Alternative Splicing in Human Tumor Viruses: A Therapeutic Target

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Introduction

At the end of the 19th century, viruses were classified as small infectious agents that are filterable. Subsequently, studies by Giuseppe Ciuffo on human warts in 1907; Ellermann and Bang in Chicken leukemia in 1908; and Rous in chicken sarcoma (RSV) in 1911, demonstrated that some viruses maybe responsible for some tumor (Kalland et al, 2009; Martin and Gutkind, 2009). In the last 30 years, evidence that viruses and involved in the genesis of human cancers has grown (Damatia, 2006). Indeed in the last three decades, viral oncology studies employing both classical virological methods and advanced molecular biology techniques have been instrumental in the development of the contemporary paradigm of molecular oncology which 'suggests that human cancers might arise from the aberrant expression or alterations in normal human genes (Martin and Gutkind, 2009; Sarid and Gao, 2010). At present, the International Agency for Research on Cancer (IARC) classifies six viruses as carcinogenic to humans (Hegel et al, 2012). Table 1 summarizes the viruses known to cause tumor in humans. Known mechanism of oncogenesis include: chronic inflammation associated with the release of reactive oxygen (ROS) and nitrogen species (RNS); integration of viral genome into host genome; alteration of protein expression and epigenetic changes; apoptosis regulation; immune modulation and genetic instability (Hegel et al, 2012). Targeting some of these pathways has been proposed as a way of eliminating chronic viral infections (Butel, 2000). In this paper, a review of alternative splicing in human tumor viruses as a therapeutic target will be undertaken.

Virus	Family	Cancer type	Cases per year [a]	Mechanism [b]	Oncogenes	Oncogene function	References
Hepatitis B virus (HBV)	Hepadnaviridae	Hepatocellular carcinoma	340 000	Chronic inflammation	X protein	Molecular-signaling dysregulation inhibition of p53	(Ganem and Prince, 2004; Guidotti and Chisari, 2006; Kao and Chen, 2002)
Hepatitis C virus (HCV)	Flaviviridae	Hepatocellular carcinoma	195 000	Chronic inflammation	None	—	(Colombo et al., 1989; Thomas et al., 2000)
Epstein–Barr virus (EBV)(HHV-4)	Herpesviridae	Burkitt's lymphoma, Hodgkin's lymphoma, Post transplantation lymphoma, Nasopharyngeal carcinoma	113 400	Oncogenic	LMP-1	Molecular signaling dysregulation NF-κB activation lymphoproliferation	(Arvanitakis et al., 1995; Brown et al., 2001; Mosialos et al., 1995)
Human papillomavirus (HPV)	Papillomaviridae	Cervical cancer, Anal cancer, Penis cancer, Head and neck carcinoma	561 180	Oncogenic	E6/E7	Inhibition of p53 and Rb Cell adhesion dysregulation	(Beaudenon et al., 1986; Dyson et al., 1989b; Scheffner et al., 1990)
Human T lymphotropic virus type 1 (HTLV-1)	Retroviridae	Adult T-cell leukemia	3300	Oncogenic	Tax	Molecular-signaling dysregulation NF-κB activation immortalization	(Matsuoka and Jeang, 2007; Poiesz et al., 1980)
Kaposi's sarcoma-associated herpesvirus (KSHV) (HHV-8)	Herpesviridae	Kaposi's sarcoma, Pleural effusion lymphoma, Multicentric Castleman's disease	102 300	Oncogenic	vGPCR, vIL-6, vBcl2, vMIPs, vFlip, vCyclin LANA, Kaposin B	Multiple-signaling events Cell cycle dysregulation Inhibition of apoptosis Immune evasion Autocrine and paracrine functions	(Arvanitakis et al., 1997; Bais et al., 1998; Cesarman et al., 1995; Chang et al., 1994; Ganem, 2006; Montaner et al., 2003; Yang et al., 2000)

Table 1: Tumor – inducing viruses, cancer type, mechanism of action and Oncogene function. It must be noted that while oncogenes are essential in tumorigenesis, other host-related and

environmental factors including nutritional status, chronic inflammation, and mmunosuppression among others are also essential. Source (Martin and Gutkind, 2010)

Theory and Principles

The notion that viruses have oncolytic potential has existed since the beginning of the 20th century (Kalland et al, 2009). Indeed, many studies undertaken in both experimental and human, settings were carried in the 1950s and 1960s using viruses. Many case reports describe the use of several viruses including rabies virus, adenovirus serotype 4, West Nile virus (strain Egypt 101) among others for direct tumor targeting (Vaha-Koskela et al., 2007). Alternative strategies which were investigated in addition to direct tumor targeting included cancer immunotherapy or tumor vaccination aimed at inducing tumor-specific immunity capable of long-term elimination of cancer cells via maintenance of immunologic memory (Vaha - Koskela et al., 2007). A different approach involved the enhancement of cancer cell recognition by dendritic cells or T-cells transduction with viruses expressing immunostimulatory cytokines. Other investigators further suggested that tumor vasculature can be a good target for viral transduction (Vaha-Koskela et al., 2007). The objective was to introduce transgenic viruses expressing oncotoxic prodrugs into endothelial cells of blood vessels in solid tumor thereby restricting angiogenesis (Vaha-Koskela et al., 2007). In addition to these approaches, other putative cellular targets for potential antiviral agents that have been investigated include: use of small interference RNAs (RNAi), targeting cellular sites of viral particle entry; assembly and exit; targeting nuclear transport; processing of viral RNA and translation of viral proteins (Hegel et al, 2012).

From the foregoing, one can clearly appreciate the fact that a large number of putative targets for potential therapies exists. <mark>In the remaining section, focus will be directed at alternative splicing, an aspect of RNA processing, as potential drug target.</mark>

Constitutive and Alternative Splicing

Whole genome sequence analyses have shown that proteomic complexity in mammalians is achieved via a relatively small number of genes (Hagiwara, 2005). For instance, the human proteome consists of about 90, 000 proteins yet the genome contains approximately 23, 000 genes (Hegel et al, 2012). This outcome highlights the importance of alternative splicing in the generation of proteome diversity. To understand this phenomenon, a brief of overview of constitutive splicing is appropriate.

Discovered first in adenovirus - 2, constitutive splicing involves the removal of introns (non-coding regions) from pre-mRNA and joining/splicing exons (coding regions) to obtain translatable mRNA (Hegel et al, 2012). A highly ordered process, splicing is mediated by a complex assembly of proteins called the splicesome machinery. The process involves pre-mRNA substrates, binding of small nuclear RNA (snRNA) to pre-RNA processing proteins (PRP) such as U1, U2, U5 and U4/U6 to form small ribonucleoproteins (snRNPs) - core of the splicesome (McFarlane and Graham, 2010). See Figure 1 below the following link for a demonstration of constitutive splicing. Competition between several auxiliary regulatory proteins such as serine/arginine-rich (classical SR proteins are named SR-splicing factor (SRSF) 1-9) (Hegel et al, 2012) proteins or heterogeneous nuclear ribonucleoparticle (hnRNP) proteins at regulatory sequences (e.g. exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs), exonic

splicing silencers (ESSs), and intronic splicing silencers (ISSs)) also determine splice site binding (Hagiwara, 2005).
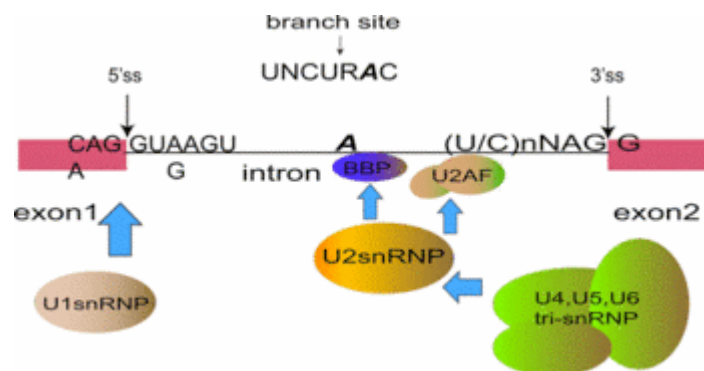

Fig 1: The basic splicing machinery (|Adopted from Hegel et al, 2012)

http://www.youtube.com/watch?feature=endscreen&v=FVuAwBGw_pQ&NR=1

On the other hand, alternative splicing involves the use of different exons within a gene to encode structurally and functionally different proteins. Known mechanisms include introns retention, introns skipping and use of alternative cassette exons (Mc Farlane and Graham, 2010). See Figure 2 and the following link. Recently, the notion that alternative splicing is important for organisms such as viruses with limited genetic material has gained a lot of support.
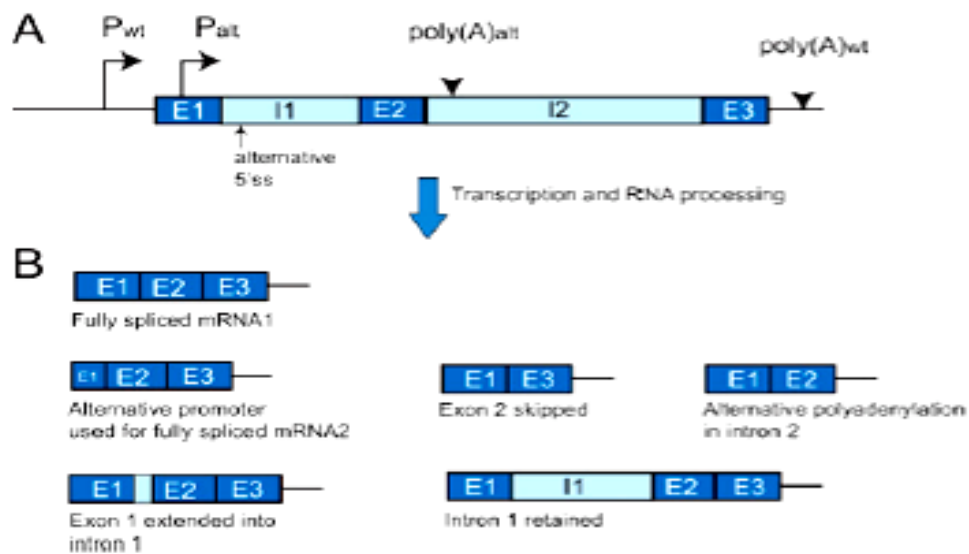
Fig 2: Mechanisms of alternative splicing showing constitutive splicing, exon skipping

and intron retention (Adopted, Hegel et al, 2012)

See link http://www.youtube.com/watch?v=p2uC5j0hK0A

Alternative splicing in Viruses

To encode a large number of proteins using a relatively small genome, viruses employ multiple strategies including multiple promoter usage, antisense transcription, stop codon read through and translational frame shifting (Hagiwara, 2005). At the same time, studies have shown that a number of viruses can interfere with cellular splicing machinery and this has been shown to be important in tumorigenesis (Hegel et al, 2012). Examples include:

Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV also called Human Herpes Virus -8 (HHV-8) is associated with a number of tumor including Kaposi Sarcoma (KS), primary effusion lymphoma (PEL) and Castleman's disease (Martin and Gutkind, 2010). See Figure 1. The virus is mainly associated with sarcoma in

immunosuppressed individuals. In HHV-8, clustered genes controlled by a single promoter are co-transcribed leading to the generation of a polycistronic pre-mRNA which can be spliced constitutively or alternatively (Butel, 2000). Alternative splicing in HHV-8 is promoted largely by ORF57, an RNA binding protein which regulates transcription, nuclear export, RNA processing and stability (Hegel et al, 2012). Studies have also shown that it can play a role in alternative splicing via its interaction with elements of cellular splicing machinery such as snRNA, U2 and hnRNP (Hegel et al, 2012).

Human Papiloma Virus (HPV)

HPV is associated with several tumors including cervical cancer, anal cancer, penis cancer and head and neck carcinoma. With over 130 genotypes existing, of particular mention are 'high risk' strains including HPV 16 and HPV 18 (Sarid and Gao, 2010; Damatia, 2006). Oncogenicity in these strains has been attributed to the expression of two co-expressed oncoproteins E6 and E7 which can alter cell cycle regulation (Butel, 2006). See Table. Studies have also shown that SRF can alter the phosphorylation of SRSF1 suggesting alteration of SR protein activity during its replication cycle (Hegel et al., 2012). Indeed, it has been demonstrated that serine protein kinase -1 (SRPK1), which can phosphorylate SRSF proteins can physically associate with HPV1, HPV16 and HPV18 E4 viral protein suggesting control of alternative splicing (p. 146). Other studies have demonstrated that expression of SRSF1, SRSF2 and SRSF3 are up-regulated in cervical tumor cells (McFarlane and Graham, 2010).

Hepatitis B and C Virus (HBV and HCV)

HBV and HCV are associated with chronic hepatitis and hepatocellular carcinoma. Known mechanism of oncogenesis by both viruses includes alteration of normal cellular micro-environment, chronic inflammation, alteration of signalling pathways and oxidative stress (Hegel et al., 2012). Oncogenesis in HBV has been linked to HBx protein. At the same time, studies have shown that alternative splicing plays a major role in the generation of proteins in HBV. HBV splice-generated protein (HBSP) and endoglycosidase H-sensitive PS (Polymerase surface) are two important proteins which are generated via this mechanism (Hegel et al., 2012). These proteins are thought to control viral cycle in some fashion. Indeed, Hegel and colleagues have suggested that severity of HBV infection may depend on 'HBSP splice variant production'.

For HCV, it is known that splicing of mRNA is absent. However, studies have shown that the cellular RNA helicase DDX3, an enzyme regulated by HCV infection, is indispensable for its replication (Hegel et al., 2012). Research appears to suggest that DDX3 may function as an SR protein or might bind to it (Hegel et al., 2012). However, others have suggested DDX3 may not function by RNA metabolism.

Others viruses

Regulation of alternative splicing is also known to play a key role in the establishment of persistent infection in HIV-1, EBV and HTLV among others (Dowling et al, 2008). Dowling and colleagues have argued that alternative splicing pathways might provide a novel target for elimination of HIV-1 in macrophages.

Human application: Alternative splicing mechanism in oncogenic viruses as therapeutic target

Targeting of alternative splicing in oncogenic virus therapeutics has explored several strategies including interference with the formation of cellular splicing machinery or inhibition

of SR activation (McFarlane and Graham, 2010). Trials of suppression of these pathways via the use of antisense oligonucleotide (AON), peptide nucleic acid (PNA) oligonucleotide, and RNAi have been reported (Hagiwara, 2005; Hegel et al, 2012). Briefly, AON and RNAi are directed at down-regulating the expression of target genes via Watson-Crick base pairing and subsequent degradation by RNA nucleases (Hegel et al, 2012).

Other molecules which can alter alternative splicing have also been identified. According to Hegel and colleagues, these molecules can be placed in the following groups: 'histone deacetylase (HDAC) inhibitors, CLK inhibitors, SRPK inhibitors, topoisomerase inhibitors, calmodulin kinase inhibitors, MAPK inhibitors and phosphatase inhibitors (Hagiwara, 2005; Hegel et al, 2012)'. Below is an overview of alternative splicing pathways targeted in current trials.

Clk inhibitors

Mammalian Clk-family of kinases are dual-specificity kinases that are known to affect splice site selection in some viruses (Hagiwara, 2005). Several inhibitors of Clk2 have been identified via screening of chemical library. Such studies have identified several indole derivative compounds (IDCs) that can inhibit SR proteins by altering their phosphorylation (McFarlane and Graham, 2010). In one such study, it was demonstrated that a benzothiazol compound TG003 can inhibit SF/ASF-dependent splicing of specific cellular mRNA in vitro (Hagiwara, 2005). It was also demonstrated that the drug can inhibit Clk-1 dependent alternative splicing in cells indicating that it's a potential candidate for suppressing conditions associated with Clk-mediated abnormal splicing (Hagiwara, 2005). Reports of the inhibition of Clk by

leucettine L41 via phosphorylation of SRSF4, SRSF6 and SRSF7 has also been described (Hegel et al, 2012).

SRPK inhibitors

Studies have shown that invitro propagation of human immunodeficiency virus 1 (HIV-1) can be inhibited by SRPIN-340 in MT-4 cells lines. It has been suggested that this observation indicates that phosphorylation of SR by SRPK play a critical role in the replication cycle of HIV-1 (Hagiwara, 2005). Other studies have also shown that tricyclic quinoxaline derivatives can also inhibit SRPK1 activity. Indeed, some investigators have argued that SRPK inhibitors are a promising line of drug against viral infections in general (Hegel et al, 2012).

Topoisomerase inhibitors

The discovery that topoisomerase 1 can mediate alternative splicing via alteration of SR protein phosphorylation suggested that it is a possible target in therapeutic strategies aimed at modulation of mRNA splicing (McFarlane and Graham, 2010). Indeed, some studies have demonstrated that topoisomerase inhibitors such as NB-506, a glycosylated indolocarbazole derivative, can inhibit topoisomerase 1 DNA relaxation and has antitumor activity (Hegel et al, 2012). It has been suggested that the antitumor activity maybe mediated via the suppression of SF2/ASF phosphorylation. A reaction catalyzed by topoisomerase I (Hagiwara, 2005).

Potential side effects

In the past, attempts to use oncolytic viruses to target tumor cells were conducted using common human pathogens such as influenza virus, adenovirus among others (Vaha-Koskela et

al., 2007). Obvious risks included increased susceptibility to opportunistic infections. Side effects associated with viral immunogenicity were also noted in some studies (Vaha-Koskela et al., 2007). However, compounds targeting selective modulation of alternative splicing of viral genes have been shown to limit the adverse effects. Indeed, micro-array based analysis of cellular splicing in animals models have shown that these drugs are well-tolerated (Hagiwara, 2005). However, to avoid possible deleterious effects on cells via disruptions of normal cellular alternative splicing pathways, such reports should be treated with caution.

Future Potential and Prospects of Success

Experimental data shows that inhibition of viral alternative splicing can be a potential target for future drugs. This conclusion has been firmed-up by the growing number of successful proof-of-principle studies (Hegel et al, 2012; Hagiwara, 2005). Indeed, the emerging critical role of alternative splicing in viral oncogenesis means that this approach can be considered for virally induced tumors. However, a number of difficulties remain. For instance, pathways involved in establishing persistent infections and viral oncogenesis, are not fully elucidated (Damania, 2006) and directed drug synthesis and screening for splicing-specific inhibitors for treatment of viruses may be a challenge (Hagiwara, 2005). More research in these areas is therefore warranted. In addition, a range of gene-based therapies are undergoing clinical trials. Some of these therapies are designed to supplement the activities of conventional anti-tumor agents (chemotherapy or immunotherapy) (Vaha-Koskela et al., 2007). Viral vectors are also being optimized as vehicles for delivery gene therapy (e.g. tumor suppressor gene replacements), oncotoxic/oncosuppresive inserts, cytokines, AON and RNAi for selective suppression of specific genes, among others

(Vaha-Koskela et al., 2007). Nevertheless, the approaches highlighted in this presentation show much promise.

Conclusion

In the last 40 years, the field of tumor virology has undergone rapid advancement. These range from discovery of oncogenic viruses to elucidation of cellular pathways that are responsible for triggering oncogenesis. More notable is the leveraging of this information to develop novel preventive and therapeutic agents for some cancers. The development of HPV vaccine and anti-viral agents for HIV-1 highlight these successes. However, while much progress has been made, much remains to be done. For instance, current anti-cancer drugs target host cell proteins rather than viral proteins. Therefore, it will be important to develop agents that can specifically target viral genes as a way of reducing cytotoxicity.

References

Butel J. (2000). Viral carcinogenesis: revelation of molecular mechanisms and etiology of human

disease. *Carcinogenesis*, 21 (3), 405-426.

Damatia, B. (2006). DNA tumor viruses and human cancer. *Trends in Microbiology*, 15 (1), 38-

44.

Dowling D., Nasr-Esfahani S., Tan C., O'Brien K., Howard J., Jans D., Purcell D., Stoltzfus C

and Sonza S., (2008). HIV-1 infection induces changes in expression of cellular splicing

factors that regulate alternative viral splicing and virus production in macrophages.

Retrovirology, 5 (18). 1-12.

Hagiwara M. (2005). Alternative splicing: A new drug target of the post-genome era,

Biochemica et Biophysica Acta, 1754, 324-331.

Hegel, R., Lopez-Hernandez & Graham S. (2012). Alternative splicing in human tumor viruses:

a therapeutic target? *Biochemical Journal*, 445, 145-156.

Kalland K-H., Ke X-S., & Øyan A, (2009). Tumor virology – history, status and future

challenges. APMIS, 117. 382-399.

Martin D, Gutkind J S., (2010). Human Tumor-Associated Viruses and New Insights into the

Molecular Mechanisms of Cancer. *Oncogene* **27**, S31-S42.

McFarlane M., & Graham S. (2010). Human Papiloma virus regulation of SR proteins.

    Biochemical Society Transactions, 38 (4), 1116-

Sarid R., and Gao S-J, (2010). Viruses and human cancer: From detection to causality. Cancer

    Letters, 305, 218-227.

Vaha-Koskela M., Heikkila J., Hinkkanen A. (2007). Oncolytic viruses in cancer therapy.

    Cancer Letters, 254, 178-216.