

Alternative Splicing in Prostate and Breast Cancer

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Abstract: Alternative splicing of mRNA precursors allows the synthesis of multiple mRNAs from a single primary transcript, thus contributing to proteomic diversity in higher eukaryotes. Multiple studies demonstrate that alternative splicing patterns are altered during cancer progression. Several different mechanisms can contribute to changes in the regulation of alternative splicing. This report will provide an overview of how splicing microarrays and large-scale sequencing are being used to identify splicing changes in breast and prostate cancer. Global analyses of splice variants have identified cancer-specific splice variant patterns that have potential use as biomarkers and potentially have prognostic value as well as may represent novel therapeutic targets. A description of specific splice variants with differential expression in these cancers and their possible functions will be presented.

Keywords: Spliceosome, exon arrays, RNASeq, androgen receptor, growth factor receptors, extracellular matrix, estrogen receptor, BRCA1.

INTRODUCTION

Alternative pre-mRNA splicing is a key molecular event that allows for substantial protein diversity [1-3]. Through this process, a single gene magnifies its coding capacity by expressing several related proteins with diverse and often antagonistic functions (Fig. 1).

Alternative splicing (AS) leads to cell or tissue specific changes in mRNA from a single gene through changes in splice site choice. As many as 80% of genes are alternatively spliced, resulting in tissue, developmental stage or disease specific mRNA expression [4]. There are many different forms of AS, ranging from inclusions or exclusions of single exons to genes like CD44 that has 20 exons and exists as multiple isoforms with alternative inclusion of 10 of the exons. Many disease states, including cancer, have increases in AS that contribute to their pathogenesis [2]. Several different mechanisms can contribute to changes in the regulation of AS. Mutations can occur in gene sequences that affect the binding of splicing factors or in splice enhancer or inhibitor sequences. Changes in expression or mutations in components of the splicing machinery also result in changes in the splicing pattern of many cancer-related genes [5]. Point mutations within splice acceptor sites or donor sites can also contribute to AS. It has been estimated that 15% of point mutations that cause human genetic disease affect splicing [4].

SPLICEOSOME

Pre-mRNA splicing is coordinated in part by the spliceosome, a macromolecular ribonucleoprotein complex that assembles on the pre-mRNA as it is transcribed (Fig. 2). The assembly of the spliceosome is a highly regulated process as

is the resulting splicing reaction. This involves coordination between binding initiators and repressors, which includes five small nuclear RNPs and over 100 proteins.

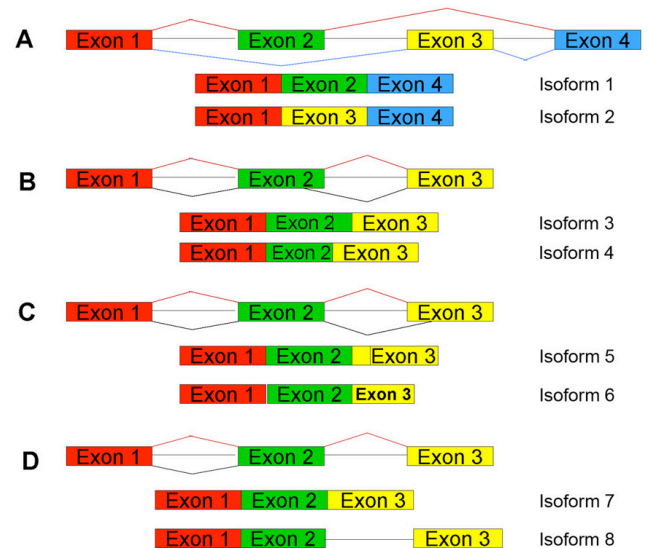


Fig. (1). Alternative splicing. Eukaryote primary transcripts (pre-mRNA) contain both intronic (thin line) and exonic (boxes) sequences. Intronic sequences are removed and alternative splicing of exons gives rise to a variety of mature mRNAs by inclusion of subsets of exons. Representative types of alternative splicing: **A.** Alternate exon usage and exon skipping; isoform 1 retains exon 2 and excludes exon 3; isoform 2 excludes exon 2 and retains exon 3; **B.** Alternate 5' splice donor; isoform 4 uses a 5' splice site within exon 2; **C.** Alternate 3' splice acceptor; isoform 6 utilizes a 3' splice site within exon 3; **D.** Intron retention; isoform 8 includes intron 2.

Intron boundaries are defined by a polypyrimidine tract at the 3' end, an AG at the 5' splice site and a branch point 5' to the polypyrimidine tract. Spliceosome components bind to these structures and direct the assembly of the other

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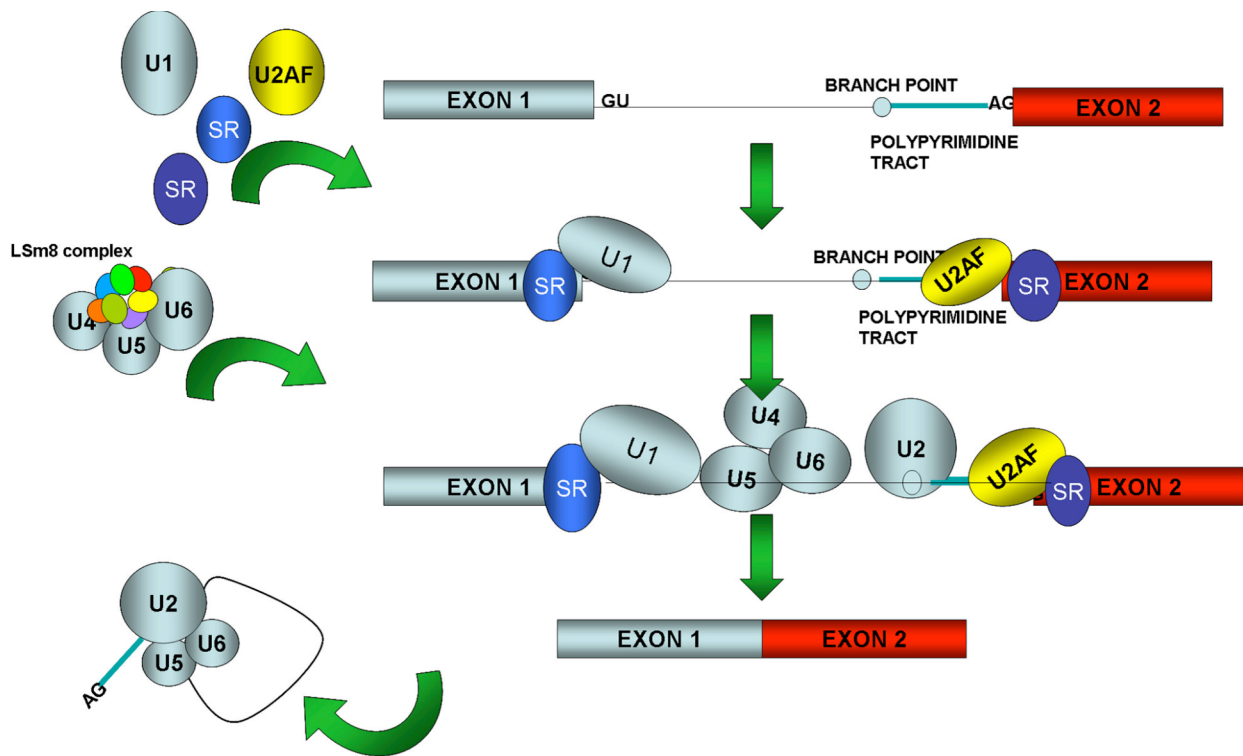


Fig. (2). The spliceosome. Schematic diagram of the steps of spliceosome assembly and intron removal. **Top:** Structures related to exon and intron definition, showing the AG sequence at the 3' end (splice acceptor), polypyrimidine tract and branch point at the 3' end of the intron and GU sequence at the 5' end (splice donor). **Middle:** The U1 snRNP binds to the 5' splice site; U2AF binds to the 3' site and the polypyrimidine tract; and the SR proteins bind the exon splice enhancer sequences. **Bottom:** U2 binds the 3' splice site. The U4/U6.U5 tri-snRNP is first bound to the LSm2-8 complex, which coordinates the assembly of the tri-snRNP which then joins the spliceosome complex. Following conformational changes, U1 and U4 dissociate from the complex and the intron is removed, resulting in the mature spliced mRNA.

components. Other sequences within the exon or intron called splicing enhancers (ESE and ISE) and silencers (ESS and ISS) also contribute to spliceosome assembly at the exon/intron boundaries. The spliceosome consists of five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6 and more than 100 other proteins that together catalyze the removal of introns [6]. The U1 snRNP recognizes the 5' splice site while U2 snRNP binds the 3' splice site. Splicing Factor 1 (SF1) binds the branch point, U2 auxiliary factor (U2AF) binds the polypyrimidine tract and the 3' splice site and SR (serine/arginine-rich) proteins bind to ESEs and contact U2AF, U1 snRNP and the branch point. The tri-snRNP, consisting of U4, U5 and U6 snRNPs joins the complex leading to conformational changes and the loss of U1 and U4 snRNPs [3]. Other proteins join the complex leading to an activated spliceosome, intron excision and ligation of the two exons. Regulation of AS is controlled by the proteins that make up the spliceosome. SR proteins bind to splicing enhancers and induce splicing, while heterogeneous nuclear RNPs (hnRNP) bind to splicing silencers and block spliceosome assembly. In addition, polypyrimidine tract binding protein (PTB) represses splicing [1]. The relative amounts of each of these proteins determine whether or not splicing will occur at the designated exon/intron junction. AS represents the suppression of constitutive splice sites and/or the use of suboptimal sites. Due to the number of

proteins involved in splicing, the diversity of pre-mRNA substrates and the multistep process of spliceosome assembly and catalysis, regulation of splicing can occur at any point in the pathway. Any mutation within the nucleotide sequence at the 5' or 3' splice site, the branch point or any enhancer or silencer can also lead to alternative splicing. Mutations at the intronic splice sites can lead to exon skipping and protein truncation. This type of mutation in a tumor suppressor gene, would result in an inactive protein [7]. Examples include a mutation in a possible intronic enhancer of *ATM* that results in a skipped exon and truncated protein in some of the transcripts and increases the risk of breast cancer in carriers. [8]. Also an AA to AG mutation in *BRCA1* creates a cryptic splice site that leads to a truncated protein in a breast cancer family [9]. AS may also regulate RNA stability through nonsense mediated decay (NMD). NMD is a pathway that targets mRNAs with premature stop codons for degradation. In mammals a stop codon more than 50 nucleotides upstream of an exon-exon junction is considered premature. A third of all alternative splicing events result in NMD [1].

TECHNIQUES TO PROFILE ALTERNATIVE SPLICING

Two types of commercial arrays are available to measure AS (Fig. 3). One platform (Affymetrix) measures the

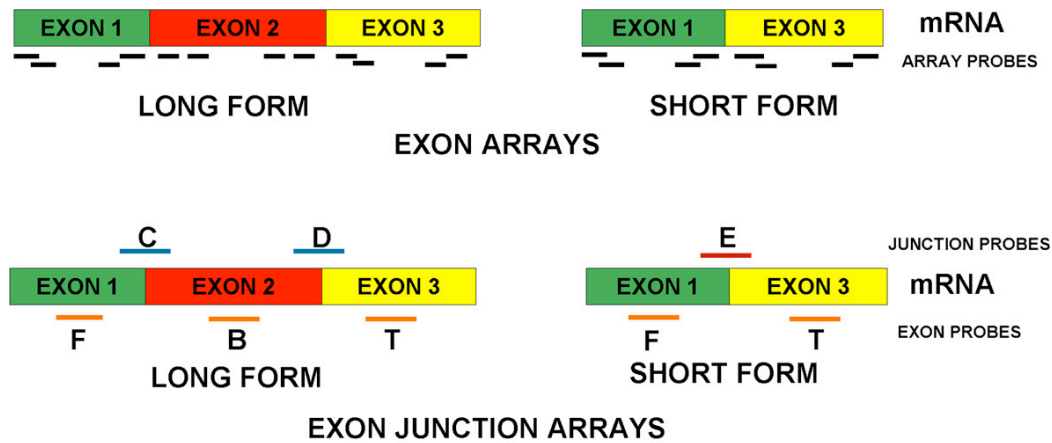


Fig. (3). Splicing array probe design. The two main splicing array platforms use different patterns of probes for detection of alternative splicing. **Top:** Affymetrix exon arrays: Probes located throughout each exon with approximately four probes per exon and 40 probes per gene, allowing unbiased measurement across the length of the gene. **Bottom:** ExonHit Probe design is based upon known splicing patterns. An exon skipping event is illustrated with a long form and short form. Two probes (T and F) are common to both isoforms. B, C and D probes are specific to the long form. Probe E is specific to the short form and overlaps the junction of exons 1 and 3.

expression of all individual exons as well as expression of the whole transcript. Exon-level analysis on a whole-genome scale detects specific AS events that contribute to disease mechanism and etiology. This is accomplished using multiple probes per exon that enable exon-level analysis, and allows one to distinguish between different isoforms of a gene [10]. This has the advantage of identifying previously undescribed splicing events; however subtle changes in exon usage may be missed when the wild-type isoform is co-expressed with the variant form.

Another commercial array (ExonHit) uses probes that are complementary to exons and to exon-exon junctions, allowing specific detection of exon skipping events [4, 11]. Since these probes are specific to known or evidenced splice events, previously undescribed exon skipping events and alternative splice site acceptors and donors will not be detected with this approach. This platform was used in a study that examined both gene expression and exon usage in benign and malignant breast tissue [12]. Comparing malignant to benign tissue defined 956 over-expressed exons in genes whose expression did not change. These over-expressed exons were in genes such as CSNK1D and VHL known to be involved in cancer biology (discussed further below).

Other published techniques to determine AS include high throughput RT-PCR [13-15] and deep sequencing or RNASeq (short-read high-throughput sequencing) [16-18]. Venables *et al.* described a RT-PCR based system that used two sets of primers specific to each identified exon-exon junction and AS events in 600 cancer associated genes [15]. This study described a total of 4,709 AS events, using an average of 30 PCR reactions per gene. The PCR products were analyzed by capillary electrophoresis and the percent splicing index, calculated as the concentration of the longer variant divided by the total of both the long and short variants, was determined and plotted as a heat map. This identified 41 splice events which showed significant differences between normal and malignant breast samples (see below). Another PCR based technique was used by Li, *et al.* [14] in the analysis of splicing patterns in prostate

cancer (PrC). Paired oligomers targeted to known splice sites are hybridized to biotinylated cDNA, affinity purified with streptavidin and amplified by PCR. These products are then hybridized to a universal bead array to quantify the expression of captured gene sequences. Deep-sequencing, uses poly-A mRNA which is reverse transcribed using random hexamers allowing the investigation of the entire transcriptome [17]. The resulting cDNA is sheared to produce short oligomers which are sequenced and mapped to the human genome. This technique using HEK293T and Ramos B cells identified over 4,000 previously unknown splice junctions in 3,000 genes that were unique to each cell type. Most splicing events were exon skipping and alternative 5' and 3' splice acceptors were also detected.

PROSTATE CANCER

Prostate cancer (PrC) is the most commonly diagnosed cancer in men, and the second leading cause of cancer death among men in the United States. The American Cancer Society estimates that 192,280 new PrC cases will be diagnosed in the US and 27,360 men will die from the disease in 2009 [19].

PrC development proceeds through defined states, including PIN (prostate intraepithelial neoplasia), clinically localized, androgen-dependent and androgen-independent metastatic cancer. The early stages of organ-confined PrC are often curable by surgery or radiation therapy, and improved early detection based to a large extent on PSA screening has led to the identification of thousands of men with localized disease. Although standard therapies initially cause regression of invasive tumor, patients eventually relapse and progress to hormone-refractory disease [20, 21].

The molecular mechanisms that drive PrC progression remain to be elucidated. Translational impact from increased understanding of the mechanisms that contribute to the development and progression of PrC includes: (1) improved and earlier detection; (2) better diagnosis and staging of disease; (3) prediction of response to specific therapeutic

approaches; (4) disease prevention and (5) novel treatment strategies.

Many factors contribute to the etiology of PrC, including age, race, dietary fat intake, androgens, environmental and genetic factors. Collectively, genetic and epigenetic alterations contribute to the multiple events that occur during the development of PrC [22, 23]. In addition to alterations that affect the function of single genes, mutations in some loci lead to alterations of multiple genes. While this concept has been best exemplified by defects in the mismatch repair genes that lead to increased genomic instability by accumulation of replicative errors in multiple genes [24, 25], it is likely that altered mRNA processing, stability and translation also impact the function of many genes.

GLOBAL ANALYSIS OF SPLICE VARIANTS IN PROSTATE CANCER

Several studies have characterized alternative splice variants in PrC compared to normal prostate (Table 1) [14, 26, 27]. In one study, splicing changes in PrC were identified using exon arrays and validated by RT-PCR. Specifically,

this group constructed a splicing array to examine approximately 1,500 mRNA isoforms from a panel of genes previously implicated in PrC [14]. PrC cell lines could be segregated from other tumor cell lines of different cellular origin using this approach. Using mRNA isolated from formalin-fixed, paraffin-embedded tissues with laser capture microdissection (LCM) of PrC and normal prostate, 49 mRNA isoforms (representing 34 genes) were found to be up-regulated and 55 mRNA isoforms (representing 22 genes) were found to be down-regulated in PrC. Three genes were validated to have significantly different expression levels between normal and PrC tissue. Microtubule-associated tau protein showed a switch to a shorter isoform in PrC and both calcium channel α -1-D and α -methylacyl-CoA-racemase showed increased expression of an alternatively spliced form in PrC [14]. To extend this study, the isoform data was used to perform average-linkage hierarchical clustering to determine whether a subset of isoforms could distinguish normal from malignant prostate tissue [27]. Using 128 signature isoforms as classifier correctly predicted 92% of the cancer cases, which was better than using mRNA expression to classify the cases.

Table 1. Gene Isoforms Disregulated in Prostate Cancer

Gene Name	Genebank Number	Exon/Introns Affected	Functional Change (if known)	References
TPM1 (tropomyosin 1)	NM_001018005.1	Alternate exon 6	Increase in metastatic PrC	[26]
ACTN1 (actinin α 1)	NM_001130004.1	alternative exon 19	Longer isoform higher in PrC	[26]
COL6A3 (collagen type VI, α 3)	NM_004369.3	exon 6 inclusion	Present in metastatic PrC	[26]
CALD1	NM_033138.3	Alternate splice donor exon 5 Exon 6 skip	Decreased in metastatic PrC Increased in localized PrC	[26]
LRRFIP2	NM_006309.2	Exon 5, 6 inclusion	Lost in metastatic PC	[26]
VCL (vinculin)	NM_014000.2	Exon 19 skip	Decreased in metastatic PrC	[26]
MAPT (Microtubule-associated tau protein)	NM_001123066.2	Exon 10 skip	Increased in PrC	[14]
CACNA1D (calcium channel α -1-D)	NM_000720.2	Alternate exon usage	Longer isoform increased in PrC	[14]
AMACR (α -methylacyl-CoA-racemase)	NM_014324.5	Alternate exon usage	Shorter isoform expressed in PrC	[14]
PMSA	NM_004476.1	Exon 1 alternate splice donor	Lack of transmembrane domain; cytoplasmic localization	[50]
		Exon 17 skip	Increased tumor invasion	[50]
		Exon 6 skip Exon 18 skip	Lack of NAALAD enzymatic activity	[106]
Survivin (BIRC5)	NM_001168.2	Exon 3 skip (Δ ex3)	Increased apoptosis	[62]
		Exon 2B inclusion	Decreased apoptosis	
TMPRSS/ERG	NM_001136154.1 (ERG)	Alternate exon fusion sites	Longer isoform increased in more aggressive PrC	[51]
BCL2L1 (BCL-X)	NM_138578.1	Alternate splice donor	Reduced apoptosis	[2, 63]
MCL-1	NM_021960	Exon 2 skip	Enhanced apoptosis	[2, 63]

(Table 1) Contd.....

Gene Name	Genebank Number	Exon/Introns Affected	Functional Change (if known)	References
AR (androgen receptor)	NM_000044.2	Duplicated exon 3 and Truncated protein	Androgen independent activation	[30]
		Alternate splice acceptor	Reduced nuclear translocation	[29]
		Intronic sequence inclusion	Loss of ligand binding domain and androgen independent gene expression	[31]
ErbB3	NM_001982.2	Alternate exon 3 with truncation	Found in PrC bone metastases	[67]
PRLZ (TPD52)	NM_001025252.1	Exon 4, 6, 7 skip	Altered protein:protein interactions	[56]
KLF6	NM_001300.5	Alternate splice site and exon usage	Tumor growth and metastasis	[70]
FAS	NM_000043.3	Exons 8, 9 skip	Inhibition of apoptosis	[65]
ITGB3	NM_000212.2	Truncated C-terminal exons	Secreted protein Inhibition of cell adhesion	[40]
BRCA1	NM_007294.3	Exon 11 partial skip	Inhibition of cell proliferation	[93]
IL-17RL	NM_153461.2	Exon 7, 18 skip Exon 7 skip	More aggressive tumor	[107]
NRG4	NM_138573.2	Exon 6 exclusion	Cytoplasmic localization and lack of ErbB4 phosphorylation	[54]
VEGF	NM_003376.4	Exon 6, 7 skip	Increased tumor angiogenesis	[71]
CYP24	NM_000782.4	Exon 10 skip	Altered vitamin D activation	[108]
NEK3	NM_002498.2	Exon 11 skip	Higher expression in PrC	[109]
ERBB4	NM_005235.2	Alternate exon 26 and exon 27 exclusion	Missing phosphorylated tyrosine and reduced colony formation	[110]
CD44	NM_000610.3	Exon 6-15 skip	Loss of hyaluronan binding; increase in fibronectin binding	[46]
TLE3	NM_005078.2	Novel exon inclusion	Negative regulation of co-repressor activity and enhanced AR function	[39]
p120 (BRD8)	NM_006696.3	Exon 2 alternative splice donor	Missing protein:protein interaction domain	[38]
FGFR2	NM_000141.4	Alternate exon usage	Altered ligand sensitivity	[53]
HYAL1 HYAL3	NM_007312.3 NM_003549.2	Exon 1 alternate splice donor and acceptor Exon 3 skip Exon 2 alternate splice acceptor	Loss of enzyme activity; increased expression in benign prostate	[57, 58]
PSA (KLK3)	NM_001648.2	Intron retention Alternate 3' acceptor Alternate 5' donor	Loss of enzyme activity in some isoforms Possible diagnostic utility over PSA alone	[69]

Affymetrix GeneChip Human Exon 1.0 ST Arrays were used to measure whole-genome exon expression in 102 normal and cancer tissue samples of different stages from colon, urinary bladder, and prostate to identify tissue and tumor specific alternative splicing. In normal tissue samples, 2069 candidate AS events were identified between the different tissues and 15 splicing events for RT-PCR validation were selected, 10 of which were successfully validated by RT-PCR and sequencing. Additionally 23, 19, and 18 tumor-specific splicing alterations in colon, bladder, and prostate, respectively, were selected for RT-PCR validation on an

independent set of 81 normal and tumor tissue samples. Seven genes with tumor-specific splice variants were identified. Many of these variants are cytoskeletal proteins, including tropomyosin 1 (TPM1), actinin $\alpha 1$ (ACTN1), collagen type VI $\alpha 3$ (COL6A3) and vinculin (VCL), that are associated with cell motility and interactions between cells and the extracellular matrix (ECM) [26]. The other three genes are caldesmon 1, an actin and calmodulin binding protein (CALD1), leucine-rich repeat (in FLI1)-interacting protein (LRRFIP2), and phosphoinositol 4-kinase, catalytic, β polypeptide (PIK4CB). Differences in expression between

spliced isoforms were observed between both normal tissue and localized tumors and between localized tumors and metastatic tumors. Metastatic PrC showed an increase in a COL6A3 isoform containing an extra exon (exon 6) with a decrease in the shorter isoform (without exon 6) compared to both normal tissue and localized PrC. Increased expression of ACTN1 containing an alternative exon 19 (19a) was observed in localized and metastatic PrC compared to normal prostate [26].

These results indicate that AS and the subsequent increase in cancer associated isoforms contributes to the malignant phenotype in PrC and understanding the pattern of these changes and the mechanism for AS are important for both the determination of cancer progression, diagnosis and possibly therapy.

ANDROGEN RECEPTOR, CO-ACTIVATORS AND CO-REPRESSORS

Androgens regulate diverse biological processes, including reproductive and non-reproductive functions. Most of the signaling effects of androgens are mediated through the androgen receptor (AR), a member of the nuclear receptor super family of transcription factors. Upon ligand binding, AR translocates to the nucleus, where it binds to androgen response elements (ARE) of androgen-dependent target genes. The majority of PrC depend upon activation of the AR by circulating androgens for growth and survival, providing the rationale for androgen deprivation therapy (ADT). Unfortunately, a variety of mechanisms are used by PrC to bypass androgen-deprived conditions. Specific molecular and cellular changes occur during the transition of hormone-naïve to castration-resistant PrC (CRPC). Significantly, CRPC remains dependent on a functional AR, AR-mediated processes, and on the availability of androgens. CRPCs acquire different molecular mechanisms that enable them to use intracellular androgens more efficiently (via AR amplification, AR protein over-expression, AR hypersensitivity), have altered co-activator and co-repressor gene and protein expression, and use alternative splice variants of the AR protein to mediate androgen-independent AR functioning. Furthermore, CRPCs can gain the ability to synthesize androgens *de novo* from available precursors through a renewed and up-regulated synthesis of steroid-hormone converting enzymes [28].

There are many mechanisms by which AS can result in production of a constitutively active AR and thus circumvent androgen ablation. One AR variant, AR23, results from AS of intron 2, in which the last 69 nucleotides of the intronic sequence are retained, leading to the insertion of 23 amino acids between the two zinc fingers in the DNA-binding domain [29]. Upon ligand binding (dihydrotestosterone [DHT]), nuclear entry of AR23 is impaired. DHT-activated AR23 forms cytoplasmic and perinuclear aggregates that partially colocalize with the endoplasmic reticulum and are devoid of genomic actions. Importantly, cytoplasmic DHT-activated AR23 remains partially active, with the ability to activate transcription from androgen-responsive promoters. Such novel cytoplasmic actions for this splicing AR variant suggest possible contribution in PrC progression [29].

Other AR isoforms also provide mechanisms for AR mediated processes to become independent from available androgens. The 22Rv1 PrC cell line was derived from a CWR22 xenograft that relapsed during androgen ablation [30]. Four AR isoforms are expressed in 22Rv1 cells: full length AR, full-length AR with duplicated exon 3 and two truncated versions lacking the COOH terminal domain (CTD). The CTD-truncated AR isoforms are encoded by mRNAs that have a novel exon 2b at their 3' end. These AR isoforms are constitutively active and promote the expression of endogenous AR-dependent genes, as well as the proliferation of 22Rv1 cells, in a ligand-independent manner. Thus, inclusion of a unique exon in the AR results in a truncated protein which is androgen independent. Significantly, this variant has been found in many PrC cell lines [30].

Collective studies demonstrate that splicing of intronic cryptic exons to the upstream exons encoding the AR DNA-binding domain gives rise to seven AR variant transcripts (AR-V1 to AR-V7) in human PrC [31]. These variants lack the reading frame encoding the ligand-binding domain. The two most abundantly expressed variants, AR-V1 and AR-V7, have 20-fold higher expression in hormone refractory prostate cancer (HRPC) (n=25) when compared with hormone-naïve PrC. Furthermore, among the hormone-naïve PrC, higher expression of AR-V7 predicted biochemical recurrence following surgical treatment. AR-V7 has been shown to be localized in the nuclei of cultured PrC cells under androgen-depleted conditions. AR-V7 is constitutively active, driving the expression of androgen-responsive genes, as revealed by both AR reporter assays and expression microarray analysis. These results suggest a novel mechanism for the development of HRPC. In addition, novel AR variants are potential biomarkers and therapeutic targets for advanced PrC.

AR function is dependent on co-expression of nuclear co-activators, including SRC-1, ARA54, ARA55, ARA70, ARA160, TRAP220, p120 and members of the TLE (Groucho/Transducin-like enhancer of split) family. In addition to differential expression [32-37], AS of co-activators has been observed in PrC. For example, a splicing variant of nuclear co-activator p120 α , designated as p120 β lacks exon 7, a region that encodes 77 amino acids containing a domain required for interaction with other nuclear receptors [38]. Transfection assays demonstrated that p120 β functions as a strong co-activator for AR, but weakly for other nuclear receptors. Thus, splicing converts a general co-activator to one that is more specific for AR. Interestingly, relative to p120 α , elevated p120 β mRNA was expressed in normal prostate, benign prostatic hyperplasia (BPH), androgen-responsive PrC and an androgen-sensitive PrC cell line, LNCaP. In contrast, p120 β expression was found to be lower than p120 α in recurrent cancers and the androgen-insensitive PrC cell lines PC3 and DU145. Thus, the ratio of 120 β /120 α may correlate with androgen sensitivity [38].

The TLE genes constitute a family of important transcriptional co-repressors involved in many cellular processes. Microarray experiments and real-time RT-PCR assays showed that alternatively spliced isoforms of TLE1, TLE2 and TLE3 were preferentially expressed in prostate in comparison to liver and kidney tissues [39]. AS of TLE3 mRNA results in a premature stop codon and would encode

for a putative shortened protein. This isoform of TLE3 was up-regulated (6- to 17-fold) in prostate tumors in comparison to matched non-tumor adjacent tissue from 7 out of 11 (64%) patients and in four prostate tumor cell lines in comparison to a normal prostate cell line. TLE splice variants may function as negative regulators of normal TLE function. Thus, over-expression of the TLE3 variant may enhance AR function in PrC [39].

EXTRACELLULAR MATRIX (ECM)

AS of $\beta 3$ integrin in human PrC gives rise to a truncated (tr $\beta 3$, truncated (tr) $\beta 3$) mRNA and protein lacking the cytoplasmic and transmembrane domains [40]. The tr $\beta 3$ is secreted by PrC cells and inhibits adhesion of PrC cells to fibronectin and vitronectin. Using confocal microscopy and time lapse live cell microscopy, tr $\beta 3$ was localized to the trailing edge of migrating cells. It has been suggested that this may represent an alternative cell detachment mechanism in these cells, supporting the notion that tr $\beta 3$ may act as an anti-integrin that plays a crucial role in cell migration, an important process in tumor invasion and metastasis [40].

CD44 is a cell surface glycoprotein that interacts with the ECM by binding to hyaluronan [41]. During cancer progression, CD44 contributes to adhesion, migration and invasion, properties that result in metastases [42]. The CD44 gene consists of 20 exons. The standard or short form of the gene, CD44s, expresses 10 of these exons, 1-5 and 16-20. Through alternative splicing, these middle 10 exons, numbered v1-v10, are variably expressed in specific tissues and developmental stages. Inclusion of the variant exons results in reduced affinity for hyaluronan [43]. In addition, the variant exons v8-v10 contains glycosylation sites which further reduces the affinity of CD44 to hyaluronan. Chondroitin sulfate and heparan sulfate sites on exons v3 and v10, respectively, also reduce binding to hyaluronan and other ECM proteins, such as type 1 collagen, laminin and fibronectin [44]. The presence of v7-v10 induces shedding of the receptor into the extracellular space, which blocks CD44 dependent tumor growth [45]. The CD44s molecule is resistant to proteolysis and subsequent shedding. PrC over-expresses a predominant variant (CD44v7-10, CD44v) isoform of CD44, and loses expression of the standard (CD44s) isoform [46, 47]. Re-expression of full-length CD44 or CD44s increased the total CD44 mRNA and CD44s protein while suppressing CD44v7-10. In addition, RNAi to CD44v decreased invasion. Thus, the over-expression of CD44v observed in PrC may contribute to cellular invasion.

SPLICING AND PROSTATE CANCER PROGRESSION

Several additional PrC-specific splicing patterns have been described that correlate with PrC progression (Table 1). Prostate-specific membrane antigen (PMSA) is an integral transmembrane glycoprotein that is over-expressed in PrC compared with benign glands. Among PrC cases, high PSMA expression correlated with tumor grade, pathological stage, aneuploidy, and biochemical recurrence [48]. Four splice variants of PMSA (PSM', PSM-C, PSM-D and PSM-E) have been described in the literature [49]. PSM' cDNA is identical to PSMA except for a 266-nucleotide region of

PSMA cDNA (nucleotides 114–380), which codes for the transmembrane region of PSMA. Compared to PMSA, PSM' is expressed higher in normal prostate and increased expression of PSMA over PSM' is found during progression from normal to tumor. PSM-C is predicted to make the same protein product as PSM'. PSM-E cDNA is identical to PSMA except for a new exon, a 97-nucleotide region, and loss of a 93-nucleotide region. Expression of PSM-E is increased from normal to BPH to PrC. This study also found that PSM-E expression correlated with increasing Gleason score; in contrast, no significant correlation was observed between total PSMA levels and Gleason score [50]. These results suggest that splice variants including PSM-E may be more useful prognostic indicators for PrC progression.

The expression of the ETS-related gene (ERG) is low or undetectable in benign prostate epithelial cells. High prevalence of ERG over-expression in PrC cells is often due to TMPRSS2-ERG fusions and supports a causal role of ERG protein in the neoplastic process. TMPRSS2-ERG fusion junctions have been extensively studied in PrC. At least 14 different splice variants for TMPRSS2-ERG have been identified. Significantly, relative TMPRSS2/ERG variant abundance is associated with pathologic features, Gleason score, and patient outcome [51].

Fibroblast growth factors (FGF1–FGF23) control embryonic development and adult tissue homeostasis by binding and activating members of the FGF receptor family (FGFR1–FGFR4). The ligand-binding specificity of FGFRs is regulated primarily through AS [52]. Most FGFs activate more than one FGFR. The FGF7 subfamily is unique among FGFs because its members (FGF7, FGF10, and FGF22) are expressed exclusively by mesenchyme and interact specifically with the b splice variant of FGFR2 (FGFR2b) present in the overlying epithelial cells. FGFR2 has several splice variants. The best characterized arise from a mutually exclusive splicing event, in which the second half of the third Ig domain is comprised of either the 148 nucleotide IIIb exon or the 145 nucleotide IIIc exon. The IIIb containing form is responsive to FGF7, whereas FGFR2 (IIIc) is not. Progression of PrC from an androgen sensitive to androgen insensitive tumor is accompanied by loss of the FGFR2b (IIIb) isoform and predominant expression of the FGFR2 (IIIc) isoform [53]. This same pattern is seen in human PrC cell lines, where loss of the FGF7 responsive FGFR2b (IIIb) isoform correlates with androgen independence in DU145, LNCaP and PC3.

The neuregulins (NRG) are the largest subclass of the epidermal growth factor (EGF) family of ligands. These molecules are synthesized as membrane-bound, biologically active growth factors that act by binding to the ErbB receptor tyrosine kinases. NRG 1, 2, and 3 genes undergo extensive alternative mRNA splicing. NRG4 is expressed in PrC and over-expression is associated with advanced-stage PrC. AS of NRG4 results in several variants with different roles in cell signaling [54]. NRG4-A1 and NRG4-A2 retain the transmembrane and EGF-like domains, are capable of activating ErbB4, and are expressed on the membrane. The NRG4-B1-3 isoforms are cytoplasmic and cannot activate ErbB4. The NRG4-A1 and NRG-B1-3 isoforms are expressed in PrC tissue and PrC cell lines; however, NRG4-A2 is expressed only in metastatic PrC cell lines [54].

PrLZ (prostate leucine zipper), a member of the Tumor Protein D52 (TPD52) family, is prostate-specific, androgen-regulated and localized at chromosome 8q21.1, a locus frequently amplified in human PrC. In a study of 100 PrC, PrLZ expression is low in normal glands and BPH, but over-expressed in 85% PIN and 75% PrC [55]. AS may contribute to higher PrLZ levels in PrC, and interaction with 14-3-3 proteins may be a mechanism by which PrLZ promotes cell proliferation and survival during PrC development and progression [56].

Hyaluronidases (HAases) are enzymes related to cancer progression. Isoforms of HAases have been described as products of AS responsible for differences in enzyme activity. The heterogeneity of HAase expression has been identified in tumors and has been related to differences in their biological behavior. Expression of the isoforms HYAL3-v1, HYAL1-v3, and HYAL3-v2 were associated with low Gleason score and non-tumor recurrence in PrC. [57, 58].

ALTERNATIVE SPLICING AND APOPTOSIS

Survivin, a member of the inhibitor of apoptosis gene family, is expressed in most common cancers. Survivin over-expression inhibits extrinsic and intrinsic pathways of apoptosis. In addition to its role in apoptosis regulation, survivin functions in mitotic progression and the spindle checkpoint [59]. Three novel alternative splice variants of survivin that differ in their anti-apoptotic properties have been identified. One of them lacks 118 bps of exon 3 (survivin- Δ Ex3), the second retains part of intron 2 as a cryptic exon of 69 bps (survivin-2B) and the third contains an additional 165 bp exon (survivin-3B) [60]. A fourth isoform, survivin 2 α has been described that consists of exons 1 and 2 and 197 bps of intron 3 [61]. Whereas the anti-apoptotic potential of survivin- Δ Ex3 is preserved, survivin-2B has decreased anti-apoptotic potential and it has been suggested to act as a naturally occurring antagonist of survivin [60]. Survivin expression in PrC was significantly greater than that in BPH or PrC after ADT. In the PrC samples, the survivin expression level was associated significantly with high-grade cancer. Furthermore, the ratio of survivin splice variant expression is also correlated with Gleason score [62]. In PrC cells, survivin and survivin-2 α and survivin-2B were expressed more than the survivin- Δ Ex3 variant. The survivin-2B/survivin ratio in high-grade cancer was lower than that in low-grade cancer. Thus, high grade tumors express less survivin-2B, the isoform with reduced anti-apoptotic potential, relative to survivin, than low grade tumors [62].

Two splice variants of the apoptosis regulator Bcl-X (B-cell lymphoma-extra) control cellular apoptotic response. While Bcl-X_L (B-cell lymphoma-extra long) has anti-apoptotic function, Bcl-X_S (B-cell lymphoma-extra short) is pro-apoptotic. Epigallocatechin-3-gallate (EGCG) and ibuprofen synergistically act to suppress proliferation and enhance apoptosis of PrC cell lines, PC3 and LNCaP. It has been shown that Protein Phosphatase 1 activity increases following EGCG plus ibuprofen combination treatment and contributes to induced AS of Bcl-x and Mcl-1 (myeloid leukemia cell differentiation protein), resulting in down-

regulation of the anti-apoptotic Bcl-2 family members, Bcl-X_L and Mcl-1, and up-regulation of pro-apoptotic variants is associated with enhanced apoptosis following treatment [63]. Anti-apoptotic members of the Bcl-2 family of proteins are over-expressed in PrC and are promising molecular targets for modulating chemoresistance of PrC. Gossypol, a natural BH3 mimetic, is a small-molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1 currently in phase II clinical trials as an adjuvant therapy for human PrC [64].

FAS, a member of the TNF receptor super family, has many differentially spliced isoforms. Some of these isoforms lack a transmembrane domain and may be soluble and act as dominant negative inhibitors of Fas-induced apoptosis. Inherited polymorphisms in the Fas gene inhibit formation of the soluble isoforms and result in lower risk of invasive PrC [65].

ALTERNATIVE SPLICING AND PROSTATE CANCER METASTASIS

ErbB3 is a member of the epidermal growth factor receptor (EGFR) family. The tyrosine kinase activity of ErbB3 is deficient, making ErbB3 incapable of forming active signaling homodimers. Phosphorylation of ErbB3 depends on dimerization with other ErbB proteins, and the ErbB2-ErbB3 complex is reportedly the most potent in oncogenic signaling. PrC cells produce an alternatively spliced transcript of ErbB3 (p45-sErbB3) that retains intron 8 sequence. Using immunohistochemical analysis, it was reported that 32% of specimens from men with PrC with lymph node metastases, and 42% of specimens from men with PrC with bone metastases expressed the truncated p45-sErbB3 [66]. Subsequent studies have shown that p45-sErbB3 produces a secreted glycoprotein that stimulates osteoblast activities in vitro, promotes bone formation in vivo, and is detected in plasma samples from patients with CRPC with bone metastasis [67].

PSA (kallikrein 3, KLK3), a highly studied PrC serum biomarker, has many splice variants, like other members of the KLK gene family. Other members of the KLK family are up-regulated in PrC and splice variant specific RT-PCR assays for KLK2 have shown some prognostic utility in lymph node biopsies [68]. A total of 12 KLK3 transcripts have been cloned. They result from AS and/or alternative polyadenylation sites and encode for eight proteins: PSA, a truncated form of PSA (PSA-Tr), five PSA variants (PSA-RPs) and one protein (PSA-LM) unrelated to PSA. All the variants share the same signal peptide and could contribute to the diversity of KLK3 proteins in prostate fluid and blood [69]. Further analysis of the different isoforms is needed to determine whether any could serve as better diagnostic markers than PSA alone.

Many other alternatively spliced genes have been shown to be associated with increased PrC risk. The Kruppel-like zinc finger transcription factor (KLF6) gene encodes a family of proteins generated through AS involved in the regulation of cancer development and progression. The full length form of the KLF6 gene acts as a tumor suppressor gene. In contrast, KLF6 splice variant 1 (KLF6-SV1) is oncogenic and antagonizes the tumor suppressor function of KLF6, promoting tumor growth and invasion in cancer

models. In addition, a common germ line polymorphism in the KLF6 gene results in increased expression of KLF6-SV1 and has been associated with increased PrC risk. Targeted reduction of KLF6-SV1 induces apoptosis in cell culture and results in significant tumor regression *in vivo*. These data make the KLF6 gene family a target for the treatment of localized and metastatic cancer [70].

Vascular endothelial growth factor (VEGF) is a critical regulator of vasculogenesis and angiogenesis. There are 3 major isoforms of VEGF of 121, 165 and 189 amino-acids in humans formed by AS of exons 6 (165) or 6 and 7 (121). Increased expression of VEGF121, relative to the combined VEGF165 and 189 expression, results in a significant enhancement of human PrC angiogenesis [71].

ALTERED EXPRESSION OF SPLICING-REGULATORY RNA BINDING PROTEINS

The RNA-binding protein Sam68 (KHDRBS1, KH domain containing RNA binding signal transduction associated 1) affects both AR-regulated transcriptional activity and AS, which may affect PrC phenotypes. Sam68 has both an RNA binding (KH (hnRNP K Homology), domain as well as a Src homology domain for protein: protein interactions. Sam 68 has been reported to be up-regulated in clinical cases of PrC, contributing to cell proliferation and survival. It has been shown to induce inclusion of the CD44 variable exon v5 in response to Ras signaling. Since CD44 is an AR-target gene and its splice variants contribute to PrC, there is the possibility that AR and Sam68 act synergistically on CD44 splicing and affect PrC development [72].

KHDRBS3 is another RNA-binding protein that participates in the regulation of AS. KHDRBS3 is located on chromosome 8q, whose amplification is associated with poor survival in PrC [73, 74].

BREAST CANCER

Breast cancer (BrC) is the most frequently diagnosed malignancy and the second leading cause of cancer deaths in American women, second only to lung cancer. This year, an estimated 192,370 new cases of invasive BrC will be diagnosed among women, as well as an estimated 62,280 additional cases of *in situ* BrC in the US, resulting in approximately 40,170 deaths [19]. In fact, every 68 seconds a woman dies of BrC worldwide. BrC mortality is almost invariably attributable to metastasis that is clinically untreatable despite aggressive chemical and radiation therapies [75]. Additional studies directed towards elucidation of the factors involved in progression should facilitate the design of molecularly based diagnostic and therapeutic approaches. The proposed molecular mechanisms underlying BrC progression include over-expression of oncogenes, such as Her2/neu or myc [76, 77] or loss of tumor suppressor genes, such as p53 [78]. The 5 year survival rate of localized disease is 98%; however, survival drops dramatically to 26% in patients with metastatic disease. Therefore, understanding the pathways and processes that are involved in metastatic progression is of the utmost importance to prolong survival in breast cancer patients.

GLOBAL ANALYSIS OF SPLICE VARIANTS IN BREAST CANCER

BrC cell lines and tissues have expression of specific splice variants that correlate with disease progression (Table 2). Profiling of 120 BrCs and 45 benign lesions on a genome-wide splice array (Splice Array, ExonHit) was used to define a molecular classifier for BrC diagnosis and to identify which exons are differentially expressed in breast cancer compared to benign lesions [12]. This study identified 37,858 exon probe sets, 18,794 junction probe sets, and 3,733 genes that were differentially expressed in malignant and benign lesions. From this data, a 1228-probe-set was defined that served as a molecular classifier for BrC. Pathway analysis of genes over-expressed in BrC samples compared to benign breast lesions identified 11 of 15 genes involved in spliceosome assembly [12]. Triple-negative (ER, PR and Her2/neu negative) or Her2-overexpressing BrCs were more likely to over-express spliceosome components. Among those associated with malignant disease, 956 exons with higher intensity and splice index values were located in genes that were not differentially expressed. These over-expressed exons were located in genes important in cancer biology, such as CSNK1D (Casein kinase 1, delta) exon 9 and VHL exon 3. Thus, these findings demonstrated that alternative transcripts (AS or short transcripts) contribute to the genomic characteristics of BrC. Furthermore, this study illustrates that analysis at the exonic level reveals biological information that would be missed by analysis of gene expression alone.

Using a PCR based array format, Venables, *et al.* [15] identified several splice variants differentially expressed between normal and breast tumors that can be used to classify tumors. Forty-one AS events out of 600 genes were validated and a subset of 12 were able to properly identify tumors [15]. The splicing changes were found in genes that contribute to an increase in cell proliferation or survival, consistent with a functional role for AS in cancer. Interestingly, a subsequent study by the same investigators demonstrated that the RNA binding protein FOX2 was an important regulator of splicing in BrC. FOX2 binding sites were found downstream of one third of the exons skipped in BrC. In addition, FOX2 is itself alternatively spliced in BrC, giving rise to an inactive isoform lacking the C-terminal region [79]. Thus, those exons upstream to the FOX2 binding sites would be excised in cells lacking FOX2.

RECEPTOR ALTERNATIVE SPLICING

Estrogen Receptor

Two estrogen receptor (ER) types, named ER α and ER β , are major mediators of a variety of biological functions of estrogens [80]. Upon binding estrogen, ER translocates to the nucleus where it binds to DNA sequences called estrogen response elements (EREs) and interacts with various co-activators and co-repressors. Both ER α or ER β homodimers and ER α / β heterodimers regulate unique sets of E2-responsive genes [81].

The ESR α gene encodes for multiple mRNAs that are translated into full-length 66kDa ESR α (ESR α -66), or alternatively spliced to produce truncated forms of the

Table 2. Gene Isoforms Disregulated in Breast Cancer

Gene Name	Genebank Number	Exon /Intron	Function (if known)	References
MCL1	NM_021960	Exon 2 skip	Enhanced apoptosis	[11]
PRKDC	NM_006904	Exon 80 skip		[11]
FKBP1A	NM_00801	Intron 4 retention		[11]
GSTZ1	NM_001513	Exon 5 skip		[11]
CSNK1D	NM_139062.1	Exon 9 retention		[12]
VHL	NM_000551.2	Exon 3 retention		[12]
PRG	NM_000926	Intron T retention Exon 1,2,3 skip Intron S retention Intron ab retention	Truncated protein	[86]
		Exon 5,6 skip Exon 6 skip	Dominant negative inhibitor	
TNC	NM_002160.2	Exon 16 inclusion Exons 15, 16 inclusion	Increased tumor invasiveness	[95]
CD44	NM_000610.3	v6 (Exon 11) inclusion	Increased lymph node metastasis; cell motility	[41, 42]
		v7-10 (exons 12-15) inclusion	Release of CD44 from cell membrane	[44, 111]
		v6 inclusion		
BRCA1	NM_007294.3	Exon 11 partial skip (Δ 11q) Exons 9, 10, 11q skip (Δ 9,10,11q) Exons 9, 10 skip (Δ 9,10)	Altered protein/protein interactions	[90, 93, 94]
HMGA1	NM_145899.2	Unique exon	Altered DNA binding domain	[15]
DNMT3B	NM_006892.3	Exons 20, 21 inclusion	Altered methylase domain	[15]
FGFR2	NM_000141.4	Exon inclusion	Increased proliferation Increased transformation potential	[15, 112]
SHC1	NM_001130040.1	Exon inclusion	Altered signal transduction	[15]
ErbB4	NM_005235.2	Alternate exon	decreased proliferation and increased differentiation	[113]
SRA1 (SRAP)	NM_001035235.2	Exon 1 skip	Increased expression of steroid responsive genes	[114]
ETS1	NM_005238.3	Exons 3-6 skip	Dominant negative DNA binding	[115]
BIRC5 (Survivin)	NM_001012271.1	Exon 3 skip	Increased sensitivity to chemotherapy	[100]
CYR61	NM_001554.4	Intron 3 retention in normal cells	Lack of angiogenesis due to hypoxia	[116]
CyclinD1	NM_053056.2	Alternate splice donor (intron 4) truncation and Exon 5 skip	Reduced sensitivity to estrogen anti-tumor therapy	[117]
ERBB2 (HER2)	NM_004448.2	Exon 1-4 skip; exon 8 retention	Reduced ERBB2 signalling	[89]
OPN (SPP1)	NM_001040058.1	Exon 4 skip	Increased invasiveness	[118, 119]
BCL2L1 (BCL-X)	NM_138578.1	Alternate splice donor	Reduced apoptosis	[2]
GHRHR	NM_000823.2	Exons 11, 12 skip	Increased cell proliferation and transformation	[120]
ESR2 (ER β)	NM_001437.2	Exons 1,2,5,6 skip	Increased sensitivity to tamoxifen Lowered expression of ER responsive genes	[85]
CASP3	NM_004346.3	Exon 6 skip	Increased apoptosis	[121]
ESR1 (ER α)	NM_000125.3	Exon 1 skip	Dominant negative regulation of ER responsive genes	[82]
CYP27B1	NM_000785.3	Exon 3-5 skip	Inactive protein and inhibition of vitamin D metabolism	[122]
ATM	NM_000051.3	Exon 11 skip – protein truncation	Increased risk of BrC	[8]

protein. One such isoform skips exon 1 and starts translation from an alternative initiation codon in exon 2, resulting in a truncated protein missing the N-terminal ligand-dependent activation domain. Thus, this ESR α -46 does not activate gene expression and can function in a dominant-negative manner by binding to ESR α -66 and blocking its ligand dependent activation function [82].

Loss of ER β is thought to enhance ER α -mediated proliferation of hormone-dependent cancer cells [83]. ER β splice variants are characterized by alternative 3' exons (ER β 2, ER β 3, ER β 4, ER β 5) or by deletion of single or multiple exons (e.g. ER β Δ 2, ER β Δ 5, ER β Δ 5/6, ER β Δ 1256). Studies on the function of an ER β isoform lacking exon 5 (ER β Δ 5) suggested that this isoform might act as a dominant negative receptor on ER α and ER β pathways [84]. The predicted proteins coded by ER β Δ 125 and ER β Δ 1256 variants do not contain the activation function 1 (AF-1) domain mediating the ligand-independent transcriptional activity of ER β and are predicted to have deletions in the ligand-binding domain (LBD). Functional evaluation of these two variants demonstrates that, like ER β , ER β Δ 125 and ER β Δ 1256 expression results in reduced cell proliferation and increased apoptosis. Cells with ER β Δ 125 and ER β Δ 1256 expression also had reduced migration ability. Differential transcriptional responses were observed as well – the ER β Δ 1256 isoform was able to strongly reduce expression of estrogen-responsive genes [85].

Progesterone Receptor

The progesterone receptor (PR), a member of the steroid/thyroid hormone-retinoid receptor super family of ligand activated nuclear transcription factors, is expressed in breast tissue and may be lost in BrC. After ligand-dependent translocation to the nucleus, PR regulates expression of target genes containing progesterone response elements (PREs). PR status is a marker for hormone responsiveness and disease prognosis in BrC as PR positive patients are more likely to respond to hormonal therapies and survive disease. PR negative tumors have a poorer prognosis than PR positive tumors [86].

PR mRNA variants are characterized by deletions of various whole, multiple or partial exons that encode differing protein functional domains. Among these variant transcripts are those that are predicted to encode proteins that lack part of the zinc finger DNA binding domain, ligand binding domain, or hinge region; thus, several of the variant transcripts could interfere with normal PR functions. Many of these variants could alter the progestin responsiveness of a tissue and contribute to the abnormal growth associated with BrC. Some of these variant proteins act as dominant repressors of wild-type PR function or compete with wild-type receptors for co-factor binding (“squelching”), which interfere with normal progestin responsiveness. Several specific mRNAs have been detected at higher frequencies in tumor compared to normal breast tissue, suggesting that the protein is functional in cancer and may be a result of a cancer associated breakdown in splicing control. Further investigation into the expression patterns and activities of PR spliced variants is required [86].

HER2/ErbB2/Neu

HER2/neu over-expression is found in approximately 30% of BrCs and has been associated with poor disease prognosis [87]. At least two HER2 alternative spliced variants have been identified, p68-HER2 and p100-HER2. P68-HER2, initially called herstatin (HST), is encoded from an alternatively spliced HER2 transcript retaining intron 8. The truncated HST protein has a unique carboxy-tail sequence. HST prevents HER2/HER3 and HER2/EGFR dimer phosphorylation and inhibits heregulin dependent growth of BrC cells [88]. HST transcripts and protein are expressed in the non cancerous breast. Although HST transcripts were found in most tumor tissue samples, HST protein could be detected in the neoplastic cells of only 46 (24.6%) of 187 tumors and was correlated with adverse prognostic parameters [89].

BRCA1

The human BRCA1 gene encodes a nuclear phosphoprotein and is a component of several different multi-protein complexes that participate in a variety of molecular functions, including transcriptional regulation, protein ubiquitination, homologous recombination and DNA repair [90]. The BRCA1 gene is comprised of 22 coding exons and encodes for a protein with a molecular mass of approximately 220 kilo Daltons (kDa). Germline mutations of the BRCA1 gene predispose individuals mainly to the development of breast and/or ovarian cancer and they also increase the risk of Fallopian tube cancer and, to a lesser extent, of pancreatic and PrC.

The BRCA1 gene transcripts include over 20 splice variants in different tissues, showing remarkably different expression patterns [90]. There are two possible exon 1 sequences, 1a and 1b, each which have unique promoters. Neither of these affect the protein sequence as the translational start site is located in exon 2. Comparing the BRCA1 orthologs in rat, mouse, dog, and human, a highly conserved region was found to contain two putative ESEs, which may regulate the AS of exons 9, 10 and 11 [91]. The four predominant mRNAs (full length, and Δ (9,10), Δ (11q), and Δ (9,10,11q) variants) each retain the same open reading frame, although the splice variants encode truncated proteins. Exon 11, the largest exon, contains many functional domains involved in protein-protein interactions; thus, loss of this exon would be expected to have the greatest effects on function compared to the full length protein. However, the BRCA1 Δ (11q) splice variant retains the ability to induce apoptosis of human BrC cells [92]. BRCA1 Δ (11q) splice variant also has been shown to inhibit growth of ER-positive and triple-negative (TN) human breast, ovarian, prostate and colon cancer cells and mouse fibroblast cells [93]. Indeed, both Δ (11q), and Δ (9,10,11q) variants are localized to the mitochondria, repress ELK-1 transcriptional activity and possess anti-proliferative activity on BrC cells [94].

EXTRACELLULAR MATRIX

The ECM protein tenascin-C (TNC) is frequently up-regulated in BrC. Two novel isoforms, one containing exon

16 (TNC-16) and the other containing exons 14 plus 16 (TNC-14/16), have been identified [95]. Over-expression of TNC-16 and TNC-14/16 in BrC cell lines significantly increased cell proliferation and invasion. Analysis of a series of BrC cell lines demonstrated increased expression of TNC-16 and/or TNC-14/16 in more aggressive lines that were also ER-negative, but not in the ER-positive cell lines.

CD44 isoforms with varied functions are expressed in BrC and are important to cancer progression as in PrC. Normal breast epithelial tissue expresses the v8-v10 isoform at higher levels than the CD44s [96], while ductal myo-epithelium also expresses the v6 containing isoform [97]. The expression of the exon v6 product is often associated with adverse clinical and morphological features, mainly with lymph node metastasis. In BrC, v6 expression correlated positively with tumor grade and steroid receptor status, but not with survival [98]. Thus, AS of CD44 results in the expression of many isoforms, each associated with different biological processes, such as lymphocyte recruitment, epithelial cell-matrix adhesion, and tumor metastasis.

ALTERNATIVE SPLICING AND APOPTOSIS

As noted above for PrC, multiple proteins in the apoptotic pathway are regulated by AS and may contribute to cancer progression and chemotherapy resistance [2]. Survivin, a caspase inhibitor, exists as four different splice variants with different apoptotic properties and intracellular localization (see above). Protein and mRNA levels of the pro- and anti-apoptotic isoforms of survivin correlate with cancer prognosis [99]. Expression of survivin-2B and -2 α was shown to correlate with resistance to chemotherapy in BrC patients, while patients expressing survivin- Δ x3 responded well to therapy. Expression of survivin-3B was associated with shorter disease free survival and overall survival [100]. The Mcl-1 splice variant lacking exon 2 has been shown to be decreased in a more invasive BrC cell line compared to less invasive lines [11]. This shorter variant is associated with enhanced apoptosis and chemosensitivity [63].

FUTURE DIRECTIONS

AS is regulated by multiple signaling pathways, an area that has been reviewed recently [101-105]. As noted above or listed in Tables 1 and 2, multiple receptors undergo AS that affect their functions as initiators of signaling pathways. AS also impacts effector molecules that are mediators of multiple signaling pathways. Protein phosphatases, which are crucial modulators of cellular phosphorylation events, undergo AS. Signaling and resultant post-translational modifications also affects the activity and sub-cellular distribution of spliceosomal regulators. Multiple studies have demonstrated an association between transcription and pre-mRNA splicing. These are a few examples of important topics for future investigation.

Advances in sequencing technology output (deep sequencing) will allow direct measurement of the abundance of individual sequences, including global analyses of alternative splicing [78]. While global assessment of mRNA splicing will continue to provide identification of alternative

mRNA products, these studies will need to be extended using proteomic technologies to distinguish and quantify protein isoforms. Two-dimensional difference gel electrophoresis, which compares protein samples labeled with different fluorescent dyes, has the potential to identify those mRNAs that are actually translated into protein. Other techniques, such as high-throughput comparative mass spectrometry and iTRAQ (isotope tags for relative and absolute quantitation), represent approaches that will be able to quantify protein variants.

Another way to extend mRNA data is to identify splice variants that are being actively translated from mRNAs, which could be provided by their preferential enrichment on polysomes. Analysis of RNA prepared from cytosolic (unbound) and polysome-bound fractions would identify a subset of transcripts that are preferentially found in polysomes, thus differences in the relative distribution of translationally active mRNAs could be quantitated.

The definition of a "splicing code" is also under investigation. The value of a splicing code is apparent in light of the observation that approximately 16% of the 31,250 point mutations listed in the data base of human disease alleles (www.hgmd.org) are located within splice sites. A splicing code should predict the effect these mutations have on pre-mRNA splicing and could also prove to be an extremely useful tool in predicting mutations that disrupt splicing [79].

While we have highlighted some of the functions of specific variant proteins, major effort will be needed to extend the current limited understanding of the biological impact of specific AS products.

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