons. This has been shown for instance for the α exon of fibroblast growth factor receptor 1 (FGFR1), which is excluded in glioblastoma cells[97]. PNAs are oligonucleotide analogs in which the nucleobases are bound to a polyamide backbone that is highly stable *in vivo*[98]. They have been tested in cultured cells and *in vivo* with promising results, inducing the splicing of Bcl-X and promoting apoptosis in cancer cells[99]. LNA antisense oligonucleotides contain a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon. This modification increases the serum stability of oligonucleotides, prevents their degradation by RnaseH, and imparts high specificity toward their target sequences[100]. LNAs have been successfully used to modulate the splicing of tumor necrosis factor receptor 2 (TNFR2) in the liver, switching the endogenous expression of the membrane bound functional form of TNFR2 to a soluble secreted form lacking exon 7 (Δ7TNFR2). The secreted form acted as a decoy receptor for TNF-α, and mice were protected from liver damage in a model of acute inflammation and injury[101]. LNAs have also been used to target and promote the degradation of specific oncogenic splice forms. This has been shown recently by Emmrich and co-workers[102], who designed LNA antisense oligo-

nucleotides directed against the *ΔEx2p73* and *ΔEx2/3p73* oncogenic isoforms of the tumor suppressor p73, which as previously described are upregulated in HCC. Specificity was obtained by targeting the splice junction of each exon deletion variant. This strategy reduced tumorigenic p73 transcripts, without affecting wild-type p73, and inhibited malignant melanoma growth. Additional examples illustrating the potential of antisense oligonucleotide-based strategies to correct splicing defects have been recently reviewed in detail by Khoo and Krainer[103]. Among other cases, these authors describe how the splicing of liver apolipoprotein B 100 (APOB100), a key player in the development of atherosclerosis, can be engineered without affecting the expression of the intestinal isoform APOB48, involved in fat absorption from the gut. Specific targeting of *APOB100* pre-mRNA with antisense oligonucleotides induced the skipping of exon 27, leading to the generation of a shorter variant that has been associated with reduced cholesterol and LDL levels[103].

Also within these targeted strategies, a recent report by Alló and co-workers demonstrated that small interfering RNAs (siRNAs) targeting gene sequences surrounding an alternative exon (the flanking introns) can modulate its alternative splicing, resulting in exon inclusion[104]. It was found that upon siRNA transfection there was an increase in the levels of facultative heterochromatin in the vicinity of the siRNA target sites. The authors proposed that the condensed chromatin structure slows down Pol II elongation and facilitates the inclusion process. Regardless of the mechanistic details underlying this phenomenon, these findings open the door to the application of siRNA technology to correct splicing defects.

Although further studies are needed, together these observations demonstrate that targeting the splicing machinery is feasible and can also be achieved *in vivo*. Moreover, the application of antisense-based therapeutic strategies may be especially effective to treat liver diseases, as suggested by the preferential accumulation of antisense oligonucleotides in this organ when administered to mice[105].

HCC is a molecularly complex type of tumor, however, the identification of key splicing defects that may be corrected or interfered with targeted molecules as outlined above could help to develop new therapeutic strategies. These strategies may be used in combination with other targeted therapies, or with conventional chemotherapeutic approaches.

CONCLUSION

The detection of splicing aberrations from early stages of hepatocarcinogenesis together with the validation of their functional significance, suggests the likely implication of splicing defects in the oncogenic transformation of the liver. The realisation of this is just a start point for future research aimed at the development of effective therapeutic interventions in a deadly disease like HCC. There are different fronts in which action is needed. For instance, a thorough knowledge of the splicing alterations in pre-neoplastic and transformed liver tissue is still lacking. The use of improved splice-sensitive microarray platforms, and the implementation of new technologies like deep sequencing of transcriptome (RNA-seq), that allows both known transcript quantification and novel transcript discovery, are likely to yield valuable information on all splicing events[106,107]. These approaches will allow the identification of new splicing defects with potential pathological significance, and the generation of splicing signatures that may have prognostic value. The characterisation of functional splice regulatory elements in the genome that can be targeted by RNA binding proteins is also a challenge. New methods combining immunoprecipitation of RNA-protein complexes with high-throughput sequencing of reverse transcribed tags are currently being devised[19,106]. From the therapeutic perspective, it is possible that alterations in pre-mRNA splicing resulting from hyperactive intracellular signalling pathways could be reversed in part by the emerging targeted therapies that block these pathways[6,15,36,37]. However, when aberrant splicing is the consequence of genetic mutations that affect splicing reactions, and are not due only to the dysregulation of signalling pathways that impinge on pre-mRNA splicing, different approaches must be used. In these cases that involve structural changes due to mutations, the inhibition of hyperactive signalling pathways may not be effective, and the antisense oligonucleotides described above can be applied. Nevertheless, some aspects such as their potential off-target effects and their efficient delivery to the target tissues still need to be further elaborated[86]. In summary, the integration of pre-mRNA processing alterations in the molecular portrait of liver cancer will surely contribute to the understanding of the disease and the development of new effective therapies.

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