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RNA Processing and Role of Alternative Splicing on Diseases and its Therapeutic Uses

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ABSTRACT

RNA processing or splicing is a method by which mRNA is produced from pre mRNA which becomes ready for translation into protein. But there is another very important process of RNA processing in eukaryotes that is called Alternative Splicing. This process is very important in these organisms particularly in human being. It is known that human has 20,000 genes but this not sufficient for such complexity and diversity in human as it was known that one gene gives one protein. There is another important process called Alternative processing by which one gene can produce more than one protein. Splicosome machinery important for RNA processing has been discussed with methods ofTrans-splicing and Alternative splicing. Any defect or dysregulation in Spliceosome and alternative processing has been found to be the main cause of many rare diseases like neurogenerative and Cancer. The origin of these diseases due to dysregulation in alternative splicing has been discussed in detail. Therapeutic uses of Antisense oligonucleotides, small molecules and small molecule modulators have been discussed. The mechanism of action of Antisense oligonucleotides used for the correction of defects in RNA processing is also discussed.

Introduction

The eukaryotic mRNA produced from DNA is longer in size when produced is called pre mRNA or heterogeneous nuclear RNA (hn RNA). It is longer in size as it contains coding genes called Exons and non-coding genes called Introns. RNA Processing is one important process by which the pre-mRNA is transformed to mature RNA. Just after the formation of pre mRNA from DNA, it gains a 5`-cap structure at one end and 3` end on the other side which has an important function of transcription termination followed by the addition of poly A tail. Now comes the RNA Processing or RNA splicing by which the non-coding regions of gene called Introns are removed and the segments having coding genes exons are united to form mature mRNA that is ready for producing proteins. This RNA processing is important for post transcriptional regulation of gene expression through RNA Splicing. Thus, the method by which Introns are removed and rejoining of exons take place in mature mRNA and then mRNA is removed from the nucleus to direct protein synthesis in the cytoplasm. This step of forming mature mRNA is the most essential step for the expression of genes in all organisms where introns are present. Any abnormalities in RNA splicing or processing led to affect the cellular function and metabolic activities causing many diseases in human and mutations due to the production of abnormal or defective proteins.

Spliceosomes

The splicing occurs through a complex molecule called **Spliceosome** consisting of numerous proteins and RNA which is located in eukaryotic nuclei. SnRNA i.e small nuclear RNA (SnRNA) forms a complex with proteins called small nuclear ribonucleoproteins (SnRNPs) which are assembled during the splicing messenger RNA known as primary transcript to excise an intron and joining of exons to form a mature mRNA which is ready for protein synthesis after coming to the cytoplasm. It has been found that human cell contains nearly 100,000 spliceosomes to remove about 200,000 different intron sequences. So, spliceosome contains both proteins and RNA. Yeasts have about 100 spliceosomal proteins and human splicosomes have 300 splicosomal proteins. Spliceosomes have assembled with U1, U2, U4 and U6 and U5 SnRNAs to form small nuclear RNPs

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(SnRNPs). U1 binds the GU consensus sequence of the 5-splice site and U5 with U4 /U6 binds to the AG consensus sequence of 3` splice site. U2 binds to the consensus sequence UACUA [A]C of branch site slightly upstream from the 3`-splice site. There are other SnRNAs also like U7, U11, U12 etc that are found also to be spliceosome contents. U7 SnRNA is added for processing of 3` ends of mRNAs of histone. U3, U6, U9 and U10 are involved in ribosome biogenesis.

Splicing mechanism for pre mRNA processing takes place with the attachment at the 5`splice site by U1 SnRNP and U2 SnRNP recognises the branch site adenosine near the 3`-splice site through cleavage of 5` site forming a lariat structure and then the cleavage of 3` site occurs followed by the ligation of two Exons. The spliceosome is then disassembled and introns are degraded [1]. The composition of the spliceosome is highly dynamic and flexible to maintain its accuracy and the method of splicing of introns and ligation of Exons is the first step for gene expression. Introns are removed from pre mRNA by two transesterification reactions. At first the 2` OH group of the Adenosine of branch site of the intron is cleaved on the 5`-splice site that is called nucleophilic attack. In the second reaction cleavage occurs on the 3`-splice site of introns by the 3`OH group of the 5` Exon followed by the ligation of 5` and 3` Exons [2]. This process of splicing is known as classic Cis splicing that is done with the help of spliceosomes.

Large number of research works are carried out in Spliceosomes of yeast and human systems. However large diversity of spliceosomes with extreme reduction of spliceosomes are found in red alga Cyanidioschyzon merolae showing the flexibility of the splicing pathway. Detailed studies to find out the spliceosome reduction in eukaryotes, have noted that independent spliceosomal reduction in the microsporidia proteins in Encephalitozoon cunicula, Giardia and C. merolae, Piptocephalis cylindrospora (fungi), E. histolytica (amoebozoa). But no reduction of spliceosomes is found in plants, green algae, animals and human [3]. The splicing mechanisms is primarily divided into **Cis** and **Trans** splicing. Cis splicing is again divided into **Spliceosome Mediated** such as **Classic** and **Alternative** Splicing There is also Spliceosome **independent** such as **Self Splicing**. The classic cis splicing has already been discussed.

Trans Splicing

Another important type of splicing in eukaryotes is Trans splicing. While cis splicing is done with primary transcripts from single gene and trans splicing is concerned with unrelated two pre mRNA molecules or genes to produce new proteins and to bring complexity in higher organisms. It is an uncommon process that joins segments from two pre mRNA molecules. The spliceosome uses 5` splice sites from one molecule and the branch site and 3` splice sites from other molecule to join two exons. Trans splicing may produce new types of mRNA to produce new proteins. In this process the mature mRNA is composed of first exon from one gene or pre mRNA and the other exon from another gene or pre mRNA producing a chimeric molecule. The method of trans splicing is found in lower prokaryotes (trypanosomes) as well as in mammals such as in octanoyltransferase mRNA in rat hepatocytes and in human estrogen receptor gene [4]. It has beenfound that trans slicing may be spliced leader independent or Spliced leader dependent where the cell replaces nucleotides at the 5`end of some pre mRNAs with special class of small nuclear RNAs called Spliced Leader RNAs [5].

Alternative Splicing

It is also one of the splicosome- mediated cis- splicing method. In this process certain exons are skipped to create more than one mRNA from the same gene through alternative splicing the eukaryotic organisms may produce many different proteins of opposing functions and structural properties. This special mechanism of RNA processing may increase the complexity of an organism and can play an important role in cellular differentiation and development. It is known that alternative splicing has an important role in cell differentiation and in the development of organ and tissues in higher organisms. With the help of this splicing method, variants of mRNAs are produced which can produce variant proteomes and protein isomers for many new cellular functions.

These various forms of mature mRNAs are produced through multiple mechanisms like i) alternative 3` and 5` splice site; ii) exon skipping; iii) exon selection; iv) intron retention (Murphy et al 2022).

Alternative splicing is effective generally in genes having multiexons and it is highly regulated. Actually, the whole process of pre mRNA processing is complex with interactions of pre mRNA, small nuclear riboproteins (snRNPs), snRNAs, spliceosomes etc. In alternative splicing different combinations of exons may take place to form the final mRNA transcript which may produce altered mRNA leading to altered proteins which can affect the normal function of cell or tissues. This abnormal function of the cell in human may be the cause of occurring many diseases which may be cancer, neurogenerative diseases and muscular dystrophies etc. [6].

Constitutive splicing is the normal process of removal of introns and ligation of the majority of exons in a gene. But the alternative splicing is a deviation from the constitutive method where certain exons are skipped resulting in various types of mature m RNA from one gene. This process is controlled by dynamic and flexible macromolecular machine called Spliceosomes.

The method of **alternative splicing** has an important deviation from Constitutive splicing where certain exons are skipped that produces various types of mature mRNA in vertebrates while in lower metazoans (sponges,corals, jellyfish etc) it shows intron retention. Intron retention is found sometimes in human transcripts of Untranslated regions (UTRs).

Alternative splicing has a role in mediating diverse biological processes in the organism. Alternative splicing genes are found mostly in higher organisms in large proportion. This method shows diverse types of gene expression in forming new proteins for the complex function of higher eukaryotes. In this way large number of variants of proteins (isomers) are formed from one gene. Non-coding RNA, microRNA and siRNAs are generally the regulators in alternative splicing. Conserved or Constitutive splicing has an important function in species differentiation and genome evolution [7].

Diseases due to defect in Spliceosome

Numerous diseases are occurring due to some mutations in splicing factors of spliceosomes. Some of the diseases will be discussed here.

Cranio-facial disorder or Cerebro Costo Mandibular Syndrome (CCMS)

This disease is found to occur due to mutations in splicosome components or regulatory factors used in pre mRNA processing. Cranio facial disorders are causing deformities in the skull and face from mild to severe. These are Cleft lip and palate, Craniosynostosis (soft spots in the skull of infant close prematurely), one side of face is undeveloped (Hemifacial microsomia), benign tumour causing red birthmark, misshapen head (deformational plagiocephaly), sometimes fusion of facial bone in abnormal way causing sometimes neurological problems. It has been identified that this Craniofacial disorder disease is due to mutations in one of the spliceosome factors (protein) that is SNRPB (Small Nuclear Ribonucleoprotein Polypeptides B). This protein is the core component of the spliceosome in regulating alternative splicing of pre-mRNA. This SNRPB protein is present mostly in an exon containing termination codon that serves as splicing sensors leading to the disease CCMS [8].

i. **Spinal muscular atrophy (SMA)**
   It shows progressive loss of spinal cord motor neurons. It has been noted that this disease is caused by mutations in the motor neuron gene (SMN1) which causes defect in the assembly of snRNPs (Small nuclear proteins) in the spliceosome leading to spliceosome dysfunction. This dysfunction is causing defect or mis-splicing of pre mRNA leading to defective protein and is finally the reason for the occurrence of disease. But this mutation in splicing proteins is heterozygous and so it is not expressed in all patients.
ii. **Retinitis pigmentosa**
   It is an inherited disease of retina showing decreased vision at night or in low light and loss of side vision. This occurs generally in childhood.
   This disease is caused by mutations in splicing factors causing altered proteins such as PRPF (PRPF 3, PRPF 4, PRPF 8, PRPF 31), pre-mRNA Processing Factor, leading to defect in the composition of spliceosomes and splicing functions [9].
iii. **Myelodysplastic Syndromes (MDS)**
   This is one type of cancers in the bone marrow with the formation of immature blood cells which may change to Acute Myeloid Leukemia (AML). It occurs with the mutation of a splicing factor (SF3B1) encoding a subunit 1 of 3b protein complex in U2 snRNP of spliceosomes. The mutation of SF3B1 may lead to inhibition of growth affecting numerous genes and pathways [8].

Dysfunction of Alternative Splicing

Alternative splicing is a method by which different mRNAs are produced from single gene or single pre=mRNA transcript. In this process cell can select which of those protein-coding parts to include in the mature mRNA or resulting protein. Any dysregulation in this process causing many problems with the resulting protein by disrupting normal function of the cell. This may cause many diseases with diverse forms of proteins having abnormal cellular function.

The deviation from normal splicing event (cis regulated or Constitutive Splicing) is the Alternative Splicing which changes the normal process of splicing in different ways like the inclusion or deletion of Exon, Intron retention etc that may lead to numerous diseases. Again, splicing mistakes can make frameshift mutations leading to sequence alterations followed by changes in splice sites in the transcript to create aberrant mature mRNAs. These altered or aberrant mature mRNA will produce altered new proteins affecting normal cellular or metabolic function of human and thus causing diseases. Alternative splicing is more responsible for causing diseases as 95%of eukaryotic genes are alternatively spliced. Alternative splicing is responsible for producing genetic diversity from a single gene as well as any splicing defects may cause diseases in human. It has been noted that many human diseases are caused by mutations that are responsible for splicing defects. When the disease- associated mutations are found in Introns, it is difficult to identify as it does not interfere coding genes but it alters the splicing pattern. Intron mutations may change the 5` and 3` splice sites by altering their sequences resulting in the skipping of Exons or retention of Introns. It has been noted that Intronic mutations may cause about 10 – 15% disease in human. Some Exonic mutations may cause silent mutations or missense mutation by disrupting splicing silencer or enhancer by making the site unrecognisable to sequence-specific RNA binding protein [10]. Splicing Silencers and Enhancers are found both in exons and introns. An Exonic Splicing Silencer (ESS) is a cis-regulatory element that inhibits the use of adjacent splice sites that contribute to alternative splicing. While Exonic Splicing Enhancers (ESE) are helping in the accurate splicing of pre-mRNA to mRNA, the Intron splicing enhancers (ISE) are regulatory elements in determining the correct exons for alternatively spliced method and Intron splicing silencers (ISS) blocks some exons not needed for alternative splicing by creating silent zones around exons. It has been noted that disorder in RNA splicing may cause gene dysfunction leading to disease. 20,000 protein coding genes are present in human so the complexity in human has been done by alternative splicing methods to produce transcript or protein isoforms i.e., more than one protein is formed from single gene. It has been found that a great number of human diseases are occurring due to defect in alternative splicing. The defects are also found in transsplicing methods but the occurrence of disease is less in trans splicing.

Diseases due to dysfunction of Alternative Splicing

With the advancement of research in alternative splicing, its role in cell differentiation, organ development and homeostasis (internal balance of the body) has been established. In this process multiple transcripts are produced from single gene to give complexity of the proteome and finally to organism. It is actually a harmonised process in combination of DNA sequence motifs, intron and exon elements, regulatory factors and signalling pathways.

Mutations in any of these components may lead to dysregulation or dysfunction of Alternative splicing methods to create numerous diseases in human including neurogenerative diseases and cancer [11].

Neurogenerative Diseases

Some organs depend mainly on alternative splicing as these organs require cellular diversity and complexity more than others.

The most common example is the Brain as it has extraordinary diversity of cell types and unparallel complexity in function with numerous neurons. When there is a defect in brain -specific alternative splicing, there is a chance of occurrence of neuro-developmental disorders leading to many neuro-generative diseases.

The data from skin, muscle, ears and eyes goes to brain and brain cells meticulously interpret the input and sends signal to different organs, muscles and glands of the body for vital functions like heart rate, digestion, homeostasis and other metabolic functions including cognitive processes through neurological system. Some of these diseases are discussed below.

a). **Familial Dysautonomia**
It is a rare recessive genetical disorder affecting autonomic nervous system and sensory neurons. This occurs due to mutations in the transcription factor 1KBKAP due to intronic substitution of the base T (Thymine) to C (Cytosine) of DNA affecting the RNA splicing resulting in the skipping of some exon (Coding genes) with a premature termination of mature mRNA. This gives abnormal or defective protein resulting in the disease [12].

b). **Spinal-Muscular Atrophy**
This disease is also a recessive disorder in human particularly in infants that brings infant mortality. This is caused by mutations in motor neuron gene SMN1 of the fifth chromosome causing loss of function of this gene leading to the degeneration of motor neurons of the spinal cord and progressive muscle wasting. Thus, the spinal cord and brainstem are not working properly. There are four types of Spinal muscular atrophy in human. SMA Type 0 results in death at birth or within one month. SMA type1 results in death by the age of 2 months with severe form of disease. SMA type 3 disease is also severe but it remains as an intermediate. Actually type 3 and type 4 did not affect life expectancy. These two types show mild to moderate muscle weaknesses, tremors and mild breathing problems.

c). **Frontotemporal Dementia**
Alterntive splicing methods may also cause mutations in the gene MAPT located in chromosome 17 leading to behavioural changes, memory and motor function changes. Sometimes they develop Parkinson syndromes. Mutations occur within regulatory elements of MAPT exon 10 that increases the ratio of tao isoform containing four microtubule –binding sites (4R) to three site isoforms (3R). This causes disease by precipitating tau aggregation. It leads to Tauopathy with respiratory failure. So, the mutation of MAPT gene causes brain disorder. Tauopathy (accumulation of abnormal Tau in brain cells) can cause brain cells to die in some regions of the brain that are important for movement, emotion and cognition. Tau is the microtubule associated protein that forms insoluble filaments in neurofibrillary tangles in brain cells causing taupathies and occasionally Alzheimer`s Disease.

d). **Parkinson`s Disease**
It is the most prevalent disease among older people and is growing throughout the world. It has been noted that six genes are mostly involved in this disease such as SNCA, Parkin, PINK1, DJ1, LRRK2 and GBA. But the gene and the mechanism of occurrence of disease is still not definitely known and so the correct diagnosis of disease takes much time causing death of 80% of striatal dopaminergic neurons in the brain of patients [13]. With the transcriptome analysis of normal healthy people and patients, the identification of splicing dysfunction or changes is found to be responsible for both Parkinson and Alzheimer`s disease.

It has been found that the accumulation of misfolded **α-synuclein** protein encoded by the gene SNCA is the main neuropathological cause of Parkinson`s Disease. This gene is located in chromosome 4 containing six exons. Through alternative splicing pre-mRNA of the gene SNCA, three isoforms of the protein α-synuclein are produced like α-synuclein 112, -126 and -140. The α-synuclein 140 isoforms show entire transcript of the gene while -126 has lower expression and -112 enhances aggregation of abnormal protein in the brain [14]. Ageing is also responsible for the occurrence of this disease. Another important cause of Parkinson`s Disease is Dopamine deficiency which is due to protein aggregation such as Lewy bodies in the neurons of the brain making neurons inactive. This symptom may be also responsible for some mutations in the gene which is not clearly known. Lewy bodies are the inclusion of aggregated protein bodies inside the neurons leading to dementia (Lewy Body Dementia), Parkinson`s disease and other neurological diseases.

Of the six genes involved in the Parkinson`s disease PARK 2 of Parkinson gene, E3 ubiquitin protein ligase from Parkin gene, α-synuclein, α interacting protein (synphilin) encoded by the gene SNCA1P, LRRK2 encoding leucine rich repeat kinase 2 (dardarin), SRRM2gene and MAPT (Microtubule -associated protein tao) are involved in aberrant alternative splicing in Parkinson`s Disease [15]. Till now there is no effective therapy for this disease but medications, physiotherapy and others can bring relief to patients.

e). **Alzheimer`s Disease**
This disease occurs due to the aggregation of β- amyloid plaques and neurofibrillary tangles in the brain leading to the deficit of memory and progressive loss of cognitive impairment. This disease with dementia is very common (about 60-80%) in aged people (older than 65 years) throughout the world. It has been assumed that this disease is due to the combination of genetic and non-genetic factors. Non-genetic factors are brain trauma, type 2 diabetes, smoking, depression, low cognitive inactivity, environmental factors etc. The environmental factors may be pollution, aluminium in drinking water, pesticides, vitamin D deficiency and occupational exposure to electric and magnetic fields [14].

It is known generally that most neurons in the brain are post-mitotic, meaning thereby that these cells will not divide again that means this state is permanent. Recently it has been noted that some neurons can re-enter the cell cycle and starts dividing. Detailed studies of 30,000 nuclei of neurons showed that these cells re-entering the cell cycle did not produce new neurons but exhibited elevated expression of senescence [16]. In patients with Alzheimer`s Disease with loss of dopamine and Lewy body (protein aggregation) dementia it has been noted that most of the neurons re-enter the cell cycle to increase senescence.

Cancer

It has been noted that there is a relationship between dysregulation in alternative splicing or aberrant splicing with cancer formation. This aberrant process can influence the cellular processes in cancer initiation and progression. This dysregulation/ dysfunction has also an impact on tumour suppressor genes and oncogenes. Aberrant alternative splicing process can affect KLF-6, a tumour suppressor gene, which inhibits cell growth through activation of p21. It is also known as cyclin-dependent inhibitor1 (CDKN1a) which is a protein that regulates cell cycle progression during G1and S phases. P21 also regulates cell death by blocking apoptotic factors or the signalling pathway of apoptosis (cell death). The aberrant splicing produces an isoform KLF6-SV1 (transcription factor) that helps in the development of cancer cells due to over expression of protein isoform KLF SV1 [12].

It has also been noted that the gene CDKN2A encodes two tumour suppressor proteins p14ARF and p161NK4a through alternative splicing. Any dysfunction of splicing creates loss of these proteins leading to a chance of disease melanoma and neurofibroma. Again, Gastrointestinal stromal tumours are due to mutations in a proto-oncogene (tyrosine receptor kinase) occurring through aberrant splicing [12]. Generally, it has been noted that some tumour suppressor genes like p53 and pRB prevent cells to become cancerous by acting on the cell cycle. Any aberrant changes in the splicing activity produce protein isomers leading to cell proliferation. Similarly, isomers of integrin subunit α6 (ITGA6) are ITGA6A and ITGA6B are produced through aberrant splicing that enhances colon cancer cells through Myc –mediated promoter activation. Again C-Myc can upregulate polypyrimidine tract-binding protein (PTB) to change the splicing of Pyruvate kinase (PKM) to isomer PKM2 leading to cell proliferation. Similarly, mutations in RNA-binding motif protein 10 (RBM10) has been seen in lung cancer cells by disrupting the splicing of NUMB that induces cell proliferation [17].

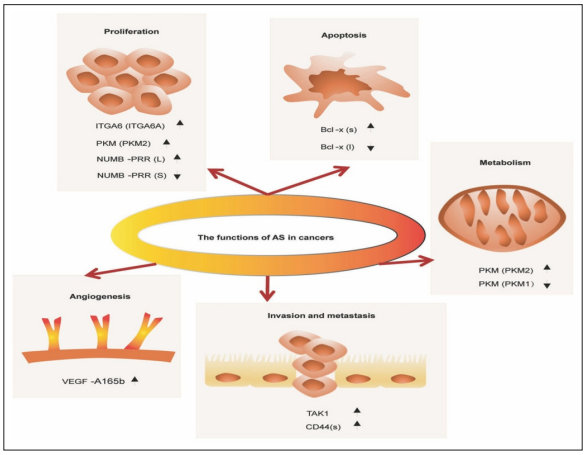

Figure 1: Role of alternative Splicing in producing Cancer. Proliferation of cells due to production of protein Isomers and finally metastasis [17].

It has been noted that splicing factors like RNA-binding motif protein (RBMP) has an important role in controlling apoptosis (cell death) through Caspase 2, B cell lymphoma (Bcl)-x, Myeloid Cell factor 1 (MCL-1) etc. But if there is an aberrant alternative splicing then Bcl-x will form two isomers Bclx and Bclxs. Bclx is inhibiting apoptosis meaning thereby that cell growth continues and metastasis will form. Bclxs will promote apoptosis [17].

Another important factor in promoting cancer is the tumour microenvironment which includes cellular and non cellular components that help in cellular progression. These non-cellular components are some growth factors, cytokines, metabolites, structural proteins and matrice cellular proteins, Hypoxia. These matricellular proteins (MCPs) are secreted into the extra cellular matrix and regulate cell functions like adhesion, migration, proliferation, differentiation, cell cycle progression and apoptosis [18]. Cellular components include fibroblasts, myofibroblasts, adipose cells, neuroendocrine cells, immune cells, blood, lymphatic cells and extra- cellular matrix etc. Hypoxia of the tumour micro-environment initiates splice in Fas. The alternative splicing of Fas (Apo-1/CD95) pre mRNA can generate membrane bound or soluble isoforms with proapoptotic and anti-apoptotic sFas isoforms. Again, SRF6 is an important regulatory protein in alternative splicing of Fas which actually meditates apoptosis. Any aberrant splicing in Fas results in the anti-apoptotic isoforms to overcome apoptosis causing unlimited cell growth (cancer). Thus, SR protein (Serine/arginine rich proteins involved in RNA splicing) is associated with cancer. Of the SR proteins, SRSF1 has been found to promote tumorigenesis and is over-expressed in cancerous cells.

Therapeutic Uses

Alternative splicing has also a role in treating several diseases including cancer as molecular therapies. There are different methods of using this technique which will be discussed below.

Antisense Oligonucleotides

As already stated, dysregulation of alternative splicing shows defective gene expression pattern leading to aberrant protein causing many diseases in human. Antisense Oligonucleotides which have the ability to bind to specific RNA targets have been used to correct splicing dysfunction. Antisense oligonucleotides (ASO) are short synthetic RNA or DNA sequences which can alter RNA or protein expression by binding to target RNA in a sequence specific manner to modulate splicing of pre mRNA, stop gene expression. ASO can bind to Aberrant m RNA will stop translation to form altered protein by binding of antisense oligonucleotides to mRNA for changing the harmful protein to functional (correct) protein leading to its use as promising treatment of many diseases like neurogenerative diseases, muscular dystrophy, cancer etc where conventional treatment is not successful [19]. Thus, Antisense Oligonucleotides may be used not only for research purposes to study gene functions or altering aberrant proteins but also in drug delivery to treat cancer and other diseases.

Aberrant alternative splicing produces abnormal proteins, called as isomers, can influence cellular processes, metabolic function etc to induce many diseases. So, these abnormal proteins may also be used as a marker for early identification correctly of cancer and other diseases.

Mechanism of Action of Antisense Oligonucleotides

a). **Targeting RNA transcript**

After binding of Antisense oligonucleotides to target RNA, a duplex structure is formed with RNA as ASO/RNA. These

duplexes then attract Ribonuclease- H1 (RNaseH1), an endonuclease that cleaves the phoshodiester bond of RNA resulting in the breakdown of mRNA to fragments (non-functional). figure.2A.

b). **Transcript Knock down**

After binding of Antisense oligonucleotides to target distinct sequences of RNA, RNA transcripts can be knocked down by gapmer Antisense oliigionucleotides (ASO) or siRNA. Gapmers are single stranded ASOs consisting of single sequence DNA core flanked by modified RNA-based structures resistant to RNase H. In other words, Gapmers are single strand DNA sequence attached with RNase H structures. It will attach with target mRNA producing from double stranded DNA during translation and immediately start the cleavage of mRNA resulting its degradation of harmful mRNA with no translated protein (figure .2B). This method is called Knock down of transcripts [20].

Again siRNA (double stranded) can also be synthesised for target m RNA, then antisense strand is incorporated into the RNA-induced Silencing Complex (RISC) leaving out the sense strand. The antisense strand binds to the complementary target mRNA causing its degradation of faulty m RNA to make it non-functional.

c). **Modulation of Splicing**

It has already been noted that single strand Antisense oligonucleotides cen enter the nucleus and binds to pre mRNA (target) in a sequence-specific manner which can block splice regulatory elements. These regulatory elements may be cryptic splice sites (that are generally dormant but can be used efficiently) or Branch points (short motif that is essential for pre-mRNA splicing) and splicing enhancers or silencers. Thus, single strand Antisense oligonucleotides can do exon skipping or can include target exon. In this way the harmful protein causing diseases can be excluded (figure.2C) or can even add new correct protein by including the target exon [20].

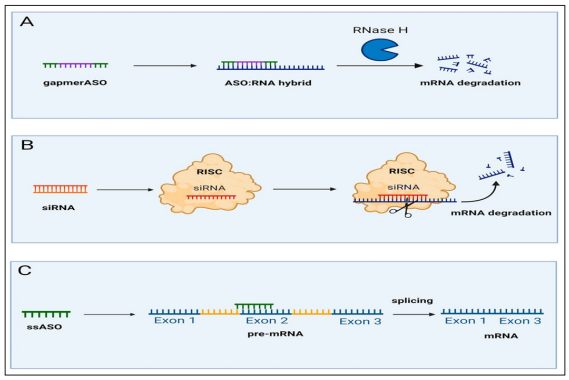

Figure 2: Mechanism of Action of Antisense Oligonucleotides (ASO). A. Showing the degradation of target mRNA forming duplexes with ASO and RNase H.
B. Cleavage of mRNA with the help of siRNA and RISC.
C. Skipping of Exon through modulation of splicing [20].

Uses of Small molecules

Small molecule is a low molecular weight organic compound which is involved in the biological process as the substrate. Monomers of nucleic acids are ribo or deoxyribonucleotides, amino acids and monosaccharides are the example of small molecules. Many drugs have already been manufactured from small molecules such as Aspirin, Penicillin G, Retrovir (azidothymidine), Xarelto (rivaroxaban) etc. Many small drugs for rare diseases have already been synthesised like Tyrosine kinase inhibitor (Imatinib) which is widely used in the treatment of cancer.

Small molecules may be used as a tool in manufacturing small molecule-drugs through modulation by alternative splicing methods for therapeutic uses in rare diseases as these drugs are most effective and safer than traditional chemotherapeutic drugs.

Small molecule modulators

Small molecules can be modulated by targeting the spliceosome. This is done by targeting trans splicing factors like Serine-Argininine rich (SR) splicing factors. These factors are very important in determining tissue-specific splicing patterns are associatedwith genetic diseases and cancer by phosphorylation of SR proteins that affect SR-protein kinases (SRPK) and CDC2 –like kinases. Diseases are originated due to abnormal SR protein expression or overexpression of this protein ue to binding of phosphorylated SRSF1 (serine rich splicing factor) to a specific regulatory element in Exon 8a. The inhibition of kinase activities of SRPK1 is possible by using inhibitors can stop abnormal expression of SR protein showing its potential as therapeutic agents. Structural studies of SRPK kinases have shown that there is an anchoring site for inhibitors leading to develop inhibitors of SRPK family such as SRPIN 340, an inhibitor characterised by a trifluoromethylphenyl group for kinase inhibition. Further development of many derivatives of SRPIN340 has been made for the development of drugs for these rare diseases. Similarly, the inhibitor of CDC2 kinases has been developed such as Chlorexidine , a memner of the cationic bisbiguanide class is also a modulator of SR protein mediated alternative splicing which is inhibiting CLK fmily members (Cdc2- like kinase). It has so been noted that splicing activators such as the splicing factor SRSF6 are overexpressed in many cancer cells through moduation of alternative splcing. Again, inhibitors of modulation have also a great potential therapeutic value. Thus, modulation of splicing methods has a great potential in molecular targeting in treatment of several diseases including cancer (Bouton etal 2024). Small molecule modulated drugs are Gleevac(BCR-ABL kinase inhibitor), Nusinersan (to treat SM[Spinal Musular Atrophy]) , Adempas(to treat pulmonary hypertension), PDES Inhibitor (to treat erectile dysfunction) etc.

Again, the splicing factors SF3B1 have the highest mutation found in various haematological malignancies where small molecules can be used as a target for cancer treatment. Some small molecules like Spliceostatin A, Pladienolide-B, GEX1A and E1707 have been found to inhibit the splicing factor SF381 found in tumour cells. Small molecule drug Amiloride is used for the treatment of edema and hypertension. This drug can change the alternative splicing of Bcl-x, HIPK3 etc by affecting the splicing factor SF2/ASF leading to the reduction of overexpression of SRP20 and other SR proteins. Recently the drug H3B-8800 is used for patients with myeloid neoplasms (MDS, CMML and AML) as a clinical trial [17].

Splice switching method

Splice switching can be done through antisense oligonucleotide of 12-30 nucleotides in length that can be designed to target

sequences to manipulate gene expression through mRNA degradation, modulating splicing, blocking translation, skipping of exons etc. In case of patients with Parkinson`s Disease, Antisense oligonucleotides have been designed for the skipping of exon 2 having defective gene LRRK2 inducing a stop codon in the transcript. By skipping the exon 2, about 50% defective protein level has been reduced in the patient. This experiment has been done by injecting targeted Antisense oligonucleotides in transgenic mouse that shows skipping of exon having LRRK2 leading to the reduction of LRRK2 kinase activity. So, this mthhod has the therapeutic potential for LRRK2-related Parkinson`s patient. Similarly skipping of other genes or inclusion of exon can also be done in changing the transcript of several diseases [21].

Thus, several diseases in human are found to be originated due to dysregulation or splicing defects of alternative splicing and this splicing method may be used to target the disease which can be helpful in its treatment. The splice switching antisense oligonucleotide therapeutics have a great potential for the development of disease-modifying treatment for several rare diseases like neurogenerative diseases including cancer.

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