Clinical Perspective on Chemo-Resistance and the Role of RNA Processing

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Abstract

Pre-messenger RNA splicing is significantly changed in cancer cells leading to the expression of cancer-specific transcripts. These transcripts have the potential to be used as cancer biomarkers and also as targets for new therapeutic approaches. In addition, the cancer-specific transcripts have the potential to alter the drug response of the cancer cells creating a chemo-resistant state. This later property of alternative splicing presents a challenge to clinicians in the design of effective therapeutic regimens. When a patient's cancer relapses it is frequently refractory to standard chemotherapies resulting in a poor clinical outcome. Therefore, understanding the mechanisms of how alternative splicing can lead to chemo-resistance is critical to the effective delivery of treatment. Here, we will discuss the impact of alternative splicing variants on drug metabolism and activation; on drug interactions with cell signaling pathways; and on cell death pathways in cancer therapeutics. In addition to the initial characterization of splicing variants, the mechanisms leading to alterations in splicing are being studied in the setting of chemo-resistance and will be discussed here. The promise of therapeutic intervention to obviate the impact of these splicing variants will significantly enhance treatment options for cancer patients.

Keywords

Multi-drug resistance · Drug metabolism · Steroid receptors

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Contents

1 Introduction

Cancer is a disease of clonal evolution which selects for cells with enhanced proliferation and survival [1]. In the face of selective pressure of chemotherapeutics, the genetic heterogeneity of the cancer clone allows for the selection and expansion of chemo-resistant cells. This may ultimately result in relapsed disease that is refractory to standard therapeutics. This is a central problem in cancer therapy [1]. Studies in bacteria indicate that selective pressure does not cause mutations, but rather selects for mutations that are advantageous to clonal survival [2]. One mechanism the cells utilize to generate diversity is alternative splicing to produce transcripts that may have a changed function or a reduced functional capacity.

Alternative splicing is a post-transcriptional mechanism for regulating the processing of pre-mRNAs such that different combinations of splice sites can be joined to form mature mRNAs. This process contributes to the functional complexity of the human proteome that is not represented by the number of genes in the genome [3]. In a tissue-specific and developmentally regulated fashion, alternative splicing regulates gene activities involved in every aspect of cell function and survival [4]. Alternative splicing can lead to functionally antagonistic products generated from the same genetic locus with both splicing isoforms being expressed in the cell. A shift in the isoform balance can lead to changes in cellular function [5].

Alternative splicing variants have been described in a number of cancers [5, 6]. Using a bioinformatics approach, Kirschbaum-Slager et al. observed a significant shift in the expression of splicing factors in tumors indicating that these factors may be involved in oncogenic pathways [7]. There is growing evidence to suggest that some cancer-specific protein expression patterns are caused by cancer-specific alternative splicing [8] indicating that splicing variants can be used as tumor markers and that alternative splicing can accompany the process of tumorigenesis [7]. Here, we will discuss the impact of alternative splicing variants on drug metabolism and activation; on drug interactions with cell signaling pathways; and on cell death pathways in cancer therapeutics. In addition to the initial characterization of splicing variants, the mechanisms leading to alterations in splicing are

being studied in the setting of chemo-resistance and will be discussed here. In summary, the control of splicing is an important component of gene regulation and alternative splicing may contribute to chemoresistance to cancer therapies.

2 Alternative Splicing and Drug Delivery

Successful drug therapy relies on the ability of the drug to enter the cell either by passive or by active mechanism and also to be retained by the cell. In passive drug uptake, molecules such as steroids can diffuse across the cell membrane without energy expenditure. Active uptake requires energy and can involve cell surface molecules such as the ATP-binding cassette transporters or ABC [9]. This process can be affected by alternative splicing. Ligand-receptor binding results in the successful delivery of drug to the cell with subsequent activation of signal transduction pathways to translate the signal of drug into cellular activity. Endocrine-based therapies are used for the treatment of a number of cancers including breast cancer, prostate cancer, and hematologic malignancies. Alternative splicing of steroid receptors has been reported in these cancers and may contribute to the resistance of the steroid-based therapies. In addition to the successful uptake of drug, the retention of drug in the cell is required for successful therapy. Mechanisms to actively remove drug from the cells result in resistance to a number of drugs and have been termed multiple drug resistance or MDR and involve the ABC transporters. The development of MDR hampers the delivery of cancer therapeutics and therefore understanding the mechanisms of MDR is important for the successful delivery of therapies. Alternative splicing has been reported to play a role in some forms of MDR [10].

2.1 Alternatively Spliced Steroid Receptors

Steroid receptors belong to a large super family of hormonally activated transcription factors. The classical activation for the steroid receptor involves binding of the steroid hormone to the hormone-binding domain allowing for translocation of the receptor monomer to the nucleus. The DNA-binding domain of the receptor binds as a dimer to its response elements allowing for the transcriptional control of gene expression. Hormonally regulated cancers have long been treated with endocrine-based therapies; however, resistance to those therapies ultimately occurs. Alterations in the steroid receptors have been described for a number of hormone-resistant cancers including alterations caused by changes in splicing. Here, we will discuss some of the reported alternatively spliced hormone receptor variants and whether they contribute to the hormone resistance observed.

Tamoxifen, a selective estrogen receptor modulator (SERM), is among the first-line endocrine therapies for estrogen receptor/progesterone receptor positive breast cancers. Analysis of tamoxifen-resistant breast cancers revealed several variants of the estrogen receptor-α (ER-α) including alternatively spliced forms [11].

Alternative splicing of ER-α with deletions for exon 2, 3, 4, 5, and 7 have been described, with the most abundant variant being the exon 7 deletion. The exon 7 deletion (Δ7) was further analyzed and found to act as a dominant negative and inhibit the actions of the wild-type receptor in transient transfection assays [12]. The Δ7 variant of ER-α has also been detected in human endometrial adenocarcinoma [13]. When the Δ7 ER-α variant adenocarcinoma is grown in a nude mouse, the tumor is not responsive to either estrogen or progesterone; however, the tumor is responsive to tamoxifen which causes an increase in the doubling time of the tumor volume. This indicates tamoxifen acts as an agonist for the Δ7 receptor in an in vivo model.

Similarly, mutations in the glucocorticoid receptor (GR) have been described in glucocorticoid-resistant hematologic malignancies. We have described an alternative splice variant of GR, GR-P, in glucocorticoid-resistant myeloma cell lines [14]. GR-P is the result of a failure to splice at the exon 7 junction and retention of intron G and is the predominant GR variant observed [15, 16]. GR-P could encode a truncated receptor with a deletion in the hormone-binding domain, a critical functional domain; however, it is not clear that GR-P is translated into protein. In transient transfection assays, GR-P is not a functional receptor, nor does it act as a dominant negative receptor to reduce the function of the wild-type GR. Further studies will be required to elucidate the potential role for GR-P in glucocorticoid resistance.

Loss of the retinoic acid receptor β (RARβ) in lung cancer cells is associated with resistance to retinoic acid-induced cell killing. An alternatively spliced form of RARβ (RAR$\beta1'$) is generated by skipping exon 2 and is expressed in lung cancer cell lines that are sensitive to retinoic acid therapy [17]. Furthermore, in paired tissue samples of normal lung and tumor tissue collected from the same patient, RAR$\beta1'$ is expressed in the normal tissue, but not in the tumor tissue. Exogenous overexpression of RAR$\beta1'$ in retinoic acid-resistant lung cancer cell lines restores retinoic acid-induced cell death. These studies indicate that identification of pharmacologic approaches to restore RAR$\beta1'$ expression could provide a basis for retinoid-based lung cancer therapy or chemoprevention [17]. Alternative splicing in association with drug resistance has also been described for other members of the steroid receptor super family including the androgen receptor [18] and the peroxisome proliferator-activated receptor [19].

2.2 Multidrug Resistance

Delivery of chemotherapeutics can also be hampered by increased activity of drug efflux. This activity allows cancer cells to sustain resistance to a number of drugs and has been termed multidrug resistance. Multidrug resistance can occur when transporters are overexpressed allowing for the efficient efflux of drug resulting in drug resistance. One such transporter is the multidrug resistance protein 1 (MRP1) which is a member of the ATP-binding cassette transporter subfamily. In a study examining tissue from ovarian cancer patients, alternatively spliced variants of

MRP1 were expressed with exon skipping detected between exons 10 and 19 [10]. Exogenous expression of three of these MRP1 variants in HEK293T cells results in expression of the variant protein in the plasma membrane conferring resistance to doxorubicin due to increased influx. The MRP1 protein undergoes alternative splicing at a higher frequency in ovarian tumors than in pair matched normal tissue from the same patient.

3 Alternative Splicing and Drug Metabolism and Activation

Several drug therapies for cancer treatment are administered as a pro-drug and require activation by the cellular metabolism to be active. For example, deoxycytidine kinase (dCK) is the rate-limiting enzyme in the activation of nucleoside analogs such as cytarabine (ara-C), gemcitabine and clofarabine [20]. Ara-C is phosphorylated by dCK to ara-C-5′monophosphate and then further converted to the triphosphate form of ara-CTP. In this form it incorporates into DNA causing chain termination, blocking DNA synthesis, and ultimately causing leukemic cell death [21]. This is the basis of the successful use of ara-C for the treatment of several leukemias including acute myeloid leukemia (AML).

A number of variants of dCK have been reported [20] including variants due to alternative splicing [22–24]. In leukemic blasts from AML patients resistant to ara-C, variants of dCK were isolated with deletions in exon 5, exons 3–4, 3–6, or 2–5 [25]. To test the functional capacity of these variants, they were introduced into dCK negative cells and their dCK activity was compared to the introduction of wild-type dCK. In each case, the alternatively spliced variants had no dCK activity and no sensitivity to ara-C. However, when the variant dCK was co-expressed with the wild-type dCK, it did not appear to reduce either dCK activity or sensitivity to ara-C. The authors conclude that resistance to ara-C may lie in a defect in the splicing machinery [22].

Another nucleoside analog which requires activation by dCK is gemcitabine (2′-2′-difluorodeoxycytidine (dFdC)). Gemcitabine is an analog that is effective against a number of solid tumors including ovarian cancer. The human ovarian cancer cell line AG6000 was found to be resistant to gemcitabine due to deficient dCK activity [24]. A dCK transcript was detected which carries an exon 3 deletion bringing into frame a premature stop codon. No gross genomic alterations were detected indicating the involvement of post-transcriptional formation of the truncated dCK transcript. Transient transfection assays indicate that the Δexon 3 transcript of dCK is not translated into protein, perhaps leading to the observed resistance to gemcitabine. When wild-type dCK transcripts were transfected into the AG6000 cells, expression of the full length dCK failed to completely reverse the resistance to gemcitabine. Parallel studies introduced the Δexon3 dCK transcript into ovarian cancer cell expressing a wild-type dCK. When tested for sensitivity to gemcitabine, there was no discernable decrease in sensitivity.

4 Alterations in the Mechanisms of Drug Action

Cancer therapeutics have been designed to target cells with abnormal growth, either through inhibition of DNA synthesis and subsequent cell division; inhibition of abnormal cell growth signals; or stimulation of programmed cell death by a number of approaches. Alternative splicing of key molecules in the drug action pathways contributes to drug resistance of chemotherapeutics.

Gastric cancers are treated with a variety of DNA damaging agents including drugs such as anthracyclines and pyrimidine analogs. Differential display to profile gene expression of the drug-resistant lines identified mitotic arrest-deficient protein 2 (Mad2) as being altered and termed Mad2-Beta [26]. Wild-type Mad2 is a key component of the mitotic checkpoint also known as spindle assembly checkpoint and functions to detect DNA damage and subsequently stop or delay chromosome segregation until repair can be effected or until the cells undergo apoptosis. Mutation of this protein in cancer cells can allow cell division to occur in the face of DNA damage, resulting in resistance to DNA damaging drugs. Mad2-Beta is generated by a deletion of the third exon which would translate into a truncated protein. Exogenous expression of the Mad2-Beta transcript in adriamycin-sensitive gastric cancer cell lines induced a decrease in adriamycin sensitivity and also reduced mitotic arrest and mitosis indicating that generation of this variant contributes to the observed drug resistance [27].

Alternative splicing variants can also contribute to resistance to targeted therapies. Chronic myelogenous leukemia (CML) expresses a specific fusion protein from the Bcr-Abl gene which causes enhanced activation of the Abl kinase activity. Imatinib, a small molecule tyrosine kinase inhibitor, has been successfully used in the treatment of CML, producing a high rate of complete remission. Unfortunately, resistance does occur usually in the form of point mutation causing substitution of critical amino acid residues in the Abl kinase domain. Among these point mutations is a C to G transversion at position 1,106 which activates a cryptic splice donor sequence [28]. Analysis of CML cells from two imatinib-resistant patients indicates the presence of the transversion at position 1,106 as well as truncated transcripts due to the alternative splicing. Detection of the splice variant may pose a diagnostic challenge when PCR product sequencing is used for detection of the resistance mutations of Bcr-Abl as it may be interpreted as mixed sequence due to reduced-quality readings and therefore withdrawn from the diagnostic procedure.

Resistance to cancer therapies can also take the form of decreased cell killing due to changes in proteins associated with programmed cell death. Acute lymphocytic leukemia (ALL) is a disease of childhood or young adults. It is frequently treated with a variety of chemotherapeutics which rely on programmed cell death for success. The extrinsic pathway of programmed cell death involves the engagement of the Fas receptor (CD95) which ultimately results in activation of the caspase cascade and cell death. Leukemic blasts isolated from infants expressed variants of CD95 that are generated by changes in splicing [29]. A

variety of variants have been characterized including deletion of exon 6, an exon which encodes the transmembrane domain of Fas. Expression of the exon 6 deletion variant results in a truncated soluble Fas protein which inhibits the membrane bound Fas receptor thus decreasing Fas ligand-induced apoptosis. Alternative splicing is also responsible for the generation of the caspase-3 short form which antagonizes the activity of full length caspase 3 resulting in chemo-resistance in breast tumors [30]. Expression of alternatively spliced inhibitors of apoptosis protein (IAPs) result in more IAPs with higher activity to inhibit apoptosis in HL60 cells leading to multiple drug resistance [31, 32]. Similarly, in hepatocellular carcinoma tissues, which are drug resistant, alternatively spliced IAPs result in enhanced inhibition of apoptosis [33].

5 Mechanisms of Alternative Splicing Associated with Resistance to Cancer Therapies

Understanding the mechanisms that result in alternative splicing may identify new drug targets for the treatment of drug-resistant cancers. This is complicated by the intricacies of the splicing reaction and the number of proteins and nucleic acids that participate in the formation and regulation of the spliceosome. Direct comparison of drug-sensitive cancer cell lines with drug-resistant cell lines of the same lineage has led to the identification of some splicing factors that appear to be differentially regulated and perhaps participate in the generation of the drug-resistant state.

As discussed earlier, the alternative splicing of the MRP1 is associated with ovarian tumors resistant to doxorubicin [10]. Two splicing factors, polypyrimidine track-binding protein (PTB) and SRp20, are overexpressed in ovarian tumors in comparison to matched normal ovarian tissues and overexpression of both of these splicing factors was associated with the increased number of MRP1 splicing forms [10]. It remains to be determined whether these two splicing factors directly participate in the splicing of MRP1 [34]. However, the overexpression of PTB may function in tumor progression. To that end, PTB expression in the A2780 ovarian tumor cell line was knocked down by siRNA resulting in impaired tumor cell proliferation, anchorage-dependent growth, and in vitro invasiveness [34]. Therefore, those tumors which overexpress PTB may benefit from reducing PTB as a novel therapeutic target in the treatment of ovarian cancer.

Pre-mRNA processing factor-4 (PRP-4) is overexpressed in several paclitaxel-resistant cancer cell lines including the multi drug-resistant ovarian cancer cell lines SKOV-3_{TR} and OVCAR8_{TR}. PRP-4 is a serine/threonine protein kinase that plays a role in splicing of pre-mRNAs. Repression of PRP-4 with shRNA constructs leads to a reversal of paclitaxel resistance in SKOV-3_{TR} cells and conversely overexpression of PRP-4 in drug-sensitive ovarian cancer cell lines leads to a modest drug resistance to paclitaxel, doxorubicin, and vincristine. These data taken together indicate an important role for PRP-4 in the development of resistance to chemotherapeutic drugs [35].

Splicing factor 45kDa (SPF45) is associated with cyclophosphamide-resistant mouse mammary tumors. A more extensive examination of tissue microarrays from several epithelial tumors indicated overexpression of SPF45 in comparison to adjacent normal tissues [36]. Overexpression of SFP45 in Hela tissue culture cells results in drug resistance to doxorubicin and vincristine, two chemotherapeutic drugs frequently used in cancer therapies [36]. In addition to generating alternatively spliced transcripts, splicing factors can also regulate transcriptional activation of the androgen receptor resulting in resistance to androgen-based therapies [18]. PTB-associated splicing factor (PSF) and p54nrb can both play key roles in regulating the transcriptional activity of the androgen receptor in prostate cancer models.

These studies open the possibility that splicing factors may form the basis of therapeutic targeting in the treatment of cancer [37]. Wilms' tumor gene (*WT*1) has been implicated in the maintenance of malignant phenotype in leukemias and a number of solid tumors [38]. Several isoforms for the WT1 transcript are produced including an alternatively spliced form skipping exon 5. In cisplatin resistant ovarian carcinoma and testicular germ cell tumor cell lines there is an increase in WT1 transcripts. Using nuclease-resistant antisense oligonucleotides which target exon 5 of WT1 reduces that transcript specifically and also induces cell death. These studies indicate that changing the ratio of exon 5+ and exon 5− WT1 transcripts affects cell viability and may be a useful approach for treating tumors that over-express WT1 [38].

Several investigators have explored modulating phosphorylation of the SR splicing factors in preclinical investigation of novel targets for cancer therapeutics. SR proteins are a family of essential factors required for constitutive splicing of pre-mRNA and play an important role in modulating alternative splicing [39]. The SR protein function is modulated by phosphorylation. While phosphorylation of the SR protein promotes spliceosome assembly dephosphorylation of the SR protein allows the transesterification reaction to occur. SR proteins are phosphorylated by Ser/Thr kinases [40]. DNA topoisomerase I (Topo I) transiently nicks DNA strands to allow relaxation of DNA supercoil which is required for transcription, DNA replication and DNA repair. In addition to these functions, Topo I also has kinase activity phosphorylating SR proteins. A Topo I-deficient murine lymphoma cell line exhibits hypophosphorylated SR proteins and an impairment of the exonic splicing enhancer (ESE)-dependent splicing. Restoration of Topo I activity in these cells restores ESE-dependent splicing leading to the hypothesis that selective targeting of the kinase activity of Topo I may provide a means to interfere with the expression of specific genes involved in cell proliferation and/or apoptosis [41]. Serine-arginine protein kinase 1 (SRPK1) also phosphorylates SR proteins. SRPK1 is expressed in ductal epithelial cells of the human pancreas and has increased expression in pancreatic tumors [42]. Decreasing the expression of SRPK1 in pancreatic tumor cell lines decreases the phosphorylation of SR proteins and enhances the sensitivity to chemotherapeutics drugs such as gemcitabine indicating that SRPK1 may be a drug target in the treatment of cancers [42]. The Cdc2-like kinase (Clk) family has also been shown to participate in phosphorylation of the SR protein family. Inhibition of Clk

activity in cell lines with a beno-thiazole compound suppressed SR protein phosphorylation and decreased Clk-dependent alternative splicing [39]. This novel inhibitor of Clk may be useful as a therapeutic to manipulate abnormal splicing associated with cancer.

6 Summary and Conclusions

In summary, alternative splicing can influence various aspects of cancer therapy. Understanding the mechanisms of alternative splicing would enable us to identify novel therapeutic targets and design new treatment modalities to enhance tumor killing and to overcome drug resistance. Here, we have provided examples where drug resistance can be traced to alterations in drug uptake, metabolism, and mechanisms of action. It is likely that increased interest in the relationship of alternative splicing will uncover additional examples of drug resistance related to cancer therapeutics.

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