Alternative Splicing in Lung Cancer

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Abstract: Alterations in alternative splicing affect essential biologic processes and are the basis for a number of pathologic conditions, including cancer. In this review we will summarize the evidence supporting the relevance of alternative splicing in lung cancer. An example that illustrates this relevance is the altered balance between Bcl-xL and Bcl-xS, two splice variants of the apoptosis regulator Bcl-x. Splice modifications in cancer-related genes can be associated with modifications either in cis-acting splicing regulatory sequences or in trans-acting splicing factors. In fact, lung tumors show abnormal expression of splicing regulators such as ASF/SF2 or some members of the heterogeneous nuclear ribonucleoprotein family. The potential significance of alternative splicing as a target for lung cancer diagnosis or treatment will also be discussed.

Key Words: Alternative splicing, Lung cancer, Bcl-x, CD44, RNA binding protein, hnRNP.

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Alternative splicing is a major contributor to transcriptome and proteome diversity. Multiple transcripts are generated from a single mRNA precursor by means of alternative splicing. The alternative use of exons in each transcript may have a profound effect on the biologic characteristics of the final protein by adding or deleting functional domains, changing its stability, controlling its location, or modifying its protein-protein interactions. Alternative splicing may also influence mRNA translation, location, and stability. Alternative splicing is not a rare event in normal cells, but a central regulatory mechanism. Recent data from genome-wide studies suggest that more than 90% of human genes undergo alternative splicing.[1] Alterations in alternative splicing have been shown to affect essential biologic processes and many disease conditions, including cancer. Mutations in splicing regulatory elements within the nucleotide sequences and alterations in the expression of splicing regulatory factors have been extensively reported in cancer. Moreover, the expression of several alternatively spliced gene products has also been linked to the development of neoplastic diseases.[2,3] In the recent years, powerful techniques for genome-wide identification and analysis of alternative splicing isoforms have been developed.[4–6] These technologies will allow for the identification of novel cancer-related markers and targets for therapy. It is, therefore, not surprising that alternative splicing in cancer is now emerging as a growing and promising field in basic and translational oncology.[7]

Relevance of Alternative Splicing in Lung Cancer

Several reviews have addressed the relevance of alternative splicing in cancer-related genes.[2,8–10] Table 1 lists genes that have alternative splice variants associated with lung cancer. Splicing variants from cancer-related genes may have a critical impact on lung cancer cell biology.[11] This is well illustrated by the significance of alternative splicing in the function of the apoptosis regulator Bcl-x. The first coding exon of this gene contains an alternative 5′ splice site (Figure 1). If the Bcl-x pre-mRNA is spliced to include the whole exon, the translated product is Bcl-xL, a protein that inhibits apoptosis. If, conversely, the pre-mRNA is alternatively spliced eliminating a 3′-end portion of the exon, Bcl-xS is produced, a shorter protein with proapoptotic activity. A higher proportion of Bcl-xL is frequently found in both non-small cell lung cancer (NSCLC) and small cell lung cancer, deregulating the balance between proapoptotic and antiapoptotic signals and potentially contributing to tumor progression.[12–14] The alternative splicing of CD44 is also biologically relevant in lung cancer. CD44 is a multifunctional surface glycoprotein with an important role in cell adhesion and migration. The human CD44 gene contains 10 variable exons (v1 to v10) that can be alternatively spliced to generate many different CD44 protein isoforms. The most common CD44 isoform is the standard CD44 (CD44s), in which all the variable exons are skipped. CD44s function as a cell-cell and cell-matrix adherence molecule, and its down-regulation has been associated with metastasis in many malignancies.[15] In fact, Pirinen et al.[16] showed that up-regulation of total CD44 and of CD44v3 were predictors of favorable outcome in NSCLC patients. Enhanced expression of other splice variants can be associated with tumor progression and metastasis. Conflicting results have been reported on the significance of CD44v6 in the outcome of NSCLC patients, with studies suggesting that its expression is related to reduced survival,[17,18] and studies showing no relationship with prognosis.[19,20] Inconsistent results on the prognostic significance of CD44 or its splice forms have been also published in other human cancers. This example underlines the importance of

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TABLE 1. Examples of Genes With Splice Variants Associated With Lung Cancer

Gene	Lung Cancer Histology	Function	References
Actinin-4	SCLC	Cytoskeleton binding	56
Bcl-x	NSCLC/SCLC	Apoptosis	12–14
CD44	NSCLC/SCLC	Multifunctional receptor	17,18
CEACAM-1	NSCLC	Adhesion	52
CAIX	NSCLC	pH regulation	57
Cyclin D1	NSCLC	Cell cycle	24,58
FHIT	NSCLC	Nucleotide metabolism	59
Fibronectin	NSCLC/SCLC	Adhesion Angiogenesis	44,60,61
KLF6	Adenocarcinoma	Transcription factor	43
MDM2	NSCLC	Proliferation	62,63
Mesothelin	Mesothelioma	Adhesion	64
NF2/merlin	Mesothelioma	Membrane stabilizing protein	65
NRSF	SCLC	Transcription factor	66
PPARγ	Squamous cell carcinoma	Nuclear hormone receptor	67
TSG101	SCLC	Receptor trafficking	68
VEGF	NSCLC	Angiogenesis	69
XAGE-1	Adenocarcinoma	Unknown	53,54

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

standardization and interlaboratory validation of reagents and protocols used to analyze cancer-specific splicing isoforms.[7]

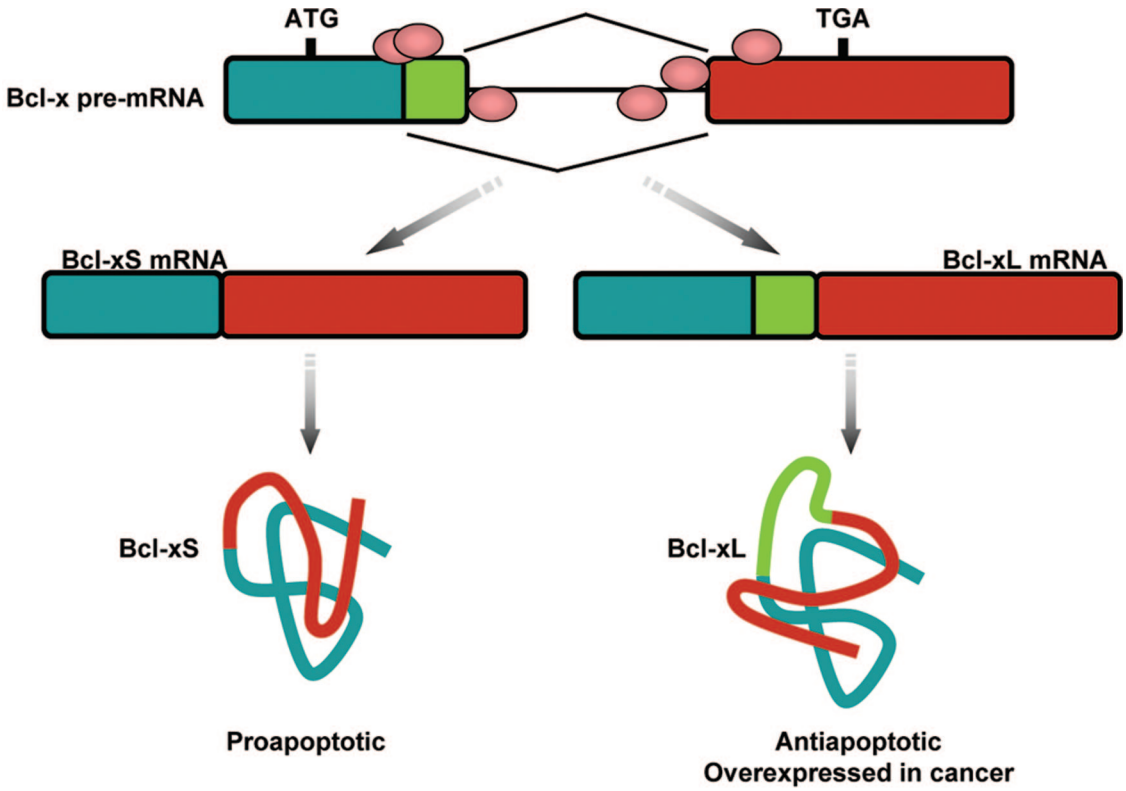
Splicing changes associated with genetic polymorphisms are also relevant for lung cancer risk. Several case-control studies have found an association between the cyclin D1 (CCND1) G870A polymorphism and an increased risk of lung cancer.[21] CCND1 is a protein that controls G1-S phase progression through its interaction with cyclin-dependent kinases (CDKs). In addition, CCND1 can act independently of the CDKs, modulating migration, invasion, differentiation, inflammation, and angiogenesis.[21] The G870A polymorphism does not change the amino acid sequence of CCND1, but modulates its splice pattern. The presence of the A870-allele favors splicing into transcript b, which encodes for the cyclin D1b splice variant. Cyclin D1b induces malignant transformation,[22,23] and its expression has been associated with poor outcome in lung cancer patients.[24]

Splicing Regulatory Proteins in Lung Cancer

The mechanisms leading to aberrant alternative splicing in lung cancer are poorly understood. Splice modifications in cancer-related genes can be associated with modifications in intronic and exonic cis-acting splicing regulatory elements or in trans-acting splicing factors. There are several examples of the importance of cis-acting splicing regulatory motifs in lung cancer, such as the polymorphism in CCND1 described above, or the somatic intronic mutations of Met kinase that lead to an alternatively spliced transcript.[25] Nevertheless, it is reasonable to assume that the majority of splicing alterations in lung cancer are due to modifications in the concentration, localization, composition, or activity of RNA binding proteins acting as splicing regulatory factors. The heterogeneous nuclear ribonucleoprotein (hnRNP) family is a group of RNA binding proteins involved in splicing and in other aspects of RNA metabolism: stabilization, nucleocytoplasmic transport, translation, and transcriptional regulation. Overexpression of hnRNP proteins has been reported in several malignancies. Abnormal expression of hnRNP proteins in NSCLC clinical samples and animal models suggests that tumors develop hnRNP profiles that may contribute to lung carcinogenesis.[26–28] In fact, overexpression of hnRNP A2/B1 was proposed in the past as a potential lung cancer early detection biomarker.[29,30] It could be argued that the overexpression of these and other RNA binding proteins mostly responds to the increased metabolic rate associated with tumor proliferation, however, some studies have demonstrated that these proteins are also involved in the malignant transformation of the cells. ASF/SF2, an important splicing regulator, is up-regulated in many tumors and can act as an oncogene.[31] On the other hand, our group has shown that hnRNP E4 (PCBP4 or αCP-4) is down-regulated in lung cancer and may function as a tumor suppressor gene.[32,33]

Impact of Alternative Splicing in Lung Cancer Therapy and Detection

Drugs that modulate alternative splicing have been proposed as potential therapeutic tools for lung cancer. Recently, the splice variant K-Ras 4A has been identified as the main mediator of the oncogenic activity of mutant K-Ras in lung carcinogenesis. This provides the rationale for designing targeted therapies specific for this cancer-associated splice variant.[34] Therapies for this and other cancer-related genes can be aimed to modify the activity of splicing factors or be directed at reversing specific abnormal splicing events. The latter can be achieved by the use of antisense oligonucleotides that shift alternatively spliced isoforms toward the therapeutically favorable one.[35] Synthetically modified oligonucleotides are favored over regular nucleotide backbones for their stability and low toxicity in vivo. Synthetically modified oligonucleotides which modify the splicing pattern of apoptosis-related genes may induce apoptosis or sensitize cells to treatment with chemo or radiotherapy. The ability to alter the Bcl-x splicing is particularly interesting, given that the two splice variants have opposing functions. In fact, antisense oligonucleotides targeting the Bcl-xL 5′ splice site to specifically inhibit Bcl-xL have been used against lung cancer cells.[36,37] Other antisense oligonucleotides which induce the expression of Bcl-xS sensitize cells to apoptosis in response to treatment.[38] Boon-Unge et al. used a different strategy to switch the splicing pattern of Bcl-x. They screened 1040 drugs searching for compounds able to regulate Bcl-x splicing in the lung adenocarcinoma cell line A549 and found that a compound named Emetine down-regulated the mRNA levels of Bcl-xL with a concomitant increase in the mRNA levels of Bcl-xS.[39] Previously, Chalfant et al.[40] showed that the chemotherapeutic agent gemcitabine increases the generation of ceramide in A549 cells, which induces the dephosphorylation of splicing factors, elevating the levels of the proapoptotic splice variants Bcl-xS and caspase-9, with a



Intervention opportunities

Detection:	*Treatment:*
▪ Overexpression of splicing regulatory factors	▪ Drugs affecting the activity of splicing factors
▪ mRNA from cancer-related splice isoforms	▪ Antisense oligonucleotides (SMOs)
▪ Proteins from cancer-related splice isoforms	▪ Drugs against cancer-related isoforms
▪ Autoantibodies	▪ Vaccines

FIGURE 1. Bcl-x is an apoptosis regulator with two main isoforms (Bcl-xL and Bcl-xS) generated by alternative 5′ splice site selection. Interestingly, the two splice variants have opposing functions: Bcl-xL is proapoptotic whereas Bcl-xS is antiapoptotic. Several transacting splicing factors regulate the balance between the two splice variants. In lung cancer, abnormal splicing of the Bcl-x mRNA gives rise to a predominant production of Bcl-xL. Various strategies for diagnosis or treatment of lung cancer can be proposed based on the predominant expression of the cancer-related splice isoform (either from Bcl-x or from any other gene with splice isoforms involved in lung cancer).

parallel reduction in the expression of the antiapoptotic forms Bcl-xL and caspase-9S. Other anticancer drugs can also induce apoptosis in association with a Bcl-x splicing switch in lung cancer cells.[41,42] Inhibition of KLF6-SV1, a splicing variant of the tumor suppressor gene KLF6, may also have therapeutic potential. DiFeo et al.[43] have recently reported that KLF6-SV1 is overexpressed in lung adenocarcinomas and is associated with poor clinical outcome. KLF6-SV1 overexpression abrogates the proapoptotic effect of cisplatin, whereas targeted reduction of KLF6-SV1, either alone or in combination with cisplatin, results in a marked increase in apoptosis.[43] Alternative splice variants can also be used as targets for imaging and selective delivery of bioactive molecules to tumors. A fibronectin splice variant (ED-B), containing an additional domain inserted by alternative splicing, accumulates around new-forming blood vessels, but is absent in normal vasculature in adult tissues. Antibodies specific for ED-B have been designed to selectively deliver therapeutic molecules to tumor neovasculature both in animal models and patients.[44,45]

Cancer specific alternative splicing forms can potentially be used as diagnostic biomarkers.[8] Any splice variant found exclusively in cancer cells may be a candidate. At the mRNA level, variants would be easily detected by polymer-

ase chain reaction using splice variant-specific primers. Antibodies that specifically detect particular splice variants could be generated and applied in immunoassays. However, to our knowledge, none of these strategies has been tested to date for diagnosis of lung cancer, which may be attributed to the absence of appropriate candidates. The use of high-throughput technologies to identify splicing patterns of many genes simultaneously, will undoubtedly push forward the discovery of lung cancer biomarkers with potential use for diagnosis.[46] In this sense, whole genome exon arrays have already been used to detect splice variants differentially expressed in several tumor types.[47–50] Xi et al.[51] have recently applied this technology to analyze alternative splicing events in lung adenocarcinoma. The authors confirmed the already reported overexpression of a CEACAM1 variant,[52] and, for the first time, found overexpression of specific variants for ERG, CDKN2A, and CDH3.[51] Exon arrays are not specifically designed to identify alternative splicing and some events may have been missed. For this reason, more powerful tools, such as splicing specific microarrays or deep sequencing technologies, should be applied in the future to characterize new splice variants suitable to be used as lung cancer biomarkers.

Finally, abnormal alternative splicing can generate protein antigens recognizable by the immune system. Autoantibody responses have been described to XAGE-1 protein in patients with lung adenocarcinoma.[53,54] XAGE-1 belongs to the family of cancer-testis antigens. Four transcript variants XAGE-1a, XAGE-1b, XAGE-1c, and XAGE-1d have been identified. XAGE-1b is the dominant antigen recognized by sera from lung adenocarcinoma patients. Immunogenicity of XAGE-1 alternatively spliced forms suggests that they might be used as vaccine targets for lung cancer.[55] Detection of these autoantibodies may be also used for early detection or diagnosis in lung cancer patients.

CONCLUSIONS

There is abundant evidence on the importance of alternative splicing in lung cancer. The impact in diagnosis and treatment is still minimal. A better understanding of the mechanisms regulating alternative splicing in lung cancer and the identification of new cancer-associated splice variants will undoubtedly lead to the development of new strategies for lung cancer detection and treatment (Figure 1). Splicing-specific microarrays, designed to explore global splicing changes, are very powerful tools to discover splice variants differentially expressed in tumors, and may be used to identify new candidates for lung cancer diagnosis, prognosis, and therapy. Finally, considering that the splicing machinery is altered in lung cancer, it is important to keep in mind that many splice variants found in lung cancer tissues would likely be "passengers" and not "drivers" of malignant transformation. Functional studies will be required to confirm the biologic relevance of the splice variants found in lung cancer.

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