Review Article

Clinical relevance of alternative splicing

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The unique phenomenon of alternative splicing is gathering concern due to its promising therapeutic potential. The human genome sequencing project suggests approximately 20,000-25,000 genes. Among these, about 35-60% of genes generate multiple mRNAs by alternative splicing mechanism and contribute to the diversity of the proteomic world. This 'gene shortfall' has ignited considerable interest in alternative RNA splicing. This process leads to expression of a single gene responsible for the transcription of different mRNA isoforms that might have multiple biological functions. The disruption of splicing pattern can produce aberrant splice variants, which are implicated in more than 50% of genetic disorders including cancer. Altered splice sites in neoplastic cell contribute to the development, progression and/or maintenance of tumorous growth. The repertoire of tumor-specific variant represents a potential marker in pharmacogenomic diagnostic relevance. Alternative splice isoforms have been analyzed serendipitously by qualitative gene profiling with *in silico* gene prediction software. Computational approach in identifying exonic splicing enhancers in genomic DNA and focus on microarray technology will elucidate differential expression of alternative splice variants. The antisense oligonucleotides modulate alternative splicing and engender the production of therapeutic gene products. Oligonucleotides have the potential to silence the mutations caused by aberrant splicing. The efficacy of the antisense oligonucleotides lies in the chemical configuration, affinity and delivery strategies. Hence the therapeutic potential of antisense oligonucleotides as modulators of aberrant alternative splicing would be a major challenge to the upcoming proteomic era.

Key words: Aberrant splice variant, alternative splicing, antisense oligonucleotides, expressed sequence tag, microarrays

A 13-year (1990-2003) worldwide effort contributed to the completion of human genome project with 2.91 million base pairs. The complete coverage of human genome indicated that it encodes only 20,000-25,000 genes for approximately 90,000 diversified protein isoforms.[1] Today, the gene count has been drastically trimmed down from our previous expressed sequence tag (EST) analysis estimates of between 100,000 and 150,000.[2,3] This 'gene shortfall' has ignited considerable interest in a mechanism called alternative splicing, a natural biological process generating multiple different transcripts from the same precursor gene.

The mechanism of alternative splicing explains the vast disparity between the predicted human genome and the highly diverse proteome.[4] Comparison of human genome with those of other organisms paved the way to realize the contribution of alternative splicing mechanism to the complexity of evolution. It accounts for much of the diversity among organisms with relatively similar gene sets. In addition, the prevalence of alternative splicing appears to increase with an organism's complexity.

Large scale bioinformatic analyses have indicated high rates of alternative splicing, with over 60% of all human genes expressing multiple mRNAs.[2] Eighty percent of these alternative spliced genes result in changes in encoded proteins revealing the proteomic expansion,[5] which also encompasses the regulatory processes for normal development.

The importance of alternative splicing is underlined by the recent discovery of deregulation, which has been documented in a diverse range of human pathologies, including neurodegenerative, cardiovascular, respiratory and metabolic diseases, as well as cancer.[6-10] The tissue- and disease-specific splice variants are analyzed by ESTs which elicit the molecular mechanism of normal cellular physiology as well as the disease states. *In silico* gene prediction tools offer biologists an opportunity to study gene expression on genomic scale. Lately, ever increasing efforts are being made to fully understand

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the functional impact of alternative splicing on physiopathological conditions and to exploit the mechanism to develop new diagnostic and pharmacogenomic tools.

Mechanism of Alternative Splicing

Alternative splicing of pre-mRNAs is a powerful and versatile regulatory mechanism that can affect quantitative control of gene expression and functional diversification of proteins. The joining of different 5' and 3' splice sites allows individual genes to express multiple mRNAs that encode proteins with diverse and antagonistic functions.[11] The regulation of splicing commonly involves the modulation of early steps in spliceosome assembly. Factors involved in the regulation of alternative splicing include the serine-arginine proteins, heterogeneous ribonuclear protein (hnRNP) and other gene-specific factors.[12] Alternative splicing is regulated by the binding of trans-acting regulatory factors to exonic and intronic splicing enhancer and inhibitor sequences. The *cis* elements comprise splicing enhancers and silencers that are located in either the exons or the introns and bind to activator and repressor proteins. There are a number of different alternative splice sites, including exon skipping, inclusion of alternative exons, use of alternative splice donor and acceptor sites and intron retention [Figure 1]. Alternative splicing not only generates segments of mRNA variability that have the potential to insert or remove amino acids, shift the reading frame or introduce a termination codon [Figure 2], but also affects gene expression by removing

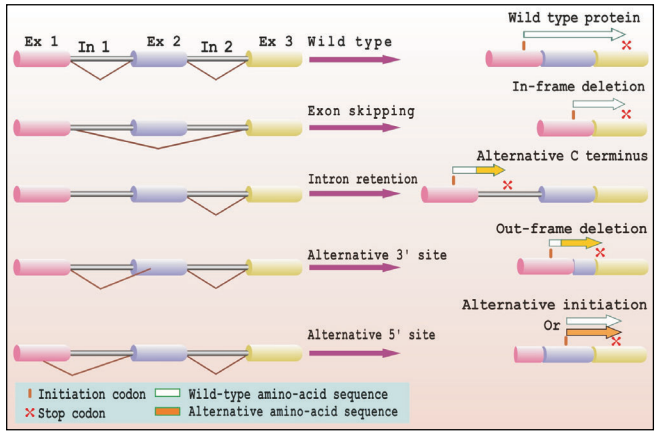

Figure 1: Types of alternative splicing and spliced isoforms

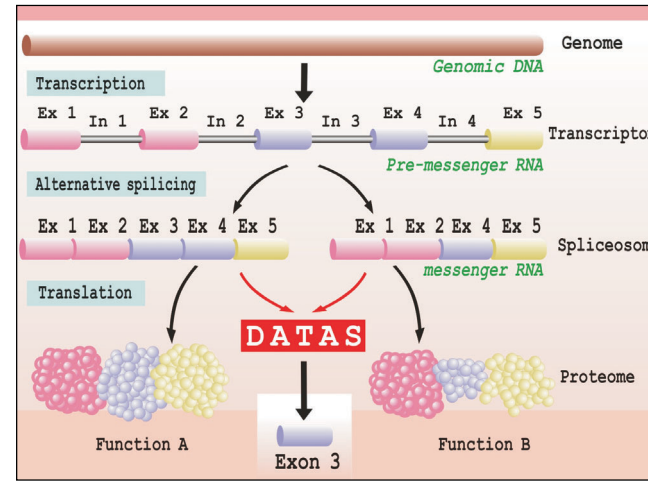

Figure 2: Differential analysis of transcripts with alternative splicing technology: Differentiates two alternative variants in comparison with two different cellular conditions

or inserting regulatory elements controlling translation, mRNA stability and localization.

Association of Alternative Splicing with Diseases

The prevalence of alternative splicing as a general mechanism for the control of gene expression renders it to be a target for alterations leading to human diseases. Deregulation of RNA splicing can be induced by mutations or polymorphisms within gene, and it becomes part of constitutive or acquired pathologies. Fifteen percent of point mutations associated with genetic disease were attributed to aberrant splicing.[13] Mutations which result in aberrant regulation of alternative splicing, causing the expression of protein isoforms that are inappropriate for a cell type, are responsible for certain diseases [Table 1].[14-37]

Pre-mRNA splicing is a natural source of cancer, causing errors in gene expression; and the affected proteins include transcription factors, cell signal transducers and components of extracellular matrix. Many cancers express specific alternative splice variants of various pre-mRNAs, suggesting that alternative splice site selection is changed in transformed cells. The change in alternative splice associated with cancer may be caused by the modification of expression of certain splicing factors. For example, the trans-acting splicing factor polypyrimidine tract binding protein was over-expressed, and the level of a specific splice variant of fibroblast growth factor, receptor-1 (FGFR-1), was

Table 1: Aberrant spliced isoforms and related diseases

Gene	Isoforms	Disease	Reference
IGHD II	Altered /skipped splicing exon 3	Familial isolated growth hormone deficiency type II	Cogan *et al* 1997[14]
WT1	Inclusion of 17 aminoacids exon 9	Wilms tumor-frasiers syndrome	Lee *et al* 2001[15]
β-globin	Cryptic 3' splice site (intron 2)	β-thalassemia	Lacerra *et al* 2000[16]
FTDP-17 (Tau)	Exon 10 inclusion	frontotemporal dementia and Parkinsonism (FTDP)	Hutton *et al* 1998[17]
CFTR	Exon 9 skipping	atypical cystic fibrosis	Cuppens *et al* 1998[18]
PRPF 31	Trans effects-exon 11	retinitis pigmentosa	Wang *et al* 1997[19]
SMN	SMN2 exon 7 inclusion	spinal muscular atrophy	Feldkotter *et al* 2002[20]
DM	Gain of function	myotonic dystrophy	Wang *et al* 1995[21]
Type 1,2	CUG/CCTG repeats		
CD44	CD44v6 (exon 11)	breast cancer,	Herrera *et al* 1999[22]
	CD44v9 (exon 12)	colorectal adenoma	Wittig *et al* 2001[23]
		GI carcinoma	Sneath *et al* 1998[24]
FGFR	FGFR 1 (exon α)	brain tumor	Jin *et al* 2000[25]
Type	FGFR 2 (exon IIIB)	prostate cancer	Kwabi *et al* 2001[26]
1,2,3,4	FGFR 3 (exon 7,8,9)	colorecter cancer	Jang *et al* 2000[27]
	FGFR 4 (exon 9)	gastric, colon, pancreatic cancer	Takaishi *et al* 2000[28]
BRCA	Exclusion of exons 9,10,11	breast, ovarian cancer	Orban *et al* 2001[29]
	(D9,10,11q, BRCA1)	breast cancer	Bieche *et al* 1999[30]
	Exclusion of exon 12 *(BRCA2)*		
XPG	Intron 1 retention *(XPG isoformI)*	lung cancer	Cheng *et al* 2000[31]
	Intron 3 retention *(XPG isoformII)*		
	Intron 6 retention *(XPG isoform III)*		
	Intron 8 retention *(XPG isoform IV)*		
BDNF	Exon 5 inclusion (exon 1,2,3,4,4I,5U)	neuroblastoma tumor	Aoyama *et al* 2001[32]
Bcl-*x*	Exon 2-Bcl-*x* L(Antiapoptotic)	leukemia, prostate,	Mercatante *et al* 2001[33]
	Bcl-*x* S (proapoptotic)	ovarian cancer	
p73	Exon 2 exclusion	breast cancer,	Fillippovich *et al* 2001[34]
		neuroblastoma tumor	Kaghad *et al* 1997[35]
Trx-1	Exclusion of exon 2, 3	lung cancer	Gasdaska *et al* 1994[36]
		colon cancer	Berggren *et al* 2001[37]

increased in more advanced brain tumors.[25] Detailed studies of specific alternatively spliced genes have revealed that splicing could be used to regulate biological processes. One of the best-characterized examples is the life and death regulatory gene, β-cell Leukemia-X, for apoptosis. Bcl-X, which is regulator of apoptosis, is alternatively spliced to produce two distinct proteins – Bcl-X (L) and Bcl-X (S); the former suppresses apoptosis and the latter promotes it.[38]

In many cases, alternative isoforms differ by small alterations of functional elements or domains. Two of the alternative RNA isoforms of the receptor, FGFR2 (IIIb and IIIc), encode altered Ig domains and show variable affinities for the different fibroblast growth factors, thereby signaling differentially and promoting prostate cancer progression.[39] Alternative splicing of pre-mRNA involved in BK potassium channels was seen to be affected by the expression of calcium/calmodulin dependent protein kinases.[40] It has also been shown that stress is able to deregulate alternative splicing of TSG101 and that the presence of certain alternative splice variants is elevated in breast cancer.[41]

Neoplastic transformations, which are known to have altered signaling pathways, may affect alternative splicing patterns. One such example of signaling pathways leading to altered splicing of pre-mRNA includes the splicing-associated factor, YT521-B, splice site selection in response to kinase activity of p59.[42]

Tools for Predicting Alternative Splice Events: EST Clustering

The mainstream methodology being adopted for the

prediction of alternative splicing is with the use of EST data. Sequence-based assembly of ESTs, containing alternative splice isoforms, provide a good means of deciphering the gene structure by gene clusters and subsequent mapping of individual genes.[43]

Several earlier studies have envisaged human ESTs to genome mapping based on the number of ESTs in UniGene clusters,[44] disease-specific or tissue-specific polyadenylation sites[45] and genes differentially expressed in normal or cancer tissues.[46,47] Hanqing[48] *et al.* developed a novel computational approach to analyze tissue information of aligned ESTs in order to identify cancer-specific alternative splicing and gene segments highly expressed in particular cancers.

ESTs from well-defined tissue sources are being used to construct sophisticated body map.[49] However, these approaches failed to consider alternative splicing estimated to occur in over 50% of human genes,[50,51] as EST clusters do not have multiple alignments.

For many genes, the number of corresponding ESTs in existing databases is low. ESTs, which are derived from the 5' and 3' ends of a cDNA and central regions of longer mRNAs are not represented adequately. Therefore, this approach might miss certain important alternative spliced isoforms.[52]

A gene Profiling Technology DATAS

Differential analysis of transcripts with alternative splicing (DATAS) allows the detection of disease-relevant changes in mRNA populations resulting from alternative RNA splicing events, providing key insight into biological causes of pathology. The technology provides a rapid method for generation of unique libraries of alternative RNA splicing transcripts between two conditions and to identify alternatively used exons and introns between these transcripts [Figure 3]. DATAS thus will identify specific mRNA variants (intron retention, exon skipping, etc.), while other gene profiling approaches do not take into account the specific nature of those mRNA variants, as they are only designed to characterize global up- or down-regulation.[53] DATAS identifies novel markers and monitors individual patient's response to therapy, opening up new horizons in target discovery and drug development.

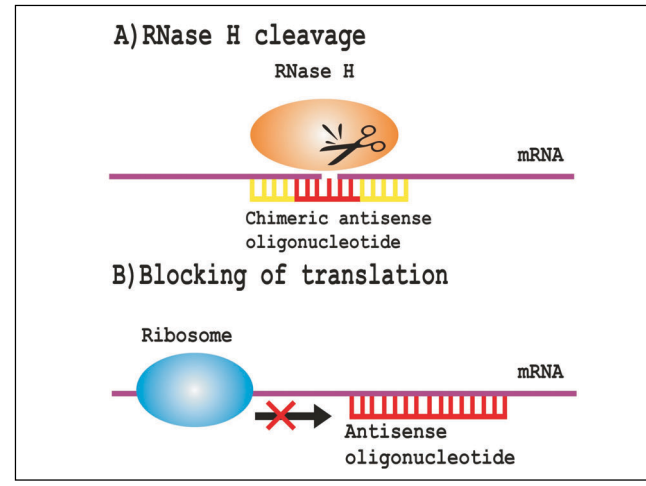

Figure 3: Mechanism of antisense oligonucleotide activity

Splice-Variant Oligonucleotide Microarray

Microarray, a high-throughput tool, monitors splice-variant expression for therapeutic studies and diagnostics. Differences in hybridization signals between two adjacent oligonucleotides from the same gene should provide isoform-specific differential expression data. Being able to measure variant-level expression is important for accurate expression profiling and consequently for obtaining a better understanding of the biological processes. Genomic tiling arrays and exon arrays are used to identify co-regulated exons, which allows the inference of variant mixtures.[54] Expression arrays with multiple probes are retrospectively analyzed to identify exons that are differentially included or skipped in a tissue-specific manner. RNA-mediated ligation combined with arrays presents a novel method for detecting exon-exon junction information of known splice variants. The development of mRNA isoform sensitive microarrays, which requires precise splice-junction sequence information, is a promising approach. Spotted oligonucleotide microarrays employing probes designed to detect unprocessed and processed RNA have been used to monitor pre-mRNA splicing in yeast[55] and the processing of non-coding RNAs in yeast and mammalian cells.[56] A fiber-optic based array method,[33] a polymerase colony assay[58] and more conventional microarray-based approaches utilizing spotted cDNA fragments or oligonucleotides[59-60] have been used for monitoring alternative splicing in mammalian cells. Complementary DNA microarray studies were

conducted to investigate development-related alternative splicing in the human testis for spermatogenesis.[61] One of the major limitations of this process is that all splice events that result in the insertion of novel sequences have to be identified prior to the initiation of the microarray study for accurate interpretation of data.

Alternative Splicing for Therapeutics

The impact of alternative splicing in disease and the knowledge that these splicing events can be regulated by drug actions have opened up completely new horizons for target discovery and drug development. Altered splicing patterns can also serve as markers of the altered cellular state associated with disease and have the potential to provide diagnostic and prognostic information. Novel therapeutic strategies are now emerging as approaches, which include (i) over-expression of proteins that alter splicing of the affected exon,[62,63] (ii) use of antisense oligonucleotides,[64,65] (iii) SiRNA-based drugs to silence gene expression,[66] (iv) use of compounds that affect phosphorylation of splicing factors[67] or stabilize putative secondary structures,[68] (v) high-throughput screens to identify compounds that influence splicing efficiencies of target pre-mRNAs[69] and (vi) a trans-splicing approach to replace mutated exons with wild-type exons.[70] Alternative splicing has been considered for its therapeutic role in designing the isoform-specific monoclonal antibody as well.[71]

The principle of antisense technology is the sequence-specific binding of an antisense oligonucleotide to the target mRNA, resulting in a translational arrest. Antisense strategies that include small interference RNA (RNAi), ribozymes and antisense oligonuleotides for gene silencing have received increased attention in functional genomics. The specificity of hybridization makes antisense strategy attractive to selectively modulate the expression of genes involved in the pathogenesis of malignant disease. The antisense oligonucleotide appears to be the promising method for identifying optimal target sequences within the mRNA of interest that has been developed.[49]

Antisense oligonucleotides (AS-ONs) usually consist of 15-20 nucleotides which are complementary to the target mRNA. Antisense oligonucleotides combine many desired properties such as broader applicability, higher direct utilization of sequence information, more rapid development at low costs, higher probability of success and higher specificity compared to alternative technologies for gene function and target validation.

Two major mechanisms of antisense oligonucleotides contribute to their antisense activity. The first is that most antisense oligonucleotides are designed to activate RNase H, which cleaves the RNA moiety of a DNA-RNA heteroduplex and therefore leads to degradation of the target mRNA [Figure 3]. In addition, AS-ONs that do not induce RNase H cleavage can be used to inhibit translation by steric blockade of the ribosome. When the AS-ONs are targeted to the 5' carboxy-terminus, binding and assembly of the translation machinery can be prevented.[49]

In the application of antisense oligonucleotides to modify splicing, three oligonucleotide backbones have been used: 2'-O-methyl- and 2'-O-methoxyethyl-oligoribonucleoside-phosphorothioates and morpholino phosphorodiamidate oligomers.[73] The first antisense oligonucleotide chemistry used was phosphodiester, followed by the more stable phosphorothioate backbone modification.[74] This provided added stability at the expense of affinity for the target. The 'second generation' nucleotides with alkyl modifications at the 2' position of the ribose increases the affinity of phosphorothioate DNA oligonucleotides and lowers the toxic side effects. More recently, many synthetic oligonucleotides have been made that have both remarkable affinity and stability.[72]

A number of methods have been developed for *in vitro* and *in vivo* delivery of oligonucleotides.[75,76] Recently, macromolecular delivery systems have been developed to mediate highly efficient cellular uptake and protect the bound oligonucleotides from degradation in biological fluids.

The phosphorothioate oligodeoxynucleotides are well absorbed from parenteral sites and get distributed to organs and peripheral tissues through improved delivery systems.[77] The intensive pharmacokinetic studies of antisense oligonucleotides with second and third generation oligonucleotides had initiated the clinical trials in the early 1990s. The antisense drug first to date is Vitravene for CMV retinitis, approved by US FDA in the year 1998.[77] Many antisense oligonucleotides have

been investigated against a broad spectrum of diseases staging in different clinical trials.

Perspectives of Alternative Splicing

The impact of alternative splicing in disease and the knowledge that these events can be regulated by drug actions have opened up completely new vistas for target discovery and drug development. Some basic questions such as drug response; specificity and selectivity of drug response; diagnostics for monitoring the disease status, progression/relapse; etc., can all be answered through more interventions / awareness on alternative splicing.

Platform technologies such as monoclonal antibodies and antisense oligonucleotides have the potential of reducing costs for discovery of new drugs. We can look forward to intriguing biology and increasing utility as we come to understand the diverse mechanisms by which disrupted splicing and splicing regulation contribute to human disease welfare. For modification of splicing, the oligonucleotides can function in two ways, either via sequestering the target sequences such as splice sites in double-stranded structures or form duplexes with RNA that are recognized by RNase H to cleave the RNA strand of the duplex, leading to degradation of targeted RNA.

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