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Advancements in Genetic Therapies for Arthritis: A Path Towards Precision Treatment

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Abstract: Arthritis, a pervasive autoimmune disease globally, lacks a definitive cure, with current treatments primarily alleviating symptoms and slowing disease progression. However, the efficacy of existing therapies is curtailed by drug short lifespan and the need for frequent systemic administration, highlighting the potential of emerging gene therapies to revolutionize healthcare for arthritis. This overview explores recent advancements in gene therapy for arthritis, with a primary focus on rheumatoid arthritis (RA). Gene therapy has begun to be employed in RA treatment, utilizing novel vectors to enhance gene transfer efficiency and induce sustained, regulated gene expression in targeted tissues. Promising strides include the utilization of different serotypes of viral vectors, such as adeno-associated virus. Additionally, the review encompasses various gene transfer strategies, vector innovations, candidate genes, and safety considerations.

Key words: Arthritis, gene therapies, rheumatoid arthritis (RA)

MECHANISMS OF AUTOIMMUNE DISEASES

Autoimmune diseases comprise a group of disorders wherein the immune system erroneously targets the body's cells, leading to self-attack. Typically, they manifest through stages of onset, progression, and eventual flare-up episodes. These conditions involve complex processes often influenced by a combination of immunological, environmental, and genetic factors [1].

Research conducted on both humans and laboratory animals aims to elucidate the environmental and genetic components that contribute to autoimmunity (Figure 1).

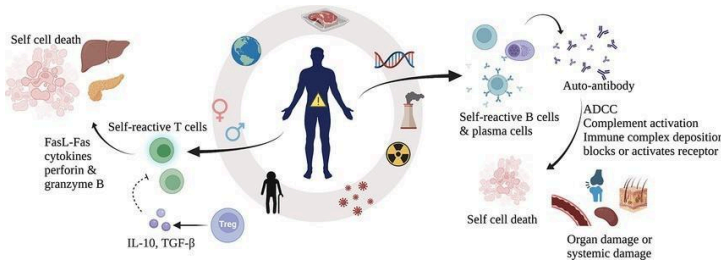

Figure 1. Mechanisms of Autoimmune Diseases [2]

Certain genetic variants are associated with a predisposition to autoimmune diseases. Specific genes can influence the regulatory mechanisms of the immune system, contributing to the initiation of autoimmune responses. While a family history of autoimmune disorders may increase an individual's risk, environmental triggers are also essential, and genetic factors alone are insufficient [2].

Environmental factors play a crucial role in the development of autoimmune disorders. In individuals with a genetic predisposition, environmental triggers such as bacterial and viral infections, chronic stress, dietary factors, and exposure to chemicals can lead to abnormalities in the immune system. Consequently, the immune system may react against the body's own cells due to this imbalance [3].

An exaggerated response of the immune system serves as a fundamental mechanism underlying autoimmune disorders. Normally, the immune system reacts to threatening microorganisms. However, in autoimmune conditions, the body's own tissues become the target of this response. This scenario varies across various autoimmune disorders; for example, pancreatic beta cells are targeted in type 1 diabetes, while joint tissues are affected in rheumatoid arthritis [4].

Impairment of immunological tolerance represents another mechanism involved in autoimmune disorders. Typically, the immune system refrains from mounting aggressive responses against its own cells due to recognition. However, disruption of this

tolerance mechanism can lead to the immune system becoming aggressive towards its own tissues [4].

ARTHRITIS

Arthritis is a broad term encompassing inflammation of the joints, with numerous distinct types, each potentially arising from different mechanisms.

Histologic Mechanism of Arthritis

Inflammatory Response: Arthritis involves inflammation of one or more joint tissues, typically beginning in the joint membrane (synovium), which produces the fluid inside the joint and its surrounding membrane. This inflammation initially arises as an immunological reaction [5].

Changes to the Synovial Membrane: Arthritis is characterized by hyperplasia, or excessive cell proliferation, and increased vascularity within the synovial membrane [6]. This can lead to the accumulation of substances and cells that promote inflammation within the joint [7].

Changes in Joint Fluid: Progression of the disease often leads to an increase in the joint's fluid content, potentially resulting in elevated joint pressure and accompanying edema [8]. Additionally, the quantity of inflammatory cells in the joint fluid may also increase over time.

Physiologic Mechanism of Arthritis

Arthritis commonly stems from an abnormal immune system reaction, wherein the body mistakenly attacks its own tissues, leading to inflammation in the joints [9]. Certain forms of arthritis are associated with a genetic predisposition, with specific hereditary factors increasing the likelihood of developing the condition [10]. Infections, particularly, play a significant role as environmental triggers in the onset of arthritis. Some types of arthritis, notably rheumatoid arthritis, are classified as autoimmune diseases triggered by external factors [11]. Age and gender are also influential factors in certain types of arthritis. For example, rheumatoid arthritis is more prevalent in women, while osteoarthritis tends to develop with advancing age [10,11].

Treatment of Arthritis: INVOSSA

The first cell-based gene therapy product developed to address symptomatic osteoarthritis is called Invossa (also known as Tonogenchoncel-L or TissueGene-C) (Figure 2). This product comprises a blend of untransformed and retrovirally transformed allogeneic chondrocytes, with a ratio of 3:1, overexpressing transforming growth factor β1 (TGF-β1) [12].

The TGF β signaling pathway is widely acknowledged for its crucial physiological role in regulating growth plate control and the formation and maintenance of cartilage.

For patients suffering from osteoarthritis of the knee joint, Invossa represents a groundbreaking cell-based gene therapy product offering promising potential for cartilage repair. Currently, it is undergoing evaluation in phase III clinical trials [13].

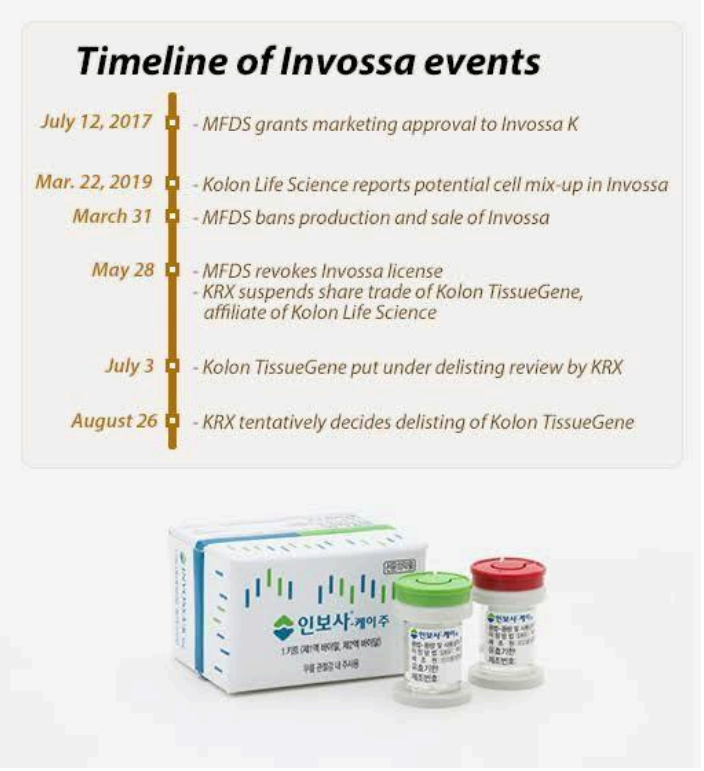

Figure 2. Timeline of Invossa events [13].

RHEUMATOID ARTHRITIS

Mechanism of RA

Rheumatoid arthritis (RA) stands as a chronic inflammatory and autoimmune disease primarily impacting the joints. The hallmark of RA is the immune system's attack on the body's cells, triggering an inflammatory response that primarily targets the lining of the joints [14]. Rheumatoid arthritis exhibits distinct differences from other forms of arthritis, such as osteoarthritis (OA), despite the broad concept of "normal arthritis." In addition to its effects on the joints, rheumatoid arthritis can lead to tissue destruction and widespread inflammation throughout the body. Internal organs like the heart and lungs may also be affected [15]. Individuals with RA commonly experience prolonged joint and muscle stiffness upon waking, often necessitating assistance to initiate regular activities [16].

Treatment Options for RA

Before the advent of gene therapy, rheumatoid arthritis (RA) was managed with various conventional methods, some of which are still utilized today [17]. Typically, the objectives of these therapies include reducing inflammation, alleviating symptoms, and delaying disease progression.

Several traditional techniques for treating RA include:
1. Nonsteroidal Anti-Inflammatory Medications (NSAIDs): NSAIDs are employed to diminish inflammation and address RA symptoms. While they do not halt disease progression, they typically provide symptomatic relief [18].

2. Corticosteroids: Drugs containing corticosteroids reduce pain and inflammation. They are usually prescribed for short durations due to the potential for adverse side effects with prolonged use [19].

3. Disease-Modifying Antirheumatic Drugs (DMARDs): DMARDs are the primary treatments aimed at slowing RA progression. By reducing inflammation, medications like hydroxychloroquine, methotrexate, and sulfasalazine help prevent joint damage [20].

4. Biologic Drugs: Targeting specific proteins or cells involved in inflammation, biologic drugs such as B cell inhibitors, interleukin-6 (IL-6) inhibitors, and tumor necrosis factor (TNF) inhibitors can manage RA symptoms by reducing inflammation [21].

5. Exercise and Physical Therapy: Crucial for maintaining muscle strength, enhancing joint flexibility, and promoting overall health, exercise and physical therapy also assist in managing RA-related joint pain and stiffness.

These therapy options collectively form a comprehensive approach to controlling disease progression and managing RA symptoms [22]. Treatment regimens are often tailored to each patient's unique needs, with adjustments possible throughout the course of care. Furthermore, novel therapeutic approaches like gene therapy are currently under development and could offer additional options for RA treatment.

Gene Therapy for RA

Research on the use of gene therapy for treating rheumatoid arthritis (RA) is ongoing, with anticipated advancements in the field leading to novel techniques in the future [23]. Currently, several specialized gene therapy techniques are being explored for RA treatment. However, these methods are still in the experimental stage and are often used alongside more established conventional treatment options.

Cytokine Management

Gene therapy proves particularly advantageous in treating rheumatoid arthritis (RA) when it targets the modulation of pro-inflammatory cytokines, utilizing genetically engineered cells to regulate the body's inflammatory response [24].

Cytokines, proteins that influence the immune system and facilitate cellular communication, play a pivotal role in RA. Various pro-inflammatory cytokines, primarily produced by immune system cells like T cells and macrophages, can exacerbate inflammation in RA. These include Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6).

Cytokine gene therapy works to reduce inflammation by focusing on the synthesis or interaction of these pro-inflammatory cytokines [25]. The following actions are typically involved in this treatment:

Vector Use

In the transfer of this gene into target cells, viral vectors are commonly utilized. Genes are transported to target cells through vectors, acting as transport vehicles. Gene transfer vectors are primarily categorized into viral and non-viral vectors. Viral vectors often lead to more sustained gene expression [24, 25].

Plasmid DNA

In arthritis research, plasmid DNA stands out as the most frequently employed non-viral vector. Apart from being straightforward and cost-effective to produce, plasmid DNA is often less immunogenic and safer compared to viral vectors. However, local distribution of plasmid DNA within the joint is less likely to be effective. The most successful application of plasmid DNA for gene delivery in arthritis involves incorporating transgenes into skeletal muscle.

In animal models of arthritis, viral vectors overwhelmingly dominate as the preferred vectors for transgene delivery [26].

Adenovirus

Adenoviruses are non-enveloped double-stranded DNA viruses capable of infecting non-dividing cells. While this vector has been extensively utilized in gene therapy research, it possesses several drawbacks that may hinder its success in clinical settings [27].

Retrovirus

Retroviruses exhibit a relatively simple genome and structure, often derived from the Moloney murine leukemia virus. Enclosed within these viruses are two identical copies of the RNA genome. A key feature of the retroviral life cycle is the capacity of the RNA genome to undergo reverse transcription into double-stranded DNA, which can then be randomly integrated into the host genome. Retroviruses are favored vectors for various reasons and are commonly employed in ex vivo studies [28].

Lentivirus

While derived from retroviral vectors, lentivirus vectors possess the ability to infect non-dividing cells [29].

AAV (Adeno-Associated Virus)

AAV, or Adeno-Associated Virus, is a small, non-enveloped, single-stranded DNA virus renowned as one of the most promising gene transfer vectors due to its broad tissue tropism [30]. There are several reasons why AAV is favored as a vector in gene transfer research: it has been shown to facilitate long-term gene expression, possess low immunogenicity, and efficiently transfer transgenes to various organs.

In numerous studies, AAV vectors have proven effective in arthritic models. For instance, in rats with LPS-induced arthritis, a single injection of AAV encoding IL-1ra into the knee joints inhibited both primary and recurring arthritis [30].

Furthermore, it has been demonstrated that administering AAV encoding IL-4 intra- or peri-articularly to CIA mice reduces paw swelling, protects against cartilage degradation, and delays the onset of CIA.

CIA (Collagen-Induced Arthritis)

By immunizing susceptible rodent species such as rats, mice, and non-human primates with type II collagen (CII), the primary protein component of articular cartilage, researchers can induce collagen-induced arthritis (CIA), an experimental autoimmune disease [31]. Following vaccination, these animals develop an autoimmune polyarthritis that closely resembles rheumatoid arthritis in numerous clinical and histological aspects.

A hallmark of the immune response to CII is the production of high antibody titers specific for both the immunogen (heterologous CII) and the autoantigen (mouse CII). In rodents, susceptibility to CIA is linked with class II molecules of the major histocompatibility complex (MHC). Histologically, the onset of arthritis in mice with CIA correlates precisely with thick synovitis.

Due to the pathological similarities between CIA and rheumatoid arthritis, extensive research has been conducted on the CIA model (Figure 3) [32].

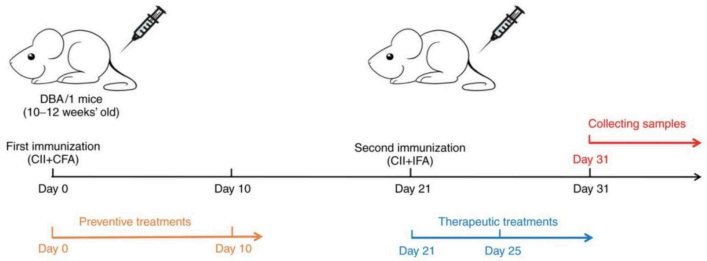

Figure 3. Application of the mouse model of collagen-induced arthritis.[32]

In gene therapy studies involving CIA mice, the following procedures are typically followed:

Model Creation: Collagen is injected into CIA mice to induce a disease resembling rheumatoid arthritis. These mice are specifically engineered to manifest symptoms characteristic of rheumatoid arthritis, including inflammation and joint degeneration.

Gene Therapy Application: CIA mice are subjected to gene therapy techniques. Often, methods involving plasmid DNA, viral vectors, or other gene delivery mechanisms are employed. These techniques aim to manipulate specific genes by silencing, altering, or adding them [33].

Impact Evaluation: Following therapy, the joints of the mice are thoroughly examined, and the impact on rheumatoid arthritis symptoms is assessed. This evaluation encompasses factors such as the mice's overall health, the extent of inflammation, joint damage, and histological investigation.

Molecular Analysis: After gene therapy, a molecular analysis is conducted to scrutinize the expression of the targeted gene and its effects on the immune system (see Figure 4) [34].

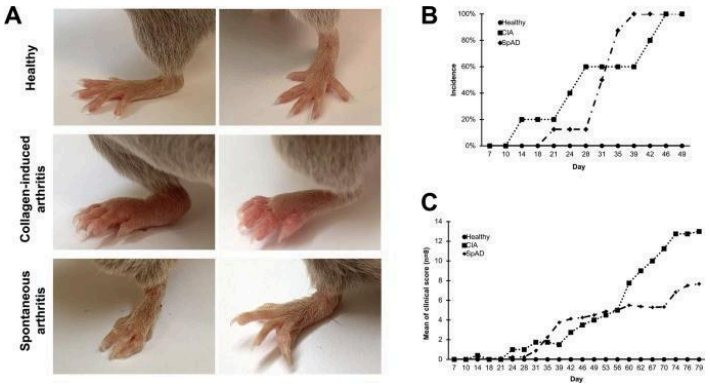

Figure 4. Differences between healthy and collagen-induced arthritis model [34].

Antigen-Induced Arthritis (AIA):

AIA models can be generated in nearly any type of animal. Ovalbumin and bovine serum albumin (BSA) are commonly employed in mice, while methylation antigens and other modified antigens can induce chronic arthritis [35].

Proteoglycan-Induced Arthritis (PGIA):

PGIA is a type of polyarthritis characterized by progressive development over time. It is typified by symmetrical synovitis, pannus formation, marginal erosion, and infiltration of immune cells into the synovium [36].

Collagen Antibody-Induced Arthritis (CAIA):

CAIA can be induced in various susceptible mouse strains, with clinical signs of arthritis typically appearing several days after antibody administration [37]. Arthritis is provoked in mice using a combination of anti-collagen type II (CII) monoclonal antibodies (Table 1).

Model	Immune cell	Cytokine
Collagen-induced arthritis	Monocytes/macrophages, dendritic cells, granulocytes, synoviocytes, T cells, B cells	TNF-α, IL-1β, IL-6, IL-17, IL-23, IL-32, MCP-1, MIP1
Antigen-induced arthritis	Monocytes/macrophages, dendritic cells, granulocytes, synoviocytes, T cells, B cells	TNF-α, IL-1β, IL-6, IL-17, IFN-γ
Proteoglycan-induced arthritis	Monocytes/macrophages, granulocytes, synoviocytes, T cells, B cells	TNF-α, IL-1β, IL-6, IL-12, MCP-1, MIP1α, MIP-2
Collagen antibody-induced arthritis	Monocytes/macrophages, synoviocytes	IL-1β, IL-6, TNF
HuTNF transgenic mice	Monocytes/macrophages, granulocytes	TNF-α, IL-1β,
IL-1 receptor antagonist knockout mice	Monocytes/macrophages, synoviocytes	IL-1, IL-17

TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; IFN, interferon; HuTNF, human tumor necrosis factor.

Table 1. Immune cells and cytokines of model organisms [37].

Tumor Necrosis Factor Alpha (TNF-α)

Tumor Necrosis Factor Alpha (TNF-α) serves as a cytokine implicated in the inflammation observed in rheumatoid arthritis when overproduced. Anti-TNF-Alpha gene therapy aims to address this cytokine's overexpression. In this therapy, genetically engineered cells are utilized to suppress TNF-α [38].

Biological medicines designed to reduce TNF-α target this cytokine directly. This class of biologics includes certolizumab pegol, adalimumab, golimumab, etanercept, and infliximab. Typically, these medications are administered via intravenous infusion or injection [38].

IL-1 Receptor Inhibitor Gene Therapy

Interleukin-1 (IL-1) serves as another significant cytokine involved in the inflammatory process. IL-1 receptor antagonists function by inhibiting the actions of this cytokine, thereby reducing inflammation. Through gene therapy, the body can produce the IL-1 receptor antagonist [39].

In autoimmune inflammatory conditions such as rheumatoid arthritis (RA), interleukin-1β (IL-1β) is a cytokine implicated in the pathogenesis. Consequently, blocking IL-1β represents a crucial approach to RA treatment. Currently, suppression of IL-1β is predominantly achieved through biological therapy rather than gene therapy. Biological medicines targeting IL-1β modify inflammatory processes to alleviate symptoms [39].

Biological medicines targeting the suppression of IL-1β include:

1. Anakinra:
Anakinra blocks the IL-1 receptor, thereby reducing inflammation by preventing IL-1β from attaching to cells and mitigating its effects. It is commonly used to treat inflammatory disorders and autoimmune diseases like rheumatoid arthritis (RA) [40].

2. Canakinumab:
Canakinumab is another biologic drug that inhibits IL-1β. By binding to IL-1β, this antibody regulates the inflammatory response and prevents its consequences. Canakinumab is primarily indicated for managing inflammatory conditions such as arthritis and recurrent fever syndromes [41].

3. Rilonacept:
Rilonacept functions by inhibiting the activity of a family of cytokines called Interleukin-1 (IL-1), which are key players in inflammatory processes [41,42].

Inhibitors of IL-1β interact with this cytokine to reduce inflammatory processes initiated by the immune system. IL-1β serves as a signaling molecule that triggers inflammation. By obstructing or altering the signaling between IL-1 receptors and IL-1β, IL-1β inhibitors aim to alleviate inflammation, particularly in the management of inflammatory autoimmune disorders.

Furthermore, inhibitors of IL-6 and IL-1, such as anakinra and tocilizumab, respectively, may also affect other inflammatory cytokines besides rheumatoid arthritis. Anakinra acts as an antagonist of the IL-1 receptor, while tocilizumab inhibits the IL-6 receptor. These medications alleviate RA symptoms by modulating inflammatory processes.

Indeed, among the biologic medications utilized to suppress IL-6 are Tocilizumab and Sarilumab.

CAR-T Cell Therapy

CAR-T cells, specifically engineered to modulate the immune system in rheumatoid arthritis, represent a potential application of gene therapy. These T cells have been genetically modified to target specific antigens [43].

While immune modulation, anti-inflammatory medications, and other conventional methods currently serve as standard treatments for rheumatoid arthritis, gene therapy holds promise for providing more targeted and efficient interventions addressing the underlying cause of the disease in the future.

Immune Deviation

Research into methods for controlling the immune system through gene therapy is ongoing. This involves exploring genetic modifications to modulate immune responses and regulate autoimmune reactions [44].

Encouraging Apoptosis

Research is actively underway on gene therapy techniques aimed at promoting programmed cell death or apoptosis. This approach targets autoimmune or excessively inflammatory cells, thereby regulating the disease process [45].

Anti-Angiogenesis

In rheumatoid arthritis (RA), research is focusing on gene therapy strategies aimed at preventing angiogenesis, which is the growth of new blood vessels. The objective is to inhibit the overgrowth of blood vessels within the joint, thereby reducing inflammation [46].

Targeting Matrix Degradation Enzymes

Research is underway to explore gene therapy strategies aimed at targeting the enzymes responsible for the deterioration of joint tissues. The goal is to mitigate the degradation of articular cartilage, which is particularly common in rheumatoid arthritis (RA) [47].

Targeting NFκB

The regulation of inflammation is significantly influenced by nuclear factor kappa B, or NFκB. Consequently, gene therapy techniques targeting NFκB are being developed [48].

Pre-clinical testing is currently underway on these experimental gene therapy approaches. While gene therapy holds promise as a potential treatment option for rheumatoid arthritis (RA) and other autoimmune diseases in the future, it's important to note that this field is still under active research. The transition to therapeutic application is an ongoing process, and further studies are needed to fully understand its efficacy and safety.

Limitations of Gene Therapy

Novel treatment methods such as gene therapy for arthritis face several challenges that may limit their widespread adoption or restrict their use to specific circumstances. Safety concerns are paramount, as genetic therapies may yield unintended side effects, necessitating thorough safety assessments before implementation. Moreover, concerns persist regarding the efficacy of gene therapy, with variability observed in patient responses, warranting further evaluation of its reliability in achieving desired outcomes. Affordability poses another barrier, with gene therapy occasionally proving costly, hindering accessibility within conventional healthcare systems. Technical complexities also arise, requiring solutions to efficiently transfer genetic material into cells, target tissues, and regulate it precisely. Immunological reactions may further compromise treatment efficacy or safety. Currently, many gene therapy applications remain in the experimental stage, necessitating additional research and clinical studies before therapeutic implementation. Personalized treatment strategies may also present challenges in management and execution. Additionally, compared to established therapies, gene therapy must demonstrate superior benefits to gain wider acceptance among arthritis patients. While these obstacles may be overcome with continued research and development, ensuring the successful and safe application of cutting-edge therapies like gene therapy for a broader patient population will require ongoing efforts [49, 50].

Advantages of Gene Therapy

Rheumatoid arthritis (RA) gene therapy holds potential benefits, including targeted interventions in specific cells or molecules, potentially reducing side effects and enhancing treatment efficiency [51]. By controlling the autoimmune response inherent in RA, gene therapy can address the underlying cause of the illness and manage the inflammatory process [52]. Certain gene therapy techniques may offer long-term effects, necessitating fewer follow-up sessions, while also modulating an overactive immune system to decrease inflammation [52]. For RA patients resistant to or intolerant of conventional treatments, gene therapy provides an alternative avenue for treatment [53]. Moreover, personalized care can be achieved by tailoring treatment plans to individual genetic profiles, considering each patient's unique biological traits [53]. However, gene therapy techniques are still in early development stages, necessitating further research and advancement in clinical settings to determine their effectiveness, safety, and long-term impacts. Consequently, gene therapy is currently employed alongside conventional therapeutic methods in the experimental stage of RA treatment [54].

Future of Gene Therapy

Although numerous obstacles remain, gene therapy holds promise as a highly effective treatment for arthritis in the future, with progress driven by completed clinical trials, safety/functionality investigations, and advancements in gene therapy technology [55]. Future expectations for arthritis gene therapy include enhanced targeting precision to provide more targeted and effective interventions with fewer side effects, alongside continued efforts to better control transgenic expression to maximize therapeutic outcomes and minimize side effects [56]. Ensuring sustained transgene expression over time is essential for enhancing treatment sustainability, while research on the effectiveness and safety of vectors, particularly adeno-associated virus (AAV), can further support gene therapy in clinical settings [56]. Ongoing efforts to enhance the safety and efficacy of AAV vectors are crucial steps toward advancing gene therapy techniques. Additionally, the development of alternative approaches such as siRNA technology, which targets specific genes

akin to gene therapy, could prove instrumental in augmenting treatment options [56]. Moreover, gene therapy holds promise for modulating the immune system to manage autoimmune reactions and inflammation in future treatments.

CONCLUSION

In conclusion, arthritis remains without a known cure, necessitating management through palliative care and disease course moderation. However, the field of arthritis management is undergoing a profound transformation with the development of gene treatments. This thesis delves into the latest advancements in gene therapy, particularly focusing on rheumatoid arthritis (RA). Enhanced gene transfer methods, facilitated by sophisticated vectors like adeno-associated virus, enable precise and sustained gene expression within targeted tissues.

As gene therapy gains traction for RA treatment, comprehensive exploration of various gene transfer methods, vectors, candidate genes, and safety considerations becomes imperative. The pursuit of extended medication half-lives and controlled gene expression presents a promising avenue to address current therapeutic limitations. The concept of personalized, gene-based therapies holds the potential to significantly reshape arthritis treatment by targeting underlying disease mechanisms and alleviating symptoms.

This review underscores the rapidly evolving landscape of gene treatments for arthritis, marking a pivotal moment in medical history where genetic interventions may redefine patient outcomes and treatment effectiveness. Beyond its scientific implications, the development of gene therapy offers hope to countless individuals grappling with the severe effects of autoimmune diseases like arthritis.

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Review of Gene Editing Approaches for Duchenne Muscular Dystrophy

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Abstract: Muscular dystrophy is a disease characterized by severe muscle weakness due to the destruction and repair of skeletal muscle cells. It can manifest from birth, potentially leading to early death, or later in adulthood. Duchenne Muscular Dystrophy (DMD) and Becker muscular dystrophy are among the types of muscular dystrophy. DMD is the most common, accounting for 85% of dystrophinopathies, but it's also the most severe, affecting approximately 1 in 3500 people. It primarily affects children under 5 years old. The disease stems from mutations in the dystrophin gene, leading to the functional and structural degradation of the 427 kDA cytoskeletal protein encoded by dystrophin. Consequently, most DMD patients lack dystrophin protein due to these mutations. This study delves into gene therapy methods for treating DMD.

Key words: Muscular Dystrophy, Duchenne Muscular Dystrophy, Gene Therapy, Zinc-Finger Nuclease, CRISPR

1. INTRODUCTION

With the developments in the field of genetics over the last century and the creation of the gene map, one of the most significant discoveries in human history, new pathways to incredible scientific discoveries have been opened. Particularly, revolutionary advances in informatics have greatly propelled scientific studies in genetics, providing momentum for further exploration.

Today, we understand that nucleic acids form the basis of hereditary factors, enabling the transmission of hereditary traits from one generation to the next. Given that heredity underlies many diseases, genetics has become directly intertwined with healthcare. Genetics, at its core, aims to safeguard human health by gradually reducing uncertainties in medicine and treating genetically inherited diseases. With the identification of mutations responsible for hereditary diseases, various diagnostic methods have been developed to aid in disease diagnosis.

Muscular dystrophies, categorized under neuromuscular diseases within neurology, constitute a group of inherited disorders characterized by abnormal muscle weakness stemming from skeletal muscle tissue involvement. These diseases are classified based on the affected muscle group, mode of inheritance, and age of onset. There are approximately forty-five types of muscular dystrophy, distinguished by autosomal dominant, autosomal recessive, and X-linked inheritance patterns [1,2].

Duchenne and Becker Muscular Dystrophy (DMD/BMD) stand as the most common X-linked recessive inherited muscular dystrophies. These conditions arise due to mutations in genes encoding the 427 kDa dystrophin protein known as dystrophin [1]. Deletions, duplications, or point mutations within the dystrophin gene, located in the Xp21.2 region, disrupt the structure of the dystrophin protein, which plays a crucial role in connecting the extracellular matrix and cytoskeleton in muscle tissue, rendering it nonfunctional [2]. Duchenne muscular dystrophy typically manifests in childhood, characterized by the absence of dystrophin protein in skeletal tissue. Conversely, patients with Becker muscular dystrophy exhibit low levels of altered dystrophin protein, presenting as a later-onset and milder form of Duchenne muscular dystrophy [3].

In today's health landscape, the pervasive influence of technology has spurred accelerated progress, offering hope to patients and their families affected by Duchenne and Becker Muscular Dystrophy. However, a definitive treatment method to eradicate the disease remains elusive. Consequently, the significance of preventive treatment strategies becomes paramount. Identifying carrier women

within affected families is crucial due to the hereditary nature of the disease and the absence of effective treatment options.

Numerous studies have delved into Duchenne and Becker Muscular Dystrophy, leading to the determination of gene regions, identification of mutations, and development of molecular diagnostic tests. The Polymerase Chain Reaction (PCR) method stands as the most common approach for mutation detection. However, PCR analyses typically focus on only the 18-22 exons, which are presumed to be the most frequently observed, thereby limiting the detection capability for duplication and point mutations. Furthermore, PCR is insufficient for detecting carriers in prenatal early diagnosis.

Fortunately, a new method called Multiple Ligation Dependent Probe Amplification (MLPA) offers a promising alternative. MLPA enables the simultaneous amplification of nearly 45 target sequences, allowing for the detection of gene deletions and duplications. In the dystrophin gene, MLPA enables the examination of all 79 exons, facilitating the detection of deletions, gene duplications, and carrier determination in women [4,5,6].

2. MUSCULAR DYSTROPHY AND DUCHENNE MUSCULAR DYSTROPHY

2.1. Muscular Dystrophies

Muscular dystrophies represent a cluster of inherited disorders characterized by progressive muscle weakness and deterioration, stemming from the cyclic destruction and repair of skeletal muscle cells. While some types manifest symptoms from birth and progress rapidly, often leading to early mortality, others may have a slower onset and remain asymptomatic until late adulthood [6]. Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are prominent examples within this category.

Distinguishing muscular dystrophies (MD) from other neuromuscular diseases lies in the presence of genetically inherited primary myopathy, marked by muscle fiber degeneration and eventual demise [6]. Classification of these conditions involves considerations such as age, rate of disease progression, mode of inheritance, affected muscle group, genetic etiology, and implicated gene [2].

Muscular dystrophies are categorized into three main groups based on their hereditary characteristics: autosomal dominant (AD), autosomal recessive (AR), and X-linked recessive (XR). However, given that some diseases may exhibit more than one mode of inheritance, they can be assessed within both groups based on their clinical manifestations [7].

2.2. Dystrophinopathies

Dystrophinopathies arise from mutations in the dystrophin gene and follow an X-linked recessive inheritance pattern. Located in band 21 of the short arm of the X chromosome, the dystrophin gene is notable for its extensive size, comprising 79 exons—the largest known gene in humans. Mutations in this gene, including deletion, duplication, and point mutations, result in either absent or severely deficient production of dystrophin, leading to dystrophinopathies [8].

Primarily affecting males due to its recessive inheritance on the X chromosome, dystrophinopathies can also manifest in carrier females. Although rare, women may experience muscle weakness associated with the disease. This occurrence can be attributed to several factors. Firstly, it may result from the inactivation of the normal X chromosome in females carrying the mutated dystrophin gene [3,9]. Additionally, muscle weakness in women can arise from translocation of the X chromosome carrying the mutated dystrophin gene with an autosomal chromosome. Furthermore, Duchenne muscular dystrophy may occur in women with Turner syndrome, characterized by the presence of a single X chromosome [10,11].

2.2.1. Historical perspective

Although muscular dystrophies have been recognized for centuries, the formal description of these conditions began with Meryon in 1852. In 1891, Erb coined the term 'muscular dystrophy' after observing a group of patients exhibiting muscle tissue damage. The specific characterization of Duchenne muscular dystrophy (DMD) was established in 1868 by Guillaume Benjamin Duchenne, who conducted muscle biopsy examinations on affected individuals. Subsequently, in 1955, Becker and Kiener reported a variant of muscular dystrophy, later known as Becker muscular dystrophy (BMD), which presented with similar yet milder symptoms compared to DMD [12,13].

The advent of genetic research in the 20th century brought significant breakthroughs in understanding the molecular basis of muscular dystrophies. In 1983, Kingston et al. demonstrated through linkage studies that the gene responsible for DMD and BMD is located on the short arm (Xp21) of the X chromosome. Subsequently, Monaco et al. achieved positional cloning of the Dystrophin gene in 1986, followed by the identification of the dystrophin protein, the product of the Dystrophin gene, by Hoffman et al. in 1987. These discoveries enabled the elucidation of the dystrophin protein's structure, paving the way for carrier detection and prenatal diagnosis [14,15].

Despite the absence of a definitive treatment for DMD, the cloning of the dystrophin gene spurred research into gene therapy, which continues to be a focus of investigation to this day.

2.2.2. Consequences of Dystrophy's Function and Abnormal Structure

The dystrophin gene is situated in the p21 region of the X chromosome, spanning a length of 2.5 Mb and comprising 79 exons along with 7 tissue-specific promoters [16]. Transcription of the dystrophin gene yields a 14 kb mRNA, predominantly synthesized in skeletal muscle, heart muscle, and brain tissues. This mRNA encodes the 427 kDa dystrophin protein specifically in skeletal muscle tissue [17].

Dystrophin serves as a crucial component on the inner surface of the sarcolemma, the membrane of muscle cells. Acting as a binding agent between the cytoskeleton and the extracellular space, it maintains the structural integrity of the sarcolemma. Dystrophin is present not only in skeletal and cardiac muscle but also in vascular smooth muscle and brain tissues. It forms a complex known as the dystrophin-glycoprotein complex with glycoproteins, offering structural support during muscle contraction.

Various isoforms of dystrophin exist, including m-dystrophin found in muscle and brain, Purkinje isoforms, the short dystrophin product Dp71, utrophin, and dystrophin-related proteins. Additionally, dystrophin contributes to the regulation of intracellular calcium concentration [18].

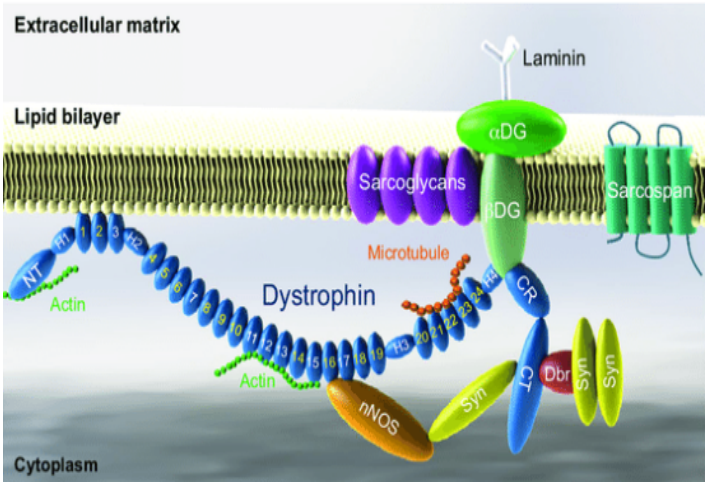

Figure 1. Functional structure of the dystrophin protein in the cell [19]

The dystrophin protein is structured into four distinct regions, each serving specific functional roles [17]. These regions include:

1. N-terminal actin-binding region: This region is responsible for binding to actin filaments and facilitating the anchoring of dystrophin to the cytoskeleton. It contains approximately 232-240 amino acids and exhibits similarity to alpha actin.
2. Rod region: Also known as the central domain, this region comprises 25 double helix repeats arranged in a spectrin-like structure. It plays a crucial role in providing structural stability to the dystrophin protein.
3. Cysteine-rich region: This domain consists of approximately 280 amino acids and contains multiple cysteine residues. These cysteine residues likely participate in the formation of disulfide bonds, contributing to the overall structural integrity of dystrophin.
4. C-terminal region: Positioned at the end of the dystrophin protein, the C-terminal domain contains approximately 420 amino acids. It serves various functional roles, although its precise functions are not yet fully elucidated [18].

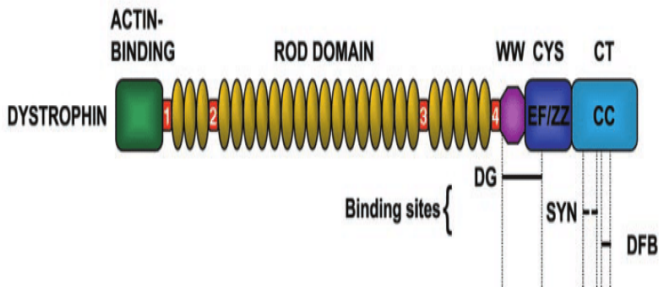

Figure 2. Schematic structure of the dystrophin protein [20]

A deletion mutation in the dystrophin gene is prevalent, affecting around 65% of patients with Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) [17]. In DMD patients, these deletions disrupt the open reading frame of the dystrophin gene, leading to premature termination of protein synthesis. Approximately 92% of mutations in DMD are deletions, resulting in the inability to produce functional dystrophin protein. Conversely, in BMD patients, the open reading frame remains intact despite mutations, allowing for the synthesis of a semi-functional dystrophin protein. While some amino acids may be miscoded, the amino and carboxy ends of the protein remain normal. This mutation pattern gives rise to Becker-type dystrophy, characterized by the production of dystrophin with impaired function and structure [18].

Partial gene duplication mutations are detected in 5-10% of DMD and BMD patients, with out-of-frame duplications being more common in DMD and in-frame mutations more common in BMD. Additionally, point mutations are observed in approximately one-third of all DMD and BMD cases [18].

Single nucleotide changes can result in various types of mutations, including nonsense mutations (34%), frameshift mutations (33%), changes in splicing sites (29%), and missense mutations. These mutations disrupt DMD gene expression and lead to the formation of faulty dystrophin protein [21,22].

2.2.3. Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) stands as the most prevalent among muscular dystrophies, constituting 85% of dystrophinopathies and representing the most severe form of the condition. Its incidence is approximately 1 in 3500 live-born male infants [18]. The vast majority of cases, over 90%, manifest in early childhood before the age of five.

DMD is primarily caused by mutations in the dystrophin gene, leading to structural and functional abnormalities in the 427 kDa cytoskeletal protein known as dystrophin, encoded by the dystrophin gene. Due to these mutations, a significant proportion of DMD patients lack dystrophin protein entirely [3].

2.2.3.1. Frequency

The incidence of Duchenne muscular dystrophy (DMD) is approximately 1 in 3500 newborn boys, while the carrier rate among women is estimated to be 1 in 2500. Although the incidence of DMD may vary across different populations, effective prenatal diagnosis has contributed to a decrease in the prevalence of the disease.

The frequency of Becker muscular dystrophy (BMD) is not precisely known; however, reports suggest it may range from 1 in 18,000 to 1 in 30,000 individuals [2,16,17].

2.2.3.2. Heredity

In Duchenne muscular dystrophy (DMD), 35% of cases result from new mutations, while 65% occur due to the transmission of the mutant gene from the mother to the affected individual [3]. As DMD is fatal in males, the mutant gene cannot be passed on to the next generation by affected males, resulting in a loss of one-third of mutant genes with each generation. However, since the incidence of DMD remains consistent across populations, it is presumed that new mutations replace lost mutant genes in society. This phenomenon is known as the Haldane Rule [21].

On the other hand, in Becker muscular dystrophy (BMD), the clinical course tends to be milder in affected boys, allowing them to maintain productivity and pass the diseased gene to their daughters. Consequently, the vast majority (90%) of BMD cases arise from the transmission of the diseased gene from a carrier mother to a male child, while approximately 10% result from new mutations [23].

2.2.3.3. Clinical Findings

Affected boys with Duchenne muscular dystrophy (DMD) typically do not exhibit symptoms at birth or in early infancy. However, difficulty in maintaining an upright head position may be an early sign of muscle weakness. While walking may be delayed, most

children can achieve this milestone around 12 months of age. To compensate for weakness in the gluteal muscles, affected children may adopt a lordotic posture while standing [24].

DMD can be diagnosed in approximately 25% of cases before the age of two, 50% between the ages of 2 and 4, 75% between the ages of 4 and 7, and almost all cases between the ages of 7 and 9 [23,25]. Children aged 2 to 5 may experience falls while walking, have difficulty climbing stairs, and exhibit a characteristic duck-like gait. Additionally, hypertrophy of the calf muscles may be observed. Weakness in the proximal arm and leg muscles is evident, often accompanied by the Gowers sign, where children use their hands to "climb up" their own bodies when rising from the ground [26].
As patients approach the age of 12, increased weakness in the lower extremity muscles and hip joints often leads to wheelchair dependence. Over time, reflexes in the arms diminish, and assistance may be required for tasks such as holding objects and eating. By the age of 20, most patients succumb to respiratory complications and cardiac disease. Approximately 75% of cases result in death from respiratory failure, 20% from heart failure, and the remainder from complications such as pneumonia, pulmonary embolism, and sudden death [27].

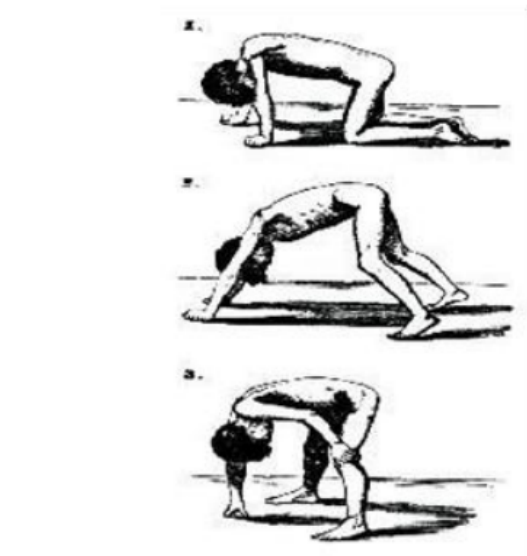

Figure 3. Gower's symptom [27]

While intellectual abilities are generally impacted in all individuals with Duchenne muscular dystrophy (DMD), approximately 20-30% of them may have an intelligence quotient (IQ) below 70. Learning difficulties are the most prevalent cognitive challenge observed. However, mental retardation is less frequent in Becker muscular dystrophy (BMD) patients compared to DMD patients [28].
BMD patients typically present with a clinical presentation similar to that of DMD patients, but the onset of symptoms tends to be later and the progression of the disease slower.

2.2.3.4. Diagnosis
Having a family history is an important diagnostic clue. It is possible to detect possible hereditary transmissions by drawing the pedigree and searching for relatives. On physical examination, pronounced muscle weakness in the proximal muscles, difficulty in climbing stairs, and gait abnormalities are the most prominent clinical signs in boys. Serum CK level 10-20 times higher than normal and muscle stiffness on EMG (Electromyography) suggest DMD/BMD. The detection of mutations by muscle biopsy sample studies and DNA analysis confirms the diagnosis of DMD/BMD.
Methods used to diagnose;
a) Creatine Kinase (CK)
It is at least 10-20 times higher in DMD. High CK values from birth should be a warning for the disease. However, creatine kinase elevation is nonspecific and its normality is incompatible with the diagnosis. Since the muscle cells destroyed in the advanced stages of the disease will be less, CK values may be found to be lower [2].
b) Electromyography (EMG)
It should be done to reveal the myopathic nature of the muscle and to exclude other neurogenic causes. While motor and sensory conduction rates are normal, myopathic changes are seen on EMG in DMD cases [2,8].
c) Muscle Biopsy
Dystrophin determination in muscle biopsy was first performed by Hoffman et al. Immunohistochemical stains are used to demonstrate the absence of dystrophin protein in a muscle biopsy sample to confirm the diagnosis of DMD. In cases where gene deletion cannot be demonstrated, muscle immunohistochemistry is valuable in determining the presence or absence of dystrophin. Even if the deletion is determined, immunohistochemical examination can be performed to determine the course of the disease [5,28].

DNA analysis plays a pivotal role in diagnosing Duchenne and Becker muscular dystrophy (DMD/BMD) and determining the genetic basis of the condition. Two main techniques are commonly utilized: Restriction Enzyme Fragment Length Polymorphism (RFLP) analysis and Polymerase Chain Reaction (PCR) combined with Multiplex Ligation-Dependent Probe Amplification (MLPA).

1. RFLP and STR Analysis:

- RFLP analysis relies on detecting differences in DNA base sequences, known as polymorphisms, at cutting sites of restriction enzymes. This method is utilized to distinguish between two identical chromosomes based on DNA sequence differences, aiding in tracing the X chromosome inherited from the patient's mother within the family [29]. However, it has limitations in application difficulty and providing informative information, thus being replaced by Short Tandem Repeats (STR) analysis.

- STR analysis involves examining short tandem repeat markers in DNA, providing better tracking of inherited genes and enabling statistically significant linkage analyses [29,30].

2. PCR and MLPA:

- PCR combined with MLPA is widely used due to its efficiency in detecting deletions in the dystrophin gene, which account for 60-65% of DMD/BMD mutations. Multiplex PCR amplifies specific regions of the dystrophin gene, allowing for the detection of deletions. Multiplex I and II target different exons associated with common deletion regions, enabling comprehensive analysis [31,32].

- MLPA is a newer method allowing for the evaluation of up to 45 specific sequences simultaneously. It involves amplifying DNA fragments, separating them using sequencing devices, and comparing the results with control samples to detect deletions/insertions. MLPA offers advantages such as requiring minimal DNA input, detecting single nucleotide changes, and being faster, cheaper, and easier to implement compared to other techniques [33,34].

Multiplex Ligation-Dependent Probe Amplification (MLPA) is a highly efficient method used to determine the relative amount of specific DNA sequences by hybridizing a probe mixture with genomic DNA [35,36]. In a single experiment, it allows for the analysis of 96 samples, making it a practical and cost-effective option widely utilized in genetics laboratories.

In MLPA, two complementary probes are initially hybridized side by side with the denatured target nucleotide sequence. Subsequently, the hybridized probes undergo ligation and amplification by multiplex polymerase chain reaction (PCR) using specially designed primers specific to the probes. Unlike standard multiplex PCR, only probes hybridizing to the target sequence are amplified, not the sequences themselves [36]. The amplified products, typically 130-480 base pairs in length, are separated by capillary gel electrophoresis, and the resulting amplification products are analyzed using software systems. The peak area of each amplification product reflects the relative copy number of that target sequence, enabling easy identification of sequences with losses or gains [35,36].

MLPA incorporates internal quality control mechanisms to prevent technical errors from affecting result interpretation. Control fragments, including DNA quantity control fragments (Q-fragments) and DNA denaturation control fragments (D-fragments), are added to all MLPA probe mixes. Q-fragments, consisting of 64, 70, 76, and 82 base pairs, are designed to amplify independently of ligation. During fragment analysis, amplification products of Q-fragments are compared with those of D-fragments and other MLPA probes. A Q-fragment amplification rate 1.5 times higher than others indicates a lack of ligation or low DNA quantity, serving as a quality control measure [35,36].

Additionally, within the MLPA probe mixture, probes of 88, 92, and 96 base lengths serve as D-fragments and undergo amplification through ligation, similar to other probes in the mixture. The amplification rates of these probes, like other MLPA probes, are assessed by plotting expected curves, assuming successful ligation, sufficient DNA quantity, and appropriate denaturation. Specifically, the 92-base-long probe targets a sequence in the 2q14 localization, while the 88-base-long fragment targets the CpG island at the head of the FANCE gene in the 6p21.3 region. Moreover, the 96-base-long fragment targets the CpG island at the head of the TP73 gene in the 1p36 region. These CpG islands contain a high percentage of C/G nucleotides, making their denaturation challenging. Consequently, the peak heights of these fragments are typically 40% or less compared to the 92-base-long D-fragment and other probes, indicating incomplete denaturation of the DNA sample [36].

In the MLPA probe mix, the 92-base-long probe, added as a D-fragment, is present in smaller amounts compared to the others and serves as a hybridization control. If the amplification product specific to this probe is significantly lower than those of the 88-base

and 96-base fragments, it suggests incomplete probe-target hybridization. This may occur due to a short hybridization time, low hybridization temperature, or high DNA quantity.

When interpreting MLPA results, several factors should be considered. Firstly, the gender of the patient is crucial, particularly in experiments involving probes related to the X chromosome. Results from males, who are normally hemizygous for these probes, should be compared with male control samples. Additionally, the proximity of probes with copy number changes is important. Probes located physically adjacent to each other and exhibiting the same type of copy number alteration (deletion or duplication) are more likely to indicate a true positive result. Conversely, changes in non-adjacent probes may suggest false results.

Furthermore, the nature of the disease under investigation plays a significant role. Diseases that commonly result from copy number alterations, such as Duchenne muscular dystrophy, are more likely to yield true positive results. Moreover, if there is a considerable physical distance between probes showing a neighborhood relationship, or if the region contains genomic repeat sequences or long introns between exons, rearrangements leading to copy number changes are more probable.bBeyond genomic DNA, MLPA enables multiplex quantification of CpG methylation patterns in mRNA studies. Presently, the MLPA method is employed in over 350 laboratories for investigating deletions and amplifications of various genes [36].

2.2.4. Becker Muscular Dystrophy

Becker muscular dystrophy (BMD) is characterized by a milder clinical course compared to Duchenne muscular dystrophy (DMD), allowing patients to live longer, sometimes up to 40-50 years [4,21]. Its incidence is approximately one in 30,000 live-born male infants, and it is associated with a deletion in the dystrophin gene [1,9].

In muscle biopsy samples, there are varying patterns of dystrophin expression. Approximately 80% of cases show a decrease in dystrophin molecular weight by 20-90%, while in 15% of cases, dystrophin is of normal size but reduced in quantity. Additionally, in about 5% of cases, dystrophin is abnormally large [1,5]. Symptoms typically manifest later than in DMD, with a duck-like gait (waddling gait) becoming noticeable around 10-15 years of age. Muscle weakness gradually increases, and calf hypertrophy develops around 15-20 years of age.

The diagnosis of BMD involves a combination of family history, elevated creatine kinase (CK) levels, electromyography (EMG), genetic examination, and muscle biopsy, similar to the diagnostic process for DMD.

Unlike DMD, BMD is not typically fatal, and individuals with BMD often have reproductive compatibility reaching 70%. However, individuals with BMD who marry have a 50% chance of passing the mutant gene to all their daughters, increasing the risk of their grandchildren inheriting the disease. Consequently, the majority of BMD cases (85-90%) are hereditary, while 10% are caused by new mutations [29,30].

2.2.5. Carriers in Dystrophinopathies

While dystrophinopathies primarily affect males, it's been demonstrated that female carriers can also exhibit symptoms to varying extents. Reports indicate skeletal muscle involvement in female carriers, with rates of 24% in Duchenne muscular dystrophy (DMD) and 20% in Becker muscular dystrophy (BMD) [37]. Symptoms in carriers may include muscle cramps and weakness, typically asymmetrical and not severe. Additionally, most asymptomatic carriers exhibit various degrees of cardiac involvement [38].

Several mechanisms contribute to the clinical manifestations in carrier women. These include the X-inactivation mechanism, X-autosome translocation, 45,X genotype, and the presence of mutant genes on both X chromosomes or uniparental disomy. While these mechanisms are theoretically possible, practical considerations often limit their evaluation.

2.3. Disease Prevention and Genetic Carriage

Disease prevention strategies for dystrophinopathies focus on early diagnosis, genetic counseling, and prenatal diagnosis. Identifying carriers within families, especially among female relatives on the mother's side of a child with Duchenne muscular dystrophy (DMD), is crucial. Early determination of carrier status in women from DMD-affected families, before they conceive, is essential.

Genetic counseling plays a vital role in providing information and support to families. Prenatal diagnosis aims to offer reassurance to families at high risk of having an affected child, allowing them to make informed decisions about their reproductive options.

Molecular analysis, particularly using methods like MLPA, facilitates the detection of carriers and enables families to pursue alternative reproductive methods if necessary, with the goal of ensuring the birth of a healthy child.

2.4. Treatment

Current treatment for Duchenne muscular dystrophy (DMD) involves a combination of steroid therapy and supportive care, with promising advancements in gene therapy under investigation. Steroid treatment has shown significant benefits in slowing disease progression and improving clinical outcomes, leading to an enhanced quality of life for patients. Additionally, ongoing research in gene therapy holds the promise of providing definitive treatment for DMD.

Gene therapy approaches include delivering intact genes to patients using vectors, exon-skipping methods to bypass faulty gene segments, and gene silencing studies to modulate gene expression. These innovative treatments offer hope for improved outcomes and potential disease modification in individuals with DMD. Efforts are also underway to develop pharmacological interventions aimed at addressing the underlying genetic causes of the disease.

3. GENE THERAPY METHODS USED IN DMD

The field of genetics traces its origins back to Gregor Mendel's groundbreaking hybridization studies with plants in 1865, which laid the foundation for understanding heredity. However, genetics emerged as a distinct scientific discipline in 1906, following the proposals of Walter Sutton and Theodor Boveri, who suggested that chromosomes serve as carriers of Mendelian factors.

Thomas Hunt Morgan's work with Drosophila melanogaster (fruit fly) in 1908 further advanced our understanding of heredity, integrating the chromosomal theory of inheritance with Mendelian genetics. Morgan's research, coupled with Hermann Muller's X-ray-induced mutagenesis experiments, contributed to the concept of a gene as a unit of mutation. Additionally, the discovery of DNA's structure in 1953 by Rosalind Franklin, James Watson, and Francis Crick was a pivotal moment in genetics.

In the late 1960s and early 1970s, the discovery of restriction endonuclease enzymes by Werner Arber, Hamilton O. Smith, and Daniel Nathans revolutionized molecular biology. These enzymes enabled molecular cloning, which facilitated the Human Genome Project initiated in 1990. The completion of this project not only ushered in a new era in medicine but also spurred the development of DNA sequencing technologies.

The advent of synthetic biology, a burgeoning field in recent years, has enabled scientists to design novel biomolecular components. Synthetic biology leverages advancements in artificial gene networks, de novo DNA synthesis, and protein engineering to manipulate cellular behavior. This interdisciplinary approach has become increasingly important with the genomic revolution and the rise of systems biology in the 1990s, opening new avenues for research and innovation in genetics and molecular biology.

Synthetic biology traces its roots back to the pioneering work on the lac operon in E. coli by Francois Jacob and Jacques Monod in 1961, which revealed the existence of circuits regulating a cell's response to its environment. The subsequent development of molecular cloning and the Polymerase Chain Reaction (PCR) technique in the 1970s and 1980s revolutionized genetic manipulation, enabling researchers to engineer gene regulation in microorganisms.

However, before the genomic era, genetic engineering was primarily focused on cloning and recombinant gene expression. It wasn't until the mid-1990s, with the advent of automated DNA sequencing and improved computational tools, that complete sequencing of microbial genomes became feasible. This paved the way for high-throughput techniques to quantify various cellular components, leading to the accumulation of a vast catalog of biological parts.

As synthetic biology expanded, genome editing techniques gained prominence. Studies by Oliver Smithies and others in the 1980s demonstrated how homologous recombination (HR) could be used to precisely alter endogenous genomic sequences using exogenous donor DNA molecules. These groundbreaking findings laid the foundation for gene targeting methodologies in mouse embryonic stem cells, for which Smithies, Mario Capecchi, and Martin Evans were awarded the Nobel Prize in Physiology or Medicine in 2007.

Initially, the efficiency of targeted integration via HR in somatic cell lines was limited. However, Ralph Brinster and colleagues refined the method in 1989, achieving more successful outcomes through direct

pronuclear microinjection into mouse fertilized eggs. Importantly, the introduction of double-strand breaks (DSBs) within the homology region of the donor DNA molecule significantly enhanced HR frequency, further advancing the field of genome editing.

The discovery of truncating endonucleases, such as the 18-base pair recognition site I-SceI in 1985, marked another significant milestone in genome editing [49]. These enzymes, which create double-strand breaks (DSBs) at specific sites in DNA, provided a crucial tool for homologous recombination (HR)-mediated DNA repair and cell remodeling.

The utility of I-SceI was demonstrated in mammalian chromosomes, where it promoted HR and facilitated genome editing. By inserting I-SceI restriction enzyme cut sites into the mouse genome via HR in embryonic stem cells, researchers were able to enhance targeting efficiency. Subsequent efforts involved cloning the I-SceI meganuclease with a vector flanking the inserted region, further optimizing the process.

Since then, various meganucleases derived from I-SceI have been developed, each targeting specific DNA sequences. These enzymes have been instrumental in inducing recombination and demonstrating effective targeting events in human, rat, and mouse embryos [50]. However, despite their utility, more efficient genome editing tools have since emerged.

Among these are zinc-finger nucleases (ZFNs), which are engineered proteins designed to bind specific DNA sequences and induce DSBs, and transcription activator-like effector nucleases (TALENs), which function similarly to ZFNs but use a different DNA-binding domain. These technologies paved the way for the development of CRISPR/Cas systems, which have revolutionized genome editing due to their simplicity, versatility, and efficiency. CRISPR/Cas systems utilize RNA-guided nucleases to precisely target and modify specific genomic loci, offering unprecedented control over gene editing processes.

3.1. Zinc-Finger Nuclease (Zfn)

A significant shift in genome editing methodology occurred in 2009 with the pioneering use of zinc-finger nucleases (ZFNs) to generate the world's first knockout mice for the immunoglobulin M (IgM) and Rab38 genes [51]. This groundbreaking study utilized ZFN technology to precisely disrupt the IgM and Rab38 genes by employing designed zinc finger nucleases.

ZFN technology harnesses the DNA-binding capabilities of zinc finger domains in conjunction with the FokI restriction endonuclease enzyme to target specific genomic regions. FokI is a type of restriction endonuclease that recognizes and cleaves DNA sequences within or near its recognition sites.

The groundwork for ZFN technology was laid in 2001 with the creation of the first chimeric protein, which resulted from the fusion of FokI endonuclease cleavage domains with DNA-binding zinc finger domains in Xenopus embryos. Essentially, the FokI endonuclease functions as a nuclease that cleaves DNA, but its cleavage activity is only activated upon dimerization. This dimerization is facilitated by the binding of zinc finger domains to specific DNA sequences, thus enabling precise targeting of genomic loci for editing purposes. Zinc finger nucleases (ZFNs) exhibit specificity to codons, a critical feature ensuring precise targeting of genomic regions for cleavage by the FokI nuclease [52]. This specificity is crucial for accurately directing the cleavage of the genome at desired locations.

In 2005, ZFN technology was further advanced when researchers corrected a mutation associated with X-linked severe combined immune deficiency (SCID) in the IL2Rgamma gene using homology-directed repair (HR) with an extrachromosomal DNA donor [52]. This milestone demonstrated the potential of ZFNs for correcting genetic mutations through precise genome editing.

The efficiency of the ZFN system was subsequently enhanced by inducing double-strand breaks (DSBs) and promoting FokI dimerization [52]. Building on these advancements, various ZFN designs were developed in 2008 for genome editing in mammalian genomes, paving the way for further applications in gene editing.

ZFN technology rapidly evolved into an efficient tool for mammalian transgenesis, with the first studies on mammalian transgenesis conducted in 2009, resulting in the generation of knockout rats [52]. This was particularly significant as classical gene targeting approaches

were not feasible in rats due to the unavailability of equivalent rat embryonic stem cells.

The widespread adoption of ZFN technology in laboratories enabled successful genome editing in various mammalian species including mice, cattle, and pigs [52]. Notably, ZFNs have had a profound impact in livestock species, where genome alterations were traditionally limited by the absence of species-specific embryonic stem cells capable of homologous recombination (HR). For instance, in 2014, genome-edited cattle with increased resistance to mastitis were generated using ZFN technology, showcasing its potential for agricultural applications [53].

3.2. Clustered Short Palindromic Repeats (CRISPR) And CRISPR-Related Proteins (Cas) in Regular Intervals

The CRISPR/Cas system's roots date back to 1987 when bacteria were observed adding a 32-nucleotide spacer sequence to a 29-nucleotide repeat sequence at the CRISPR locus upon encountering phage DNA [54]. Initially, the function of these repeat sequences, present in 90% of archaea and 40% of bacterial genomes, remained unclear. However, subsequent discoveries revealed that spacer sequences actually originated from phage genomes, leading to the hypothesis that the CRISPR system serves as an adaptive immune defense mechanism against phage attacks in bacteria and archaea.

Further research uncovered various components of the CRISPR system, including CRISPR-associated genes (Cas genes), protospacer adjacent motif (PAM), CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA) [54]. These findings shed light on the molecular mechanisms underlying the CRISPR/Cas system's function in prokaryotes.

In 2013, the CRISPR/Cas system was successfully tested in cultured mammalian cells, marking its entry into the realm of genome editing [54]. Soon after, it was utilized for genome editing in mice, demonstrating its potential for precise and efficient genome modifications. CRISPR technology quickly gained traction due to its simplicity, ease of use, and high efficiency compared to other genome editing techniques such as meganucleases, zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALEN) [54]. It emerged as the preferred choice among researchers for its powerful capabilities and versatility, making it accessible to laboratories worldwide.

The CRISPR system comprises two key components: a guide RNA (gRNA) that identifies the target DNA sequence and a Cas endonuclease enzyme that induces a double-strand break (DSB) at the target site. Originally, prokaryotic systems utilized two separate RNA molecules (crRNA and tracrRNA) to achieve this, but in 2012, researchers combined them into a single synthetic RNA molecule (sgRNA or gRNA), simplifying the process. Once the gRNA recognizes the protospacer adjacent motif (PAM) sequence on the target DNA, the Cas9 nuclease cleaves the DNA, mimicking the cell's natural defense against viral infection. For the widely used Cas9 from Streptococcus pyogenes, the PAM sequence is typically 5′-NGG-3′. The CRISPR/Cas system, often referred to as CRISPR/Cas9, requires three basic elements to function: gRNA, PAM sequence, and the Cas enzyme.

Since its inception, CRISPR technology has been instrumental in generating a wide range of specific genomic modifications in various mammalian species. Initially used for small insertions and deletions (indels) in 2013, CRISPR has since been adapted for large-scale genomic alterations, including deletions, insertions, inversions, and chromosomal rearrangements. In 2015, researchers utilized CRISPR to investigate the role of DNA regulatory elements in non-coding genomic regions, and in 2017, it was demonstrated that CRISPR could achieve the largest known deletions and chromosomal rearrangements (<24.4 Mb) in rats and mice [55].

In recent years, CRISPR technology has been increasingly explored for gene therapy applications. In 2015, CRISPR tools were successfully employed to generate or correct mutations in human induced pluripotent stem cells (iPSCs) [56]. Building on this advancement, in 2016, CRISPR reagents encapsulated in adeno-associated virus (AAV) vectors or non-viral particles were used to partially restore gene function in animal models of human genetic disorders, including Duchenne muscular dystrophy, retinitis pigmentosa, and human hereditary tyrosinemia [56].

Additionally, CRISPR has opened up new possibilities for producing interspecies chimeras. In 2017, experiments were initiated to explore the feasibility of generating human organs within other

species for potential transplantation. These experiments involved using CRISPR to trigger the differentiation of human pluripotent cells, resulting in distinct progeny that contributed to various cell lines and organs during the development of CRISPR-edited pig embryos [57].

The latest breakthrough in genome editing, discovered as "prime-editing" in 2019, represents a significant advancement in CRISPR technology. One of the primary challenges in genome editing techniques has been the occurrence of off-target double-strand breaks (DSBs). Prime editing aims to minimize this issue by enabling nucleotide substitutions without creating DSBs. This technique utilizes Cas9 nickase (H840A)-reverse transcriptase (RT) fusion proteins along with a site-specific pegRNA for editing. Developed in Liu's labs, prime editing has been successfully tested on various variations in human and mouse cells, accomplishing 175 different genome edits. Notably, it has demonstrated high efficiency in creating and correcting mutations associated with diseases like sickle cell anemia and Tay-Sachs disease. Anzalone et al. reported that prime editing could potentially correct 89% of known pathogenic human genetic variants [58].

3.3. DMD Gene Editing Treatment in Animals: Proof of Principle and Obstacles

With the advent of Cas9 transgenic mice, organ-specific changes have become feasible by encoding the relevant guide RNAs (gRNA) targeted by AAVs. This advancement holds promise for addressing hereditary diseases such as DMD using Cas9-mediated genome editing. DMD has emerged as an ideal target for genome editing due to the presence of mutations causing the disease and the potential for functional restitution with minimal impact on disease progression [59].

Studies have shown the feasibility of repairing exon 23 loss-of-function mutations in mdx mouse zygotes using Cas9 paired with gRNA and single-stranded oligodeoxynucleotide. HDR was effective in providing correction in 4 out of 11 mdx mice, while NHEJ was successful in 7 mice. These findings indicate that HDR correction rates as low as 17% could be sufficient to restore dystrophin expression, with up to 47-60% of muscle fibers expressing dystrophin [60].

Large animal models better mimic the clinical scenario and offer insights into dosage, toxicity, and administration methods. In one study utilizing a beagle model of DMD, vector injections containing AAV9-Cas9 and sgRNA-51 induced dystrophin expression in skeletal muscles and the heart. Systematic administration of varying dosages resulted in a significant increase in dystrophin expression, reaching up to 92% in the heart [60].

In a study by Louise R et al. (2007), multiple strategies were explored for gene therapy in muscular dystrophy, emphasizing the need for optimizing vascular delivery pathways and understanding the immunogenicity of AAV serotypes and transgenes. DMD gene therapy was highlighted as a promising approach with the potential to improve muscular dystrophy outcomes [62].

Another study emphasized the urgency of addressing the unmet needs of patients with muscular dystrophy while ensuring safety and efficacy in gene therapy development. The importance of maintaining scientific rigor alongside expediency was underscored, aiming to establish a comprehensive DMD gene therapy program and enable broader clinical studies [62].

Regarding DMD gene therapy methods, Ramos (2015) highlighted the efficacy of AAV vectors in delivering microdystrophin to body muscles. This approach prioritizes the safe and efficient transfer of microdystrophin without eliciting an immune response, positioning AAV as a leading method in DMD gene therapy [63].

4. CONCLUSION

Gene therapy, conceived as early as 1970 by Martine Cline during studies on retroviral RNA, gained significant momentum in 1990 with landmark applications by Michael Blaese and William French Anderson. These pioneers successfully treated severe combined immunodeficiency (SCID) by introducing a functional ADA gene via a retroviral vector, marking a major breakthrough in medical history.

The essence of gene therapy lies in rectifying defective genes by delivering normal counterparts into target cells, thus restoring proper protein production. This approach, aiming to tackle diseases at the nucleotide level, typically involves inserting functional genes into specific genomic loci using vectors. Other strategies, such as zinc

finger and homologous recombination, enable direct mutation correction. Gene therapy primarily targets monogenic disorders like cystic fibrosis, hemophilia, muscular dystrophy, and sickle cell anemia. However, the intricate biology of human gene therapy remains poorly understood, necessitating advancements in genetics, bioinformatics, and molecular biology.

Before gene therapy can be widely adopted in clinical settings, a thorough understanding of genetic disease mechanisms and the development of safe gene transfer techniques are imperative. Successful gene therapy outcomes hinge on ongoing progress in these fields, paving the way for transformative treatments for a myriad of genetic disorders.

The discovery of the CRISPR/Cas9 technique in 2013 stemmed from investigations into the intricate interactions between bacteria and viruses. When viruses infect bacteria, they inject their DNA into the bacterial cell, hijacking its genetic machinery to replicate. In response, bacteria have evolved a defense mechanism to combat viral invasion. At the heart of this defense system is the Cas9 enzyme, which acts as a molecular scissors. It scans the bacterial genome for viral DNA sequences and, upon recognition, cleaves the viral DNA, neutralizing the threat.

Crucially, bacteria store snippets of viral DNA within their own genome as a memory of past infections. These viral DNA fragments are integrated into specific sequences known as CRISPR sequences. When the same virus attacks again, the bacterium can rapidly recognize and mount a defense against it, thanks to the stored memory in its CRISPR sequences. In essence, CRISPR serves as the bacterial immune system, bolstered by the action of the Cas9 enzyme.

The CRISPR/Cas9 technique harnesses this natural bacterial defense mechanism for precise genome editing. By guiding the Cas9 enzyme to specific locations in the genome using synthetic RNA molecules, researchers can remove mutated gene sequences or replace faulty genes with correct versions. This groundbreaking technology holds immense potential for treating genetic disorders and advancing scientific research.

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Advancements in Gene Therapy for Immunodeficiency Diseases

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Abstract: Over the past three decades, gene therapy has made significant strides in treating immunodeficiency diseases. This review article provides an overview of gene therapy's historical development, spanning multiple phase trials and clinical applications. It discusses various delivery vectors, both viral and non-viral, and evaluates their success rates. The article briefly touches on the immune system before delving into primary and secondary immunodeficiency disorders. Specific primary immunodeficiency diseases, such as ADA-SCID, X-SCID, ART-SCID, Wiskott-Aldrich Syndrome, X-CGD, and LAD-1, are explored in terms of their causation, treatment options, with a focus on gene therapy, and the latest advancements in clinical trials. While Strimvelis' success in treating ADA-SCID is highlighted, ongoing research for other PIDs is also discussed, outlining their path to success through various trials. By addressing challenges such as long-term effects, off-target effects, and immune responses to gene therapy treatments, the review provides insight into the complexities of this therapeutic approach. However, it also underscores recent achievements and advancements, illustrating not only the future of gene therapy but also its potential to transform healthcare as a whole.

Key words: Gene therapy, Immunodeficiency diseases, Clinical trials

1. GENE THERAPY

1.1. History of Gene Therapy

For a medication to be classified as gene therapy, it must meet two conditions: firstly, it must be administered to the patient using a recombinant nucleic acid carrying the active substance, and secondly, the therapeutic effect of the medication must originate from this recombinant nucleic acid. This therapeutic effect can be achieved through several mechanisms: a) replacing a disease-causing gene with a healthy copy, b) inactivating a malfunctioning disease-causing gene, or c) introducing a new or modified gene into the body to aid in disease treatment (1).

Gene therapy can be divided into two main categories: somatic cell therapy and germ line therapy. Somatic cell therapy involves introducing the gene therapy into the patient's somatic cells, where it remains localized within the patient's body. On the other hand, germ line therapy entails introducing the gene therapy into the patient's germ line cells, potentially allowing it to be passed on to future generations (2).

Gene therapy remains a subject of numerous safety and ethical concerns worldwide, with human gene therapy trials facing significant hurdles in approval due to the complexities of DNA alteration. Germ line cell gene therapy is universally prohibited, leaving somatic cell gene therapy as the only viable option to avoid genetic changes in future generations (2)(3). However, even somatic cell therapy carries risks of uncontrolled genetic changes that could potentially be transmitted to offspring, particularly raising ethical dilemmas in fetal somatic cell therapy.

To trace the origins of gene therapy, one must delve into history, beginning in 1928 when Frederic Griffith introduced the concept of the transforming principle, ultimately leading to the discovery and extensive research of DNA (2)(4). In 1962, Szybalski's work on hypoxanthine-guanine phosphoribosyl transferase revealed the potential of transferring healthy foreign DNA to address genetic issues, thus opening the door for gene therapies to impact future generations. Additionally, Temin's discovery in 1961 highlighted the bidirectional flow of genetic information between DNA and RNA,

laying the foundation for carrier vectors, which would later become instrumental in gene therapy (2)(5).

Edward Tatum first proposed the use of viruses as vectors for gene therapy, but technological limitations hindered progress until Rogers and Pfudderer successfully executed the first human therapy trial (2). In 1990, Cline attempted the first gene therapy using recombinant DNA, although these treatments were not sanctioned by regulatory boards (2).

The first approved gene therapy treatments occurred on September 14, 1990, for ADA-SCID patients, sparking increased interest and success stories throughout the 1990s. However, a tragic setback occurred in 1999 when Jesse Gelsinger passed away following a viral vector transfer (2). Notably, in 2003, China became the first country to approve a gene therapy product (Gendicine), followed by the European Union's approval of Europe's inaugural gene therapy drug, Glybera, in 2012 (2) (Figure 1).

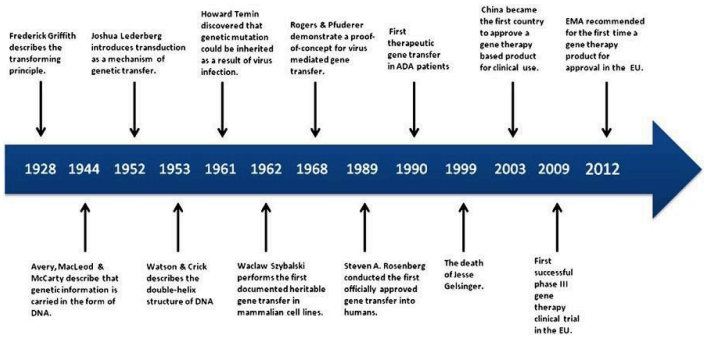

Figure 1: The history of gene therapy until the approval of the first gene therapy in europe (2)

Gene therapy drugs can be categorized based on their mechanism of action into four main groups: naked plasmids/DNA, non-viral vectors carrying RNA-I drugs, viral vectors, and cell-mediated therapies.

Initially, naked plasmids garnered attention from researchers due to their ease of handling and perceived safety, despite offering limited gene expression. Meanwhile, RNA interference (RNA-I) drugs, such as Onpattro developed by Alnylam Pharmaceuticals, have shown promise in gene silencing, making them attractive for therapeutic purposes (6). Viral vectors have gained popularity in gene therapy due to their efficient infection capabilities. Among these, adenoviruses are commonly used as delivery vehicles for gene therapy interventions. The fourth category, cell-mediated therapies, also known as cell therapy, is a relatively newer approach compared to other gene therapy delivery methods. In this method, cells are extracted from the patient, genetically modified, and then reintroduced into the patient's body.

1.2. The Stance of European Countries and Current Gene Therapies

In terms of the legalization of gene therapy drugs, the stance of European countries can be summarized as follows (Figure 2):

The United Kingdom and Germany are the most advanced countries in this regard. All listed countries, except Turkey, rely on the European Medicines Agency (EMA) for research and drug approval. However, Portugal and Spain do not have any nationally approved gene and cell therapy practices. Turkey lacks specific authority or regulations for gene and cell therapy methods but closely follows both the EMA and the FDA (7).

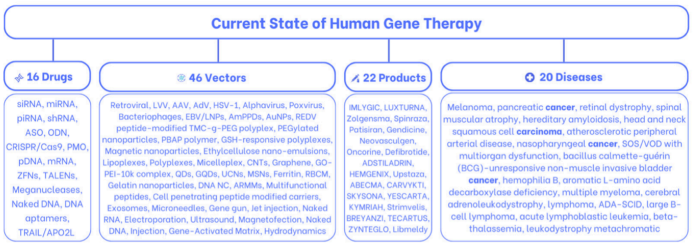

Figure 2: Current approved gene therapy drugs, which disease they cure, what delivery methods they use and which vectors are used (As of 2023)(8).

As of 2019, there were only approximately 20 approved gene therapies worldwide. The first approved gene therapy in America was Macugen, while in Europe it was Glybera. Gendicine, approved in China, was the world's first-ever approved gene therapy. In recent years, the use of gene editing tools such as CRISPR has become prevalent in gene therapy (9).

CRISPR technology has expanded the scope of gene therapy to target various conditions including cancer, neurological disorders, rare genetic diseases, cardiovascular diseases, and some infectious diseases. However, it's worth noting that the field is predominantly focused on cancer treatment (10)(11).

As of 2023, there are over 2,000 gene therapy clinical trials worldwide. It is anticipated that in 2024 alone, the FDA (the Food and Drug Administration) will approve 59 new gene therapy products (12).

1.3. Delivery Vectors

In the early 2000s, it was observed that some patients developed additional diseases such as leukemia or granulomatous disease after receiving gene therapy, due to uncontrolled genome integration (2)(13). To mitigate these risks, targeted integration during vector development became crucial (2).

This rationale led to the creation of targeted carrier vectors, designed to transport treatment precisely to the required location in vivo. Carrier vectors must fulfill several conditions, including being unrecognizable to the immune system, non-allergenic, and non-inflammatory. They should correct deficiencies, restore normal functions damaged by the disease, inhibit unwanted activities, and maintain therapy expression until the end of the patient's life (14-15).

Carrier vectors delivering gene therapy can be categorized into viral and non-viral vectors. While viral vectors are commonly favored, non-viral vectors offer advantages such as general safety, easy administration, low toxicity, and no size limitations for DNA inserts. However, their main drawback is low gene transfer efficiency. Natural polymers, preferred over synthetic ones, can trigger an environmental response. For a delivery method to be accepted, it must fulfill three conditions: immunological dormancy, prevention of genetic material degradation, and continuous expression of therapy at the target site (16).

Viral vector delivery systems can be classified into five major categories: retroviral vectors, adenoviral vectors, adeno-associated vectors (AAV), lentiviral vectors, and herpes simplex virus (HSV) vectors (16).

- Retroviral vectors can transfect both somatic and germ line cells, target dividing cells, and be used in situ.
- Adenoviral vectors can transfect both dividing and non-dividing cells and are suitable for a wider range of tissues.
- Adeno-associated vectors (AAV) share properties with adenoviral vectors but have limited transgene capacity.
- Lentiviral vectors, a category of retroviruses, can naturally integrate with non-dividing cells.
- Herpes simplex virus (HSV) vectors can carry large DNA material and are ideal for delivering therapy to cancerous and tumor cells (16).

2. THE IMMUNE SYSTEM AND IMMUNODEFICIENCY

2.1. The Immune System

The immune system is a complex network of organs, cells, and cytokines that constantly communicate to protect the host from harmful organisms encountered through inhalation, ingestion, or contact (Figure 3). It can be categorized into two main responses: innate and adaptive.

The innate response, provided by cells like macrophages and monocytes, offers immediate defense against invading organisms. On the other hand, the adaptive response, mediated by T lymphocytes and B lymphocytes, generates antigen-specific reactions that take time to develop in response to a threat (17).

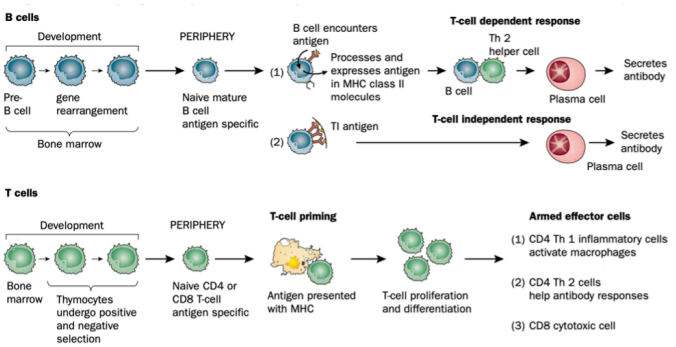

Figure 3: The immune response working principle of B cells and T cells (17).

2.2. Immunodeficiency

When essential elements of the immune system, such as lymphocytes and phagocytes, are lacking or fail to function properly, a condition known as immunodeficiency occurs (18). The origins of immunodeficiency diseases can be traced back to the early 20th century, with conditions like Ataxia Telangiectasia (1926) and Wiskott-Aldrich syndrome (1937) emerging nearly a century ago.

The first recognized case of an immunodeficiency disorder was documented in 1950, when Eduard Glanzmann and Paul Riniker linked Candida albicans infections to abnormally low lymphocyte levels in a patient. Subsequently, in 1970, this condition was officially termed severe combined immunodeficiency (SCID) by the World Health Organization (WHO) (19).

Immunodeficiencies can arise from deficiencies in T cell lymphocytes, B cell lymphocytes, phagocytes, and Immunoglobulin A (IgA). For instance, a halt in B cell development at the pre-B receptor stage leads to reduced mature B cell numbers and subsequent immunoglobulin production deficiency. This deficiency can be caused by mutations in genes such as Igα, BLNK, or Bruton's tyrosine kinase (17).

B cell deficiencies are often associated with X-linked disorders, frequently involving mutations in the tyrosine kinase protein. Immunoglobulin A deficiency is relatively common and can result in severe lung infections. T-cell deficiencies predominantly predispose individuals to fungal and viral infections (20).

Disease	Gene	Ref.
Innate immunity		
Severe congenital neutropenia (subset)	*GFI1*[a]	6
Shwachmann syndrome	*SBDS*[a]	7
Susceptibility to *Streptococcus pneumoniae* (subset)	*MASP2*	8
Susceptibility to pyogenic bacterial infections (subset)	*IRAK4*	9,10
Susceptibility to mycobacteria and viral infections	*STAT1*	11
Adaptive immunity		
Common variable immunodeficiency	*ICOS*	12
HIGM syndrome	*UNG*	13
SCID (subset)	*CD3D*	14
T cell deficiency with IFN-γ unresponsiveness	*STAT5B*	15
T cell deficiency with anhidrotic ectodermal dysplasia	*NFKBIA*[b]	16
Familial hemophagocytic lymphohistiocytosis (subset)	*UNC13D*[a]	17
Undetermined[c]		
Epidermodysplasia verruciformis	*EVER1, EVER2*[a]	19
WHIM syndrome	*CXCR4*[b]	20

Figure 4: Genes that cause inherited immunodeficiencies (17).

Primary immunodeficiencies represent some of the rarest chronic conditions within the spectrum of immunodeficiencies. They are categorized into nine groups, including antibody deficiencies, combined deficiencies, phagocytic defects, complement deficiencies, disorders of innate immunity, nuclear factor kappa B pathway defects/anhidrotic ectodermal dysplasia, toll-like receptor signaling pathway deficiencies, herpes simplex encephalitis, and natural killer cell deficiencies (19-20).

Severe combined immunodeficiency disorders, or SCID, constitute a primary immunodeficiency that predominantly affects infants and often leads to death before the age of two (21). SCID is exceedingly rare, with an estimated prevalence of 1 in 58,000, particularly when compared to more common conditions like Immunoglobulin A deficiency, which affects approximately 1 in 1000 individuals (19).

Most SCID cases stem from mutations in the gamma chain of the interleukin-2 receptor, although a few are attributed to adenosine deaminase (ADA) deficiencies (22). Secondary immunodeficiencies arise due to underlying conditions, such as primary immunodeficiencies, resulting in reduced immune cell counts (16)(19). Notably, HIV infection stands as one of the most significant disorders associated with secondary immunodeficiency (22-23).

3. IMMUNODEFICIENCY DISEASES WITH APPROVED GENE THERAPY

3.1. Adenosine Deaminase Severe Combined Immunodeficiency

Adenosine deaminase (ADA) is a crucial enzyme involved in purine salvage pathways. Decreased ADA levels can lead to toxic accumulation of secondary purine degradation products, such as adenosine, 2'deoxyadenosine, and deoxyadenosine triphosphate (dATP). These products can severely impact T-lymphocytes and B-lymphocytes, potentially resulting in lymphocyte death (24).

ADA is expressed in various cell types, with the highest expression observed in rapidly dividing lymphocytes. Consequently, the deficiency of lymphocytes due to ADA deficiency can lead to adenosine deaminase-deficient severe combined immunodeficiency, also known as ADA-SCID (24)(25). Eloise Gilbert identified ADA deficiency in two patients with CID and absent ADA enzyme in their red blood cells in 1972, marking ADA-SCID as the most common type of SCID. ADA-SCID typically manifests if overall ADA activity is less than 1% and is often detected at birth. While ADA deficiency can cause neurodevelopmental issues, hearing loss, and skeletal defects, its most devastating effects are observed in immune system dysfunction (24).

Untreated ADA-SCID can be fatal if not diagnosed early and treated promptly. There are currently three treatment options available: enzyme replacement therapy (ERT), allogeneic hematopoietic stem cell transplant (HSCT), and autologous gene therapy (GT) using the drug Strimvelis. In cases where gene therapy or suitable donors for HSCT are unavailable, ERT is recommended. HSCT has the highest survival rates with sibling donors (86%), followed by other family members (81%), unrelated donors (66%), and haploidentical donors (43%). However, matched donors represent only 20% of all ADA-SCID patients (24, 26).

Diagnosing ADA-SCID typically involves biochemical testing for ADA activity and levels of purine degradation products, along with genetic testing to identify mutations in the ADA gene. Reduced levels of immunoglobulin, T-lymphocytes, and B-lymphocytes support the diagnosis. Symptoms of ADA-SCID in infants may include recurrent infections, diarrhea, and dermatitis (25, 27).

Without treatment, infants with ADA-SCID typically do not survive beyond the first few years of life. The disorder can lead to various side effects, including neurological, skeletal, cognitive, and behavioral problems. ADA-SCID may present as late-onset, gradually worsening over the first decade of life or even into adulthood, particularly with hypomorphic mutations. Long-term data on late-onset ADA-SCID is currently lacking, but some patients with partial ADA deficiency may exhibit normal immune system function despite low ADA levels (25).

Early initiation of ERT is crucial for managing ADA-SCID symptoms, allowing patients to reside at home while awaiting definitive treatments such as HSCT or gene therapy. Before the advent of these treatments, patients relied on weekly injections of PEGylated bovine ADA as ERT. Clinical trials demonstrated promising results, with a 78% survival rate observed after two years of treatment (28).

Recipients of treatment who survived the first six months had a 90% probability of surviving for the next 12 years. However, after three years, a gradual decrease in lymphocyte numbers was observed, leading to lymphomas in some patients. ERT not only regulates immune system functions but also improves certain abnormalities and neurologic injuries caused by ADA deficiency (28).

Currently, ERT is recommended as a precursor to HSTC and HSC-GT treatments. While there is no defined time limit for ERT, patients opting for HSC-GT should discontinue injections 2 to 3 weeks before the procedure, whereas ERT should be continued for a period after HSTC (28).

HLA-matched sibling and family donors have shown efficacy in correcting metabolic and immune system activities in ADA-SCID patients. Among 54 patients undergoing HSTC, 46 survived, with three deaths due to treatment-related causes, and four requiring repeated procedures (28).

ADA-SCID was the first immunodeficiency disorder treated with autologous gene therapy. All patients who received HSC-GT are alive, but a small percentage required subsequent ERT, HSTC, or additional rounds of HSC-GT. To mitigate side effects, patients typically undergo 3 to 6 months of ERT before HSC-GT (28).

One major challenge of HSC-GT is the need for prompt transfer to the patient after transduction, requiring patients to relocate to treatment centers for up to six months. Lentiviral vectors and cryopreservation are being explored to address this logistical issue (28).

3.1.1 Strimvelis

In 1972, Friedmann and Roblin posed the question of whether gene therapy could effectively treat human genetic diseases, sparking the exploration of gene therapy trials for primary immunodeficiency diseases in the mid-1980s. As previously mentioned, vector selection is a crucial aspect of gene therapy, influenced by factors such as target cells, gene expression duration, and genetic materials. Currently, retroviruses are the chosen vectors for ex vivo gene therapy (29)(30).

There are approximately 20 different disorders associated with SCID, with one of the most prevalent variations being ADA-SCID. ADA deficiency is an autosomal recessive disease and represents the first PID to be treated with gene therapy. The initial gene therapy trial occurred in 1990 on a four-year-old child, utilizing her own T-lymphocytes transduced and delivered with a retrovirus (31). Although the therapy showed efficacy, it was insufficient to discontinue the patient's ERT treatment. In 1993, an infant was treated at just four days old, and gene expression persisted until he was 18 months old. However, he still required ERT supplementation thereafter. Despite these partial successes, the widely publicized trials encouraged further research in gene therapy (29).

A significant milestone in gene therapy was achieved with the application of nonmyeloablative conditioning to retrovirus-transduced CD34 and HSCs, leading to the discontinuation of ERT. This treatment method, later approved by the EMA in 2016 under the name Strimvelis, followed a test trial involving 18 patients spanning seven years (29).

Strimvelis emerged from a collaboration between Fondazione Telethon and GlaxoSmithKline (GSK), initiated by the Telethon Foundation in Italy. In 2010, the San Raffaele Telethon Institute for Gene Therapy (SR-Tiget) and GSK joined forces for cell-based gene therapy research due to funding limitations faced by the Telethon foundation (32).

Ultimately, GSK and SR-Tiget developed the final version of the gene therapy drug, with GSK handling industrial expertise, marketing, and economic aspects, while SR-Tiget oversaw research, laboratory work, and trials (32).

Following the seven-year trial journey, all 18 patients remained alive in June 2017, with modified genes still present in their circulating cells (26). While three patients in the trial group experienced unsuccessful outcomes with Strimvelis, improvements were generally observed, as evidenced by increases in CD+ and T cell counts (33).

Through the PASS (Prospective Postauthorisation Safety Study) registry, patients receiving Strimvelis will undergo regular check-ups for up to 15 years post-treatment, with consent from the patients (28). Concerns about leukemic transformation during trials were not observed in the follow-up of treated patients (32) (Figure 5).

Monitors	
Effectiveness Assessments • Survival • Intervention-free survival[a] • Use of medications/treatments of interest[b] • Immune reconstitution[c] • Growth[d] • Systemic metabolite detoxification[e] • Vector copy number[f] • Number and proportion of patients with severe infections[g] and associated hospital length of stay • Additional parameters reflecting the nonimmunological manifestations of ADA-SCID[i] • Paediatric development and quality of life data • Patient (or proxy) reported outcome measures and development questionnaires[j]	Safety Assessments • Frequency of adverse events and serious adverse events related to medical or surgical procedures associated with Strimvelis administration • Frequency of immune reactions • Frequency of oncogenesis • Frequency of reported adverse events and serious adverse events • Laboratory blood test results[h] • Fertility- and pregnancy-related outcomes • Data from RIS and RCR analysis

Figure 5: Areas of check-ups for the efficiency and the safety assessment of Strimvelis (28).

In the initial gene therapy trials, peripheral blood lymphocytes were transduced with a γ-retroviral vector to deliver the ada gene, which were then administered to patients via T lymphocytes or cold blood cells. Despite this approach, patients continued to receive ERT treatment post-gene therapy (29, 32). However, it was observed that ADA activity remained insufficient, and transduction rates were low (29).

Subsequent trials adopted different strategies. In the second set of trials, bone marrow (BM) or umbilical cord blood progenitors were administered to patients without conditioning. Unfortunately, this approach yielded inefficient results.

In the third set of trials, a significant breakthrough occurred with the removal of ERT and the introduction of busulfan conditioning. This approach led to sustained lymphoid reconstitution with altered T cells and improvements in immune function, positively impacting patient survival rates (29, 32) (Figure 6).

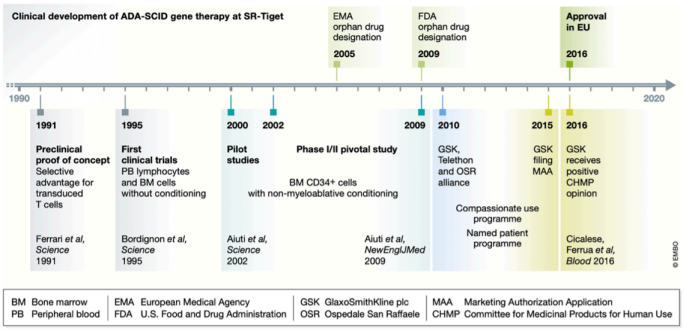

Figure 6: Milestones in the gene therapy treatment for ADA-SCID (32).

Strimvelis stands out as the first ex-vivo gene therapy to receive approval anywhere in the world. However, its cost, estimated at around 400,000 to 500,000 dollars/euros for a one-time treatment with lifelong benefits, presents a significant challenge (29, 32). Moreover, due to its short shelf life and the need for specialized gene therapy expertise, Strimvelis is exclusively administered at San Raffaele Hospital in Milan, Italy, necessitating patients to relocate temporarily for treatment (32).

Approximately 80% of rare diseases worldwide involve genetic alterations. Successful and legally approved gene therapy methods for these conditions fall into four main categories:

1. Direct modification of somatic cell DNA (in vivo) (34).
2. Modification of DNA in differentiated somatic cells prior to reimplantation (34).
3. Modification of DNA in stem cells prior to reimplantation (34).
4. Utilization of nucleic acid technology to modify post-transcriptional RNA and translation (34).

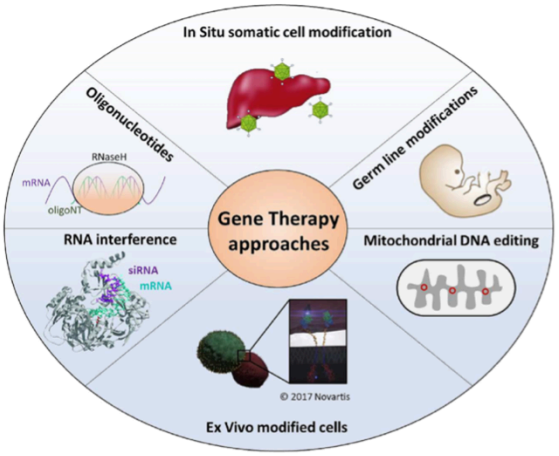

Figure 7: Different approaches for gene therapy (34).

The ex-vivo approach of altering stem cells represents a particularly effective method for blood-related gene therapy, as exemplified by Strimvelis (Figure 7). This treatment, available exclusively in Milan, Italy, requires patients to donate a sufficient number of CD34+ cells (approximately 4 million CD34+ cells/kg) to facilitate the creation of personalized Strimvelis therapy and additional stem cells as a backup in case the gene therapy is not successful (33).

In this treatment process, CD34+ hematopoietic stem cells (HSCs) are isolated from the patient's bone marrow and then modified using FLT3L, KITL/SCF, THPO, IL3, and IL6 to express ADA. These modified cells are transduced using a leukemia virus vector and then re-infused into the patient following non-myeloablative busulfan conditioning (33). Once reinfused, these cells engraft in the bone marrow and populate the hematopoietic system (35).

Insertional mutagenesis, a significant concern associated with all vectors, is particularly feared with γ-retrovirus vectors. Previous gene therapies targeting X-linked SCID, Wiskott-Aldrich Syndrome, and Chronic Granulomatous Disease (CGD) using γ-retrovirus vectors have resulted in leukemia in some patients. However, due to the continuous regulation of the ADA enzyme, gene expression is not

required, significantly reducing the risk of leukemia associated with Strimvelis therapy (35-36).

3.1.1.1 Challenges of Strimvelis

Like many drugs developed for treating extremely rare disorders, Strimvelis encountered several challenges before receiving approval from the EMA. Due to the small size of the subject group in the trials, concerns arose among scientists about the drug's precision when applied to a larger patient population. Additionally, none of the trial patients had active viral infections, raising questions about the representativeness of the success rates. This led the Evidence Review Group (ERG) to scrutinize potential biases in the study outcomes.

Another point of contention was the duration of enzyme replacement therapy (ERT) received by UK patients, which exceeded expectations. There was speculation that this prolonged ERT might have contributed to Strimvelis' apparent success. Moreover, discrepancies in estimating survival rates emerged, as all surviving patients were initially counted as successful, despite incomplete success in some cases. Consequently, both the ERG and the EMA opted to calculate an intervention-free survival rate, resulting in a revised estimate of 82.7%. This figure is considered a more accurate representation of the treatment's efficacy (33, 37) (Figure 8).

Base case	Strimvelis®	HSCT MUD	HSCT haploidentical
Costs			
Product	£505,000		
Screening pre-procedure	£161	£45,127	£45,127
Initial hospitalisation/transplant	£92,217	£81,973	£108,760
Rescue PEG-ADA/transplant	£339,955	£203,973	£815,893
Severe infection	£12,786	£9310	£8600
IVIG	£17,977	£12,863	£15,147
GvHD	£635[a]	£5089	£6376
Follow-up	£65,457	£46,792	£58,502
Travel to HSR-TIGET in Milan	£1412	£0	£0
Total	£1,035,601	£405,126	£1,058,405
Effects			
QALYs	31.3	22.8	19.7
Cost effectiveness			
ICER per QALY gained		£74,430	Dominated

Figure 8: Cost-effectiveness analysis of Strimvelis (33).

As previously mentioned, survival rates of patients living intervention-free were deemed the most accurate measure of treatment efficacy, prompting detailed analysis of these cases. Among these patients, all ten exhibited stable multilineage gene marking up to eleven years post-treatment. Notably, lymphocytes carrying the gene therapy demonstrated higher gene marking compared to granulocytes. Nine patients remained off enzyme therapy, with the three patients exhibiting the highest gene marking showing the most significant correction of ADA-SCID symptoms.

Unlike other gene therapy treatments utilizing gammaretroviral vectors, Strimvelis showed rare instances of insertional mutagenesis. Only one patient out of at least fifty treated with autologous gene therapy (Strimvelis) since 2016 reported leukemia in fall 2020. The reason for the comparatively lower incidence of leukemia development in ADA-SCID patients treated with gammaretroviral vectors remains a mystery.

In the years following the Strimvelis trials, gene therapy has made significant strides. Lentiviral vectors are now preferred over gammaretroviral vectors for PID treatments, including ADA-SCID, due to their more natural insertion pattern and shorter transduction time.

After years under the ownership of the Telethon Foundation and GSK, Strimvelis found a new home at Orchard Therapeutics in 2018. This move marked a significant step forward, as it was the first time a non-profit organization had engaged in such a transfer. However, Orchard Therapeutics faced financial challenges, leading to the discontinuation of gene therapy research, including Strimvelis, in 2022. As Strimvelis remains the sole gene therapy cure product available for ADA-SCID, the Telethon Foundation reacquired its license and resumed distribution in 2023.

4. OTHER IMMUNE DEFICIENCIES AND TREATMENTS

As of 2023, three successful gene therapy options have been reported for SCID disorders. The first, Strimvelis, has been extensively discussed as it is currently the only authorized drug on the market for treating adenosine deaminase deficiency severe combined immunodeficiency (ADA-SCID) in infants under the age of two. The other two successful therapies target X-linked severe combined immune deficiency (SCID-X1) and Artemis combined severe immune deficiency (Art-SCID).

However, SCID disorders are not the only primary immune deficiency disorders benefiting from gene therapy advancements. Three other PID's—Wiskott Aldrich syndrome (WAS), X-linked chronic granulomatous disease (CGD or XCGD), and leukocyte adhesion deficiency (LAD-1)—have also shown promising results in their respective trials. These diseases and their progress in gene therapy will be discussed in this section of the article.

4.1 X-Linked Severe Combined Immunodeficiency

X-linked SCID is a primary immunodeficiency disease caused by alterations in the IL2RG gene, located on the X chromosome. Without treatment, patients typically do not survive beyond their first year of life, making early detection critical. Newborn screening programs are in place across the United States to facilitate early intervention.

X-SCID accounts for around one-third of all cases of severe combined immune deficiencies, making it the most common subtype among them. Like ADA-SCID, patients with X-SCID are unable to produce functional T and NK cells, although B cells are present but non-functional. Treatment options are limited to either hematopoietic stem cell transplantation from a matched donor or autologous gene therapy.

Gene therapy trials for X-SCID began in 1999, showing promising results with patients achieving normal T cell counts within the first six months of treatment. However, in the following years, some patients developed leukemia attributed to the use of gamma retrovirus vectors in the gene therapy process. Subsequent trials using safer lentiviral vectors showed no instances of leukemia development in patients up to eight years after treatment. These self-inactivating (SIN) vectors based on lentiviruses have demonstrated improved safety profiles compared to their predecessors (Figure 9).

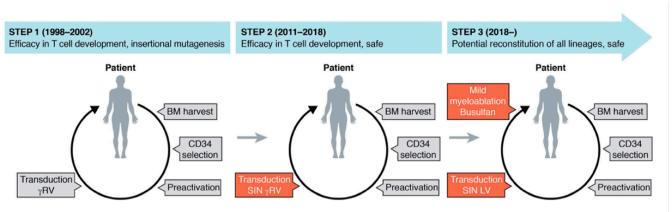

Figure 9: Treatment approaches that were taken with SCID-X1 gene therapy trials over the years (45).

The autologous gene therapy approach for SCID-X1 involves isolating CD34+ cells from the patient's bone marrow or peripheral blood stem cells. These cells are then transduced with a vector carrying the corrected IL2RG gene. Conditioning, such as low-dose busulfan, may be performed on the patient to facilitate engraftment of the transduced cells. Once transduced, the cells are reintroduced into the patient's system.

One advantage of this treatment method compared to ADA-SCID is that the extracted cells of SCID-X1 patients can be cryopreserved, allowing for treatment at multiple centers. Ongoing studies are investigating the efficacy and safety of gene therapy for SCID-X1, with no vector-related complications reported thus far. This approach holds promise for providing a potentially curative treatment option for patients with SCID-X1.

4.2 Artemis Combined Severe Immunodeficiency

Artemis combined severe immune deficiency (ART-SCID) is caused by mutations in the DCLRE1C gene and is inherited as an autosomal recessive trait. Patients with ART-SCID lack functional T and B cells, but typically have normal levels of natural killer (NK) cells. A notable characteristic of ART-SCID is radiosensitivity due to the inability to repair DNA double-strand breaks, which can lead to severe consequences upon exposure to radiation.

Traditionally, hematopoietic stem cell transplantation from a matched sibling has been the primary treatment option for ART-SCID, but results have been suboptimal. However, recent advancements in gene therapy have shown promising results for treating ART-SCID. Similar to gene therapy approaches for other forms of SCID, autologous gene therapy involves transducing the patient's CD34+ stem cells with a self-inactivating (SIN) lentivirus carrying the corrected DCLRE1C gene. These transduced cells are then reintroduced into the patient, allowing for production of functional Artemis protein and correction of the immune deficiency.

In clinical trials, patients who received this gene therapy showed improved lymphocyte proliferation rates and recovery from

infectious diseases. Some patients experienced transient anemia as a side effect, but this resolved once T cell immunity was restored. Conditioning with busulfan prior to gene therapy has been shown to enhance treatment efficacy.

While gene therapy for ART-SCID is still in its early stages compared to other primary immunodeficiency diseases, the initial results are promising and suggest that this approach may offer a viable treatment option for patients with ART-SCID. Ongoing research aims to further optimize and improve the effectiveness of gene therapy for this condition.

4.3 Wiskott Aldrich Syndrome

Wiskott Aldrich Syndrome (WAS) is an X-linked immune disorder characterized by symptoms such as bleeding, eczema, and susceptibility to infections. The syndrome results from mutations in the Wiskott Aldrich Syndrome protein (WASp), which plays a crucial role in regulating the functions of the actin cytoskeleton within hematopoietic cells (55).

The actin cytoskeleton is essential for various cellular processes, including phagocytosis, intracellular signaling, cell movement, and immune synapse formation. Dysfunctional WASp proteins severely limit cellular abilities, leading to rapid cell death. Moreover, malfunctioning WASp can trigger autoreactive B cells, leading to autoimmune reactions and secondary diseases like anemia, arthritis, inflammatory bowel syndrome (IBS), or nephropathy (57).

The dysfunction in WASp is believed to originate from disturbances in the 12 different exons located by the C terminus, resulting in various symptoms (58). Patients with WAS typically have a lifespan of up to fifteen years, but those who undergo stem cell therapy before the age of five, regardless of whether the donor is a sibling or unrelated, can live longer.

Treating WAS with gene therapy poses challenges compared to other primary immune deficiency diseases because sufficient gene expression is required not only in lymphocytes but also in myeloid and megakaryocytic cells (59). Initial gene therapy trials for WAS began almost two decades ago in 2006. However, only ten patients were enrolled in phase one, and busulfan conditioning was not performed. In phase two, after the addition of busulfan conditioning, patients received isolated CD34+ cells transduced to have normal cDNA. Unfortunately, seven out of ten patients developed leukemia due to the use of gammaretroviral vectors (60).

In current trials of WAS gene therapy, self-inactivating (SIN) lentiviruses carrying corrected WAS cDNA are used. Various preconditioning methods, including myeloablative, non-myeloablative busulfan conditioning, and fludarabine usage, have been adapted in clinical trials across the United States and Europe. These new methods have shown survival rates of 91.5%, and even when WASp levels did not return to normal, immune system functions improved. As of 2021, no patients have developed leukemia after receiving SIN lentiviral vector treatments, and even one adult has received the therapy without complications (44).

4.4 X-linked Chronic Granulomatous Disease

X-linked Chronic Granulomatous Disease (XCGD), also known as Chronic Granulomatous Disease (CGD), is a rare immune deficiency disorder that affects approximately one in every 250,000 individuals (63). The disease is characterized by impaired leukocyte enzyme function, specifically in the production of superoxide compounds necessary for killing microorganisms in monocytes and macrophages.

Mutations in the CYBB gene located on the X chromosome are frequently observed in CGD patients, with at least 65% of cases being X-linked (62). This gene encodes a component of the enzyme complex responsible for oxidizing NADPH, and mutations lead to a deficiency in superoxide production, making patients susceptible to infections, particularly those caused by the Staphylococcus family of bacteria.

Interestingly, CGD patients typically have normal antibody production, but the disease manifests as a late response with T cells.

Allogeneic human stem cell transplant (HSCT) can be used to treat CGD, but finding a suitable donor can be challenging and is not always guaranteed (63). As a result, gene therapy trials for CGD have been ongoing since 1990, although as of 2021, there is no approved gene therapy for the condition.

Early gene therapy trials involved using G-CSF mobilized CD34+ stem cells retrieved from peripheral blood and transduced with a gamma retroviral vector (64). However, this approach led to myeloproliferation in patients. Subsequent trials utilized lentiviral vectors adapted to express the gene during myeloid formation, which helped mitigate vector-related complications.

While studies in mice treated with bone marrow cells and lentiviral vectors have shown the development of leukemia, this has not been observed in human gene therapy studies for XCGD. Thus far, there are no reports indicating leukemia development in human patients undergoing gene therapy for XCGD.

Overall, gene therapy holds promise as a potential treatment for XCGD, with ongoing research aimed at optimizing treatment strategies and ensuring patient safety.

4.5 Leukocyte Adhesion Deficiency 1

Leukocyte adhesion deficiency-1 (LAD-1) is another autosomal recessive primary immune deficiency disorder characterized by abnormalities in an essential leukocyte adhesion molecule, ITGB2, which encodes CD18. CD18 is crucial for neutrophil adhesion to intercellular adhesion molecule (ICAM) molecules, facilitating the migration of blood cells to infection sites (66).

Infants with LAD-1 may experience delayed umbilical cord separation after birth. Patients are prone to more frequent and severe skin and mucosal infections. However, research has shown that blocking interleukin-12 (IL-12) and interleukin-23 (IL-23) can prevent these infections (67).

Hematopoietic stem cell transplant (HSCT) has demonstrated success in curing LAD-1, particularly with a matched sibling donor. Improvements have also been observed when using unrelated donors for transplantation. However, the first gene therapy trial for LAD-1 in 1992, using a gamma retrovirus vector without conditioning, did not yield significant improvements in treatment (68).

In a more recent gene therapy trial conducted by Rocket Pharmaceuticals, LAD-1 treatment transitioned from gamma retroviral vectors to lentiviral vectors carrying normal ITGB2 cDNA. Within one year of treatment, patients showed no infections and were able to discontinue antibiotic medications, indicating promising outcomes for future gene therapy trials targeting LAD-1 (69-70).

5. CONCLUSION

This review highlights the remarkable advancements in gene therapy for immunodeficiency diseases, offering hope to patients with previously incurable conditions. The review discusses six different immunodeficiency diseases, including ADA-SCID, X-SCID, ART-SCID, Wiskott-Aldrich Syndrome, X-CGD, and LAD-1 disease, showcasing the potential of gene therapy in clinical applications. The use of two delivery vectors underscores the pivotal role of technology in driving medical progress.

Through studies of various immunodeficiencies, researchers have gained insights into the immune system and genetic disorders, leading to the development of effective gene therapy approaches. The success of treatments like Strimvelis for ADA-SCID demonstrates the promise of gene therapy in addressing these diseases.

Despite significant advancements, challenges persist in gene therapy, including the long-term stability of corrected genes, off-target effects, immune responses, and the risk of leukemia formation. Additionally, the high costs of therapies and logistical complications, such as relocation requirements for treatments like Strimvelis, pose barriers to accessibility for patients.

In conclusion, gene therapy offers a viable solution for immunodeficiency diseases, but ongoing research is crucial to improve safety, efficacy, and accessibility. The field continues to evolve rapidly, and future studies hold promise for further enhancing treatments in this area.

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Advancements and Challenges in Anticancer Gene Therapy

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Abstract: Gene therapy has emerged as a significant approach in the realm of anticancer treatments, offering targeted and personalized therapies for carcinoma. Approved novel anticancer gene therapies work by damaging and altering detected carcinoma cells, impeding tumor cell growth, and enhancing the body's immune system response to cancer. Major advancements in anticancer gene therapy include modifications to oncolytic viral therapies, CAR-T therapy, and RNA interference therapy. Furthermore, CRISPR-CAS9 technology enables precise and highly efficient alteration of cancer-related genes, facilitating the development of anti-cancer immunity. This study focuses on recent advancements in anticancer gene therapies and the challenges encountered in the field of gene therapy for cancer treatment. It highlights the potential of these innovative approaches and their associated risks for patients, aiming to advance anticancer treatments and improve patient outcomes.

Key words: Anticancer gene therapy, Targeted therapies, CRISPR-CAS9 technology

1.CONCEPT OF CANCER AND GENE THERAPY

1.1 The Overview of Cancer and its Treatments

Cancer occurs when cells grow uncontrollably, spreading to other tissues and organs in the body. Mutations in the human genome, evasion of apoptosis, and changes in signaling pathways can lead to the development of cancer [1, 2]. All cells in the body undergo cell cycles for metabolism and division, regulated by checkpoints. Alterations in these checkpoints can result in cancerous cells, primarily due to abnormalities causing uncontrolled cell proliferation. Abnormalities in the human genome can also lead to changes in the proteins secreted by cells [1, 2].

In the medical industry, various treatments target cancerous cells to eliminate them. The choice of treatment depends on the type of cancer a person has. The main goal of anticancer treatment is to eradicate detected cancerous cells or tissues from the body. Treatment options include chemotherapy, hormone therapy, immunotherapy, and gene therapy, among others [3, 4].

1.2. Limitations for the Anticancer Therapies

In today's world, there are significant limitations in cancer treatment. According to the World Health Organization, approximately 10 million deaths were reported due to cancer throughout 2020, making it the leading cause of death alongside Coronavirus cases. However, all cancer treatments come with severe side effects, some of which may even lead to the death of the patient undergoing anticancer therapy. The primary aim of anticancer therapies is to prevent the uncontrolled growth of cancer cells while sparing healthy cells. However, treatments such as chemotherapy, surgery, and radiotherapy face limitations, including side effects and drug resistance in malignant cells [5, 6, 7].

Surgical removal of cancer cells is often limited by factors such as the size and depth of the tumor within the tissue. If a tumor is too large or deeply embedded, it may not be entirely removable, leading to damage to surrounding functional tissues and organs. This limitation is commonly encountered in cancer therapy, with many cases worldwide reporting damage to surrounding tissues during the removal of cancerous tissues. For instance, removal of cancer from the Frontal Lobe of the brain may impair the patient's speech or cognitive functions. Therefore, the location and size of the tumor

significantly impact the feasibility of surgical removal therapy for cancer [5, 6, 7].

Chemotherapy, another common treatment modality, can be hazardous to patients due to the use of chemotherapeutic agents. This treatment is typically administered to patients with advanced cancer, where metastasis has occurred, making it difficult to target specific areas. Chemotherapy affects the entire body with toxic agents, leading to high toxicity levels that may harm both cancerous and normal cells. The growth of non-proliferating cells can also be affected, potentially causing damage to tissues, organs, or bones. Hence, selecting the appropriate chemotherapeutic agent is crucial to avoid damaging tissue function [5, 6, 7].

Radiotherapy, involving the exposure of cancer cells to radiation, aims to reduce tumor size or completely remove the tumor. However, this treatment is associated with both early and late side effects. While early side effects, mainly skin diseases, are treatable, late side effects may be irreversible and untreatable, including damage to neurons and blood vessels [5, 6, 7].

1.3. Advantages and Disadvantages of Gene Therapy for Cancer

Gene therapy, like other anticancer therapies, comes with its own set of limitations, advantages, and disadvantages. It is currently providing safer treatment options for cancer patients and shows promise for treating other diseases in ongoing medical research. Gene therapy offers several advantages, particularly in the early stages of cancer treatment. Over the years, numerous cancer cases have been successfully treated using various gene therapy technologies, providing a new avenue for curing cancer when conventional treatments are ineffective. Unlike chemotherapy, which lacks specificity, gene therapy targets cancer diseases specifically, offering targeted treatment options.

Gene therapy has the potential to alleviate long-lasting impacts on the body with just one dose of treatment. However, while gene therapy is beneficial in early-stage cancer treatment, its long-term side effects remain uncertain due to its relatively recent development. It cannot guarantee a certain cure for cancer as its efficacy is still being studied. Additionally, gene therapy carries risks, particularly the risk of infection. Inactivated agents, such as viruses used in gene therapy, may infect the body, leading to unforeseen side effects within the immune system. Furthermore, gene therapy may interfere with or prevent the application of other types of treatments [8, 9].

2. APPROVED GENE THERAPY TYPES FOR CANCER

2.1. CAR-T Cell Therapy

2.1.1. Introduction to Chimeric Antigen Receptor (CAR) Technology

Chimeric Antigen Receptor, also known as CAR-T cell therapy, is a groundbreaking approach used against cancer cells by genetically altering the genomes of a patient's T-cells. This treatment falls under the umbrella of immunotherapy for various cancer types. Genetically modified T-cells are engineered to target and inhibit the growth of cancer cells within the human body, offering a more efficient method of combating cancer. Some T-cells within the immune system fail to recognize the receptors present on cancer cells, many of which have unknown receptors. By altering the genome of T-cells, receptors can be produced that recognize and bind to these cancer cells. Each T-cell receptor is customized based on the type of cancer being targeted, as not all cancer types possess the same receptors.

The process begins by collecting specimen T-cells from the patient's blood serum. These T-cells are then loaded with the Chimeric Antigen Receptor, allowing them to form bonds and bind to the specific receptors of cancer antigens. The modified T-cells are then cultivated in the laboratory until they reach adulthood. CAR-T cell therapy has shown significant efficacy in treating leukemia and lymphoma, particularly those expressing CD19 B antigens. This technology has the potential to destroy hard-to-treat cancer antigens. However, like all treatments, CAR-T cell therapy comes with its own set of side effects. One major side effect is Cytokine Release Syndrome (CRS), wherein a large number of cytokines are secreted into the bloodstream by the activated CAR-T cells. This can lead to symptoms such as high fever, nausea, breathing difficulties, and high blood pressure. Patients undergoing this therapy require close monitoring at every stage to mitigate the risk of side effects on the

nervous system. Imposing the altered T-cells into the bloodstream can result in balance loss, seizures, and various other effects [10, 11].

2.1.2. Chimeric Antigen Receptor (CAR) Technology

The structure of Chimeric Antigen Receptors (CARs) includes extracellular domains built by single-chain variable fragments (scFv), which are derived from monoclonal antibodies (mAb) (Figure 1). These scFv domains enable target cells to recognize CARs through binding interactions. Meanwhile, the intracellular part of CARs functions in activating T cells via signaling pathways. The presence of Major Histocompatibility Complex (MHC) further enhances the advantages conferred to T cells.

Over the years, there have been significant advancements in the target specificity of CAR-T cells. Nowadays, this therapy has expanded beyond cancer treatment and can be utilized for other diseases as well. Clinical studies have demonstrated the success of CAR-T cell therapy in various diseases, reflecting the continuous development and refinement of this therapeutic approach [12, 13, 14].

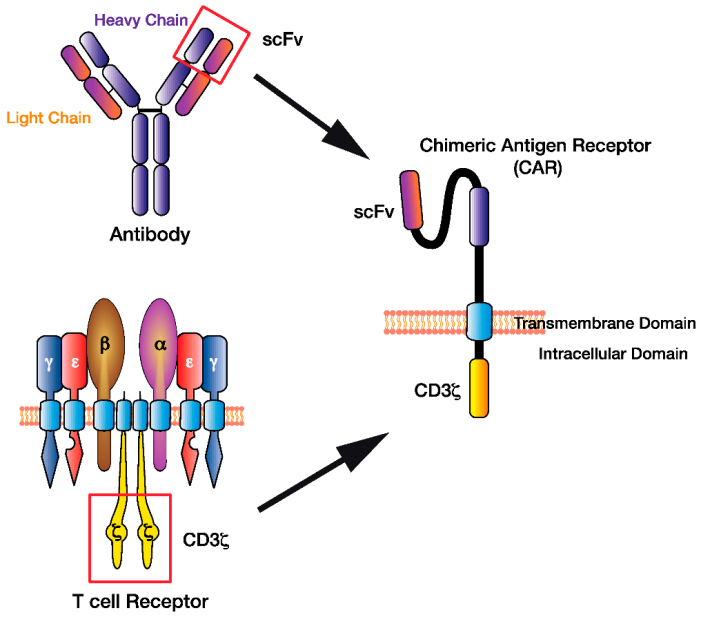

Figure 1. The structure of Chimeric Antigen Receptor with the single-chain fragment variable (scFv) which is taken from monoclonal antibody (mAb) [15].

2.1.3. CAR-T Cell Therapy and its Future Perspectives

CAR-T cell therapy has revolutionized the field of anti-cancer therapy. This innovative approach involves editing the genome of T cells obtained from the patient, enhancing their specificity and targeting capacity before administering them back to the patient. As a result, there has been a significant increase in the success rate of this therapy, opening up new avenues for anticancer immune therapies, particularly in specific types of leukemia and lymphoma.

The future outlook for CAR-T cell therapy is promising, with ongoing advancements aimed at ensuring safer and more effective treatment processes. Interventions involving pharmaceuticals can help minimize side effects, thereby enhancing therapy outcomes for patients. Additionally, combining CAR-T therapy with the CRISPR system holds potential for further improving success rates.

Recent developments also indicate progress in extending the application of CAR-T cell therapy to solid tumors. Strategies such as targeting multiple antigens and modifying CAR-T cells to secrete cytokines for the elimination of cancerous tissues represent futuristic perspectives and developments for this therapy.

Efforts are also underway to address challenges related to the persistence of CAR-T cells within the body. Adjustments in CAR-T cell design and durability, along with the integration of multiple therapeutic approaches, are being explored to enhance the effectiveness of CAR-T cell therapy [16, 17, 18, 19, 20, 21].

2.2. Oncolytic Viruses

2.2.1 Overview of Oncolytic Viral Therapy

Oncolytic viral therapy harnesses genetically engineered viruses to target cancer cells while sparing healthy cell lines (Figure 2). This therapeutic approach bolsters the immune defense against tumors, delivering transgenes to the cells and safeguarding them against cancer. The primary objective of oncolytic viral therapy is to design viruses capable of entering cancer cells and replicating within them. Successful replication ultimately leads to the death of the tumor. It is crucial to meticulously design the virus's genome to prevent harm to surrounding tissues.

Over the years, molecular oncology has witnessed the development of four types of oncolytic viruses engineered to combat cancer. Among these, talimogene laherparepvec (T-VEC) has gained global approval as an anticancer therapy by the Food and Drug Administration (FDA). T-VEC is specifically utilized in the treatment of metastatic melanoma.

In research centers, efforts are ongoing to develop more genetically engineered oncolytic viruses following the success of T-VEC. Some studies have revealed that certain tumors produce a protein known as TGF-ß, which shields the tumor from immune attacks. Researchers have engineered oncolytic viruses to suppress the production of TGF-ß. To prevent the destruction of these engineered oncolytic viruses by the immune response, they must be injected directly into cancer cells [22, 23].

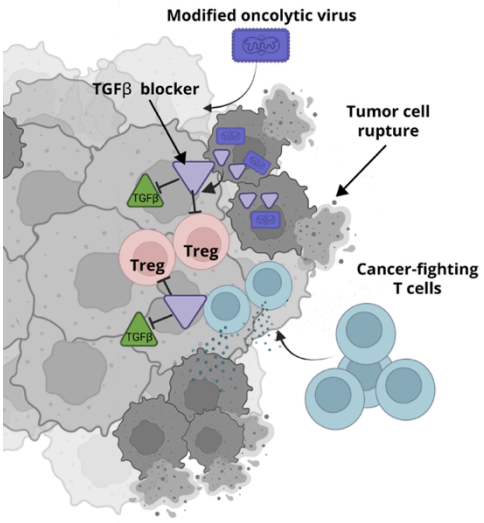

Figure 2. A destruction mechanism of tumor cells by injection of OV to block TGF-ß. [24].

2.2.2. Examples of Approved Viral Therapies and Their Mechanisms on Cancer Cells

In the field of molecular oncology, numerous therapies have been developed, ranging from chemical-based to viral-based treatments. While nonviral vectors exist, they are not as efficient as viral vectors. Viral vectors offer higher efficiency and target specificity in gene delivery. In viral vector therapy, genes encoding pathogenic proteins in the viral genome are replaced with therapeutic genes. These modified vectors are typically administered to patients via injection. Alterations to the viral genome increase target specificity and transduction efficiency. Modifications are made to viral therapies to prevent the secretion of pathogenic proteins, ensuring patient safety. Several viral therapies, such as Gendicine, T-VEC, and Kymriah, have received approval from the Food and Drug Administration (FDA). These vector-based products are utilized in treating neck cancer cells, melanoma, and acute lymphoblastic leukemia, respectively.

Viral therapies can be categorized into two main types: replication-defective viral vectors and replication-competent viral vectors. These categories operate differently within tumor cells. Replication-defective viral vectors lack essential genes for replication, preventing replication within tumor cells. In contrast, replication-competent viral vectors can replicate within tumor cells and may even induce the secretion of antitumor immune factors to combat tumors. Commonly used viral vectors in anticancer viral therapies include Adenovirus (AdV), Herpes Simplex Virus (HSV), and Retrovirus (RV), all of which are globally approved for cancer treatments.

Oncolytic Adenoviruses (OAdVs) are engineered to express p53 to induce apoptosis in cancer cells. Additionally, OAdVs interact with suicide genes to convert nontoxic products into cytotoxic ones, effectively killing cancerous cells. Immune-stimulating genes are also delivered by OAdVs to enhance the body's response against tumors. Combining OAdVs with CAR-T cells can further increase therapy efficiency [25].

2.2.3. Future Approaches of Oncolytic Viral Therapy on Cancer Diseases

Oncolytic viral (OV) therapy holds promise for treating patients suffering from various cancerous diseases. This therapy utilizes genetically engineered viral strains to target and eliminate cancerous tissues and cells within the patient's body. In addition to directly killing cancer cells, OVs also boost the patient's anti-cancer immune response, thereby mitigating the effects of cancer in the body.

However, like all gene therapies, OV therapy comes with its own set of side effects. The immune system may eliminate oncolytic viruses administered to the body, hindering their ability to target tumors effectively. To address these off-target effects, researchers have developed and continue to develop various strategies.

The future outlook for oncolytic viral therapy is promising, with ongoing research shedding light on its potential applications in cancer treatment. Future directions for OV therapy include combining it with other therapies such as radiation, which can synergistically improve the body's response against cancer. Combining therapies can help eliminate off-target effects associated with OV therapy.

Personal genetic data play a crucial role in OV therapy, as tailoring and designing OVs based on an individual's genetic profile can enhance treatment outcomes. These personalized approaches can increase OV specificity against tumor cells and lower toxicity levels, thereby overcoming resistance to therapy. Additionally, developments in this research area may lead to the identification of new types of oncolytic viruses for cancer treatment [26, 27, 28, 29, 30].

2.3. RNA Interference (RNAi)

2.3.1 Introduction to RNAi As a Therapeutic Approach

RNA interference (RNAi) technology facilitates gene silencing, holding immense potential for suppressing genes implicated in cancer development. Anticancer treatments utilizing RNAi have identified specific genes associated with tumor formation, demonstrating enhanced efficacy in cancer therapy. Research indicates that targeting cancer-causing genes through RNAi-based therapies is more effective in combating cancer.

RNAi operates within the non-coding region of RNA, which consists of small regulatory RNAs and long non-coding RNAs (ncRNAs). Small regulatory RNAs, including small interfering RNA (siRNA) and microRNA (miRNA), play a crucial role in RNA interference. SiRNA, with its 20-22 nucleotide length, regulates gene expression by silencing target genes and is a fundamental component of transcriptional regulation mechanisms across eukaryotic cell lines. Additionally, RNAi is involved in various cellular processes, including cell growth regulation and transposon protection within the genome.

RNAi technology can block mRNA translation and degrade mRNA, inhibiting the overproduction of carcinogenic proteins and suppressing cancer-causing single-gene diseases. RNAi-based anticancer therapies have garnered recognition for their remarkable efficacy and potential, demonstrating high efficiency in targeting specific genes associated with cancer progression. Furthermore, RNA interference exhibits the capability to transmit gene silencing to subsequent generations and offers a cost-effective alternative to other gene therapy methods.

In anticancer therapy, RNAi targets oncogenes, mutated tumor suppressor genes, and other carcinogenic genes involved in cellular pathways leading to tumor cell proliferation. Concurrent targeting of multiple genes presents an effective strategy for anticancer therapy, reducing the risk of drug resistance often associated with chemotherapy. Personalized drug development based on RNAi technology offers a tailored approach to controlling and preventing the growth of cancerous cells, contributing to more efficient cancer management.

Studies on animal cell lines have shown promising results with specific siRNAs targeting proteins involved in cell cycle regulation, such as kinesin spindle protein (KSP) and polo-like kinase 1 (PLK-1), demonstrating significant antitumor effects in various tumor cell types, including subcutaneous and hepatic tumors [31, 32, 33, 34, 35, 36, 37].

2.3.2 Approved RNAi Based Treatments for Cancer

RNA interference (RNAi) based cancer treatments have gained widespread acceptance worldwide, offering higher efficiency and lower risk compared to other types of anticancer therapies. These treatments primarily target cancerous cells, leveraging pivotal regulatory molecules involved in cellular pathways such as cell proliferation and apoptosis (Figure 3).

Efficient delivery of therapeutic genes to lung tumor cells is achieved through RNAi therapies using nanocarriers and specific biomarkers tailored for lung cancer cells. Nanoparticle-mediated RNA interference agents show promise in specifically silencing oncogenes and multidrug-resistant genes, overcoming the limitations of traditional chemotherapy. Combining nanoparticles with the understanding of lung cancer biology has significantly enhanced the efficacy of RNAi-based anticancer therapies.

Pancreatic cancer, particularly pancreatic ductal adenocarcinoma (PDAC), poses a significant challenge due to its aggressive nature. RNAi-mediated target gene knockdown therapy shows considerable promise in PDAC treatment. Combining RNA interference with radiotherapy and chemotherapy has shown to reduce the resistance of pancreatic cancer cells to these treatments.

Breast cancer, affecting approximately 68% of women, presents another significant challenge in oncology. RNAi-based therapies offer a novel approach to target mutations or gene overexpression associated with breast cancer progression. Gene implantation incorporating small interfering RNA (siRNA) and microRNA (miRNA) into targeted cells shows potential for genetic modification.

In preclinical investigations, RNAi-based therapeutic approaches for human epidermal growth factor receptor 2-positive (HER2+) breast cancer have shown promise. Combining siRNA targeting polo-like kinase 1 (siPLK1) with peptide fusion proteins incorporating HER2 single-chain variable fragment (scFv), alongside the use of PEG-PLA-based nanocarriers designed for precise siRNA delivery, has demonstrated effective targeted administration. Additionally, researchers are exploring the delivery of siRNA in a targeted manner to address chemoresistance in breast tumors, leveraging miRNA as a functional indicator to modulate biological functions [38, 39, 40, 41, 42, 43, 44].

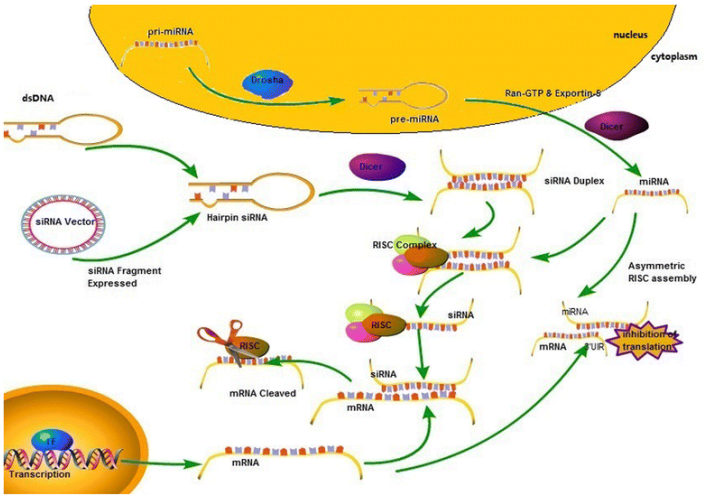

Figure 3. A sketch of an RNA interference mechanism on cancer treatment [45].

3. GENE EDITING TECHNIQUES WITH CRISPR-CAS9

3.1. CRISPR-Cas9

3.1.1. Fundamentals of CRISPR-Cas9

The advancement of next-generation sequencing (NGS) methods has significantly reduced the costs associated with genetic diagnostics, enabling the clinical identification of rarely seen mutations linked with pathology. Gene editing technology has experienced a remarkable surge since the inception of CRISPR-Cas9, which was first introduced in 2012. The widespread adoption of the CRISPR method in clinics has ushered in a new era of gene editing techniques, offering hope to patients grappling with genetic disorders.

The CRISPR-Cas9 system revolves around the Cas9 enzyme and RNA molecule, which serves as a guide. This technology utilizes RNA-guided endonucleases to edit genes within cell lines. The Cas9 enzyme mediates a double-stranded break at the targeted location on the template DNA. The guide RNA (gRNA) directs the Cas9 enzyme via Watson-Crick base pairing to the sequence of interest.

The gRNA comprises two connected components: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The crRNA recognizes the protospacer, or the targeted sequence, while the protospacer adjacent motif (PAM) follows the protospacer sequence. The Cas9 enzyme identifies and activates the PAM, which determines the specific location for cleavage.

The editing of the genome occurs during the repairing process facilitated by the CRISPR-Cas system (Figure 4). The system is responsible for two primary repair mechanisms: homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR requires a donor template with homology to the surrounding sequence of interest. Studies have indicated that the CRISPR-Cas system enhances the efficacy of HDR in gene editing. HDR aids in inserting a gene of interest into its proper sequence and correcting unwanted mutations within the gene sequence [46].

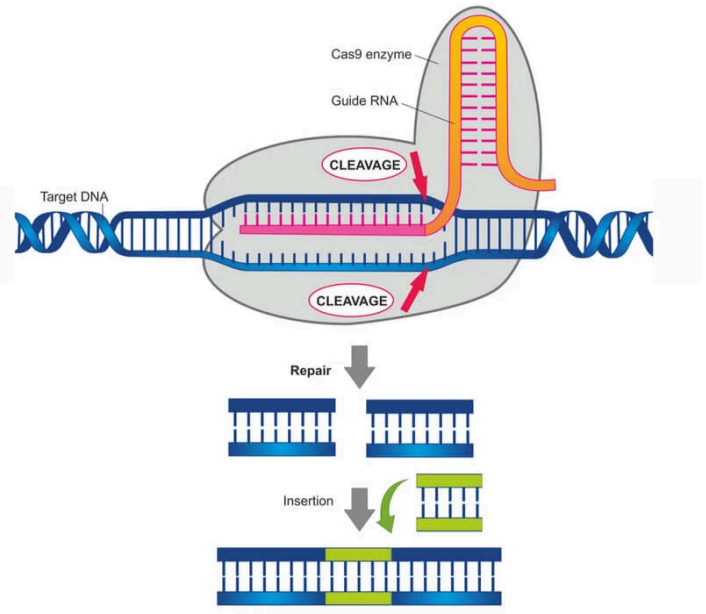

Figure 4. The gene editing modification by the CRISPR-Cas system. The ds break occurs by the Cas9 enzyme and the cut cleavage is repaired by either NHEJ or homology-directed repair [47].

3.1.2. Clinical Applications in Cancer Treatment of Crispr-Cas9 System

Since the 2010s, the CRISPR-Cas9 system has emerged as a promising tool for anticancer treatment. With its advanced technology, this system enables the monitoring of tumor progression and the manipulation of the cancer genome throughout its development. The programmable nature of CRISPR-Cas9 allows for the replacement of mutated genes with healthy ones and the removal of carcinogens, offering potential therapeutic benefits for cancer patients.

Personalized CRISPR-Cas9-based precise medicines can be tailored to target specific compartments within cancer cells, providing a targeted approach to anticancer therapy. This system shows promise in treating various types of cancer, including brain cancer, renal cell carcinoma, and colorectal cancer, where other therapies may be less

effective. In particular, CRISPR-Cas9 has demonstrated efficacy in addressing the genetic components of brain cancer, a challenge that other treatments struggle to overcome.

Clinical research studies have contributed valuable insights into the use of CRISPR-Cas9 technology for cancer therapy, paving the way for further advancements in personalized and targeted treatments [48].

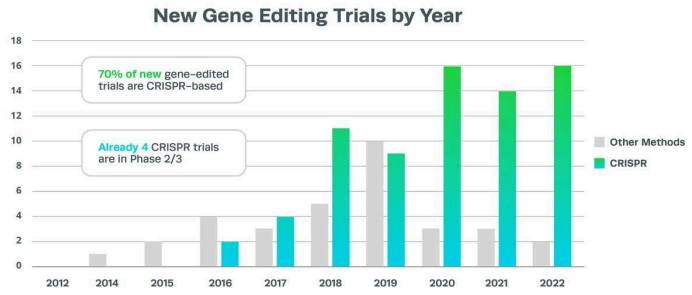

Figure 5. The rates of new gene editing clinical trials throughout the years (since 2012). Graph also demonstrates that newly gene editing techniques are based on CRISPR-CAS9 system [49].

3.1.3. Futuristic Approaches of CRISPR-Cas9-Based Applications

The CRISPR technology is rooted in prokaryotic cells' adaptive immunity responses to plasmids and phages, offering a foundation for enhancing the Cas9 gene editing system. This advancement holds promise for the treatment of carcinomic diseases due to its remarkable accuracy.

Prokaryotic cells employ a defense mechanism known as CRISPR when threatened by phages and viruses. Within the CRISPR locus, short repeating DNA sequences interspersed with fragments are found, originating from bacteriophages. These sequences encode small non-mRNAs that aid in blocking viral infections and fostering adaptive immunity. Cas genes are also present in the CRISPR locus, contributing to the system's effectiveness in combating cancer.

Clinical trials have demonstrated the efficacy and safety of CRISPR-based immune cell administration in patients with severe lung carcinoma, highlighting its potential as a precise and reliable therapy for cancer. Moreover, ongoing improvements, such as the development of the very fast CRISPR technology in 2020, allow for the generation of double-stranded breaks at extremely small scales, enabling accurate investigations into DNA repair processes with enhanced genomic resolution [50, 51]. With its precise genome editing techniques, the CRISPR system offers a promising avenue for the treatment of cancer-based diseases [52, 53, 54, 55].

3.1.4. Ethical and Moral Consideration of CRISPR-Cas9 System

The development of the CRISPR system has sparked profound ethical and moral discussions worldwide, particularly regarding genomic engineering in human genetics and the emergence of a new generation of CRISPR babies. These debates center around the editing of the human genome to prevent hereditary and carcinogenic diseases, raising fundamental questions about human nature and the sanctity of life.

Central to these moral dilemmas is the notion of enhancing and altering human functions, particularly the immune system's ability to mount a stronger response against cancer. The ethical implications of altering the human genome for future generations provoke contentious discussions about who holds the authority to conduct such editing and under what circumstances.

Preserving individuals' rights to make informed decisions about gene editing applications without coercion is paramount to upholding humanistic values. However, concerns arise about the potential for societal division and inequality if access to gene editing technologies is not equitably distributed across different social classes.

Moreover, the prospect of creating disparities within society raises broader questions about social justice and equality. The CRISPR debates extend beyond individual rights to encompass societal choices and considerations.

While CRISPR technology holds promise for medical advancements, concerns persist regarding off-target side effects and associated health risks. Moral deliberations must weigh these potential consequences, considering the well-being of future generations in the decision-making process [56, 57, 58, 59].

3.2. Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALEN)

3.2.1 Overview of ZFN and TALEN Technologies

Zinc Finger Nucleases (ZFNs) play a crucial role in genome editing technologies by targeting and cleaving specific DNA sequences. Structurally, ZFNs comprise a DNA-binding zinc-finger protein (ZFP) located at the N-terminus and the Fok1 nuclease cleavage domain at the C-terminus. The ability of ZFNs to heterodimerize allows the Fok1 domain to cleave the DNA into fragments, facilitating targeted genetic modifications.

Zinc Finger Proteins consist of repeating units of Cys2His2 zinc fingers, each recognizing three base pairs of DNA. This specificity enables ZFNs to precisely bind to their intended genomic targets. However, compared to other genetically programmed nucleases, ZFNs exhibit a broader range of off-target effects and lower binding quality, potentially leading to cytotoxicity in targeted cells.

To deliver ZFNs to targeted cells, Adeno-Associated Viruses (AAVs) are utilized as vectors due to their small size and programmable nature. Notably, ZFNs have been administered to human patients in clinical trials aimed at cancer treatment [60].

In contrast to ZFNs, Transcription Activator-Like Effector Nucleases (TALENs) offer a promising alternative in genome editing. TALENs consist of a DNA-cleaving enzyme lacking specificity, paired with a reprogrammable DNA-binding domain. This feature allows TALENs to target any sequence without difficulty, enabling rapid and precise alterations to the gene of interest, particularly in anticancer therapies.

TALENs are composed of transcription activator-like effectors originating from Xanthomonas bacteria, with closely preserved repeating sequences in the DNA-binding region. This unique structure makes TALENs a favorable choice in biotechnology, especially as an alternative to ZFNs [61].

3.2.2. Applications of ZFNs and TALENs in Cancer Gene Therapy

In recent years, Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) have emerged as promising technologies in clinical trials, particularly in the field of cancer research. These programmable nucleases are utilized to target and modify oncogenes, offering potential therapeutic benefits for cancer treatment.

In specific applications, ZFNs and TALENs are programmed to collaborate in eliminating oncogenes, thereby altering their functions within cancerous cells. For instance, in a clinical trial, CompoZr ZFN was paired with the E6 gene of Human Papillomavirus 16 (HPV-16) genome. Both TALENs and ZFNs were programmed to target the E6 and E7 genes within the sequence. To monitor the activity of these programmed nucleases on the targeted genes, techniques such as Reverse Transcription Polymerase Chain Reaction (RT-PCR), western blotting, and immunofluorescence were employed. These methods allowed researchers to assess the effectiveness of ZFNs and TALENs in knocking out the oncogenes in carcinoma cells. The results of the study demonstrated that the use of TALENs and ZFNs was successful in disrupting the activity of oncogenes within cancer cells, highlighting the potential of these technologies as therapeutic agents in cancer treatment [62].

4. CHALLENGES AND FUTURE DIRECTIONS

4.1. Potential Risks and Unfavorable Effects

Gene therapy, while promising as a potential treatment for cancer, carries inherent risks that need to be carefully considered and managed. One significant risk is the potential for the patient's immune system to mount an excessively strong response against the foreign vector used in the therapy, leading to rejection of the genetically engineered cells or vectors. This immune reaction can undermine the efficacy of the treatment and pose challenges for its success.

Moreover, there is a risk that the administration of genetically engineered cells could inadvertently promote the growth of cancer or trigger the development of another type of cancer in the patient. Allergic reactions to the therapy components are also a concern, potentially causing adverse effects and complications for the patient.

Additionally, the therapy may inadvertently damage healthy tissues or organs, compromising their function and overall health. Of utmost concern is the risk of disrupting or altering the normal functioning of the patient's genome when therapeutic DNA is introduced into their genome. Such unintended alterations could have serious consequences and must be carefully mitigated.

Toxic effects are another potential risk associated with gene therapy, particularly if the therapeutic agent leads to overexpression of certain genes, resulting in harmful side effects. These risks underscore the importance of thorough monitoring and careful assessment of the therapy's safety profile.

Given the relatively limited data available on the long-term effects of gene therapy, there is a need for continued research and comprehensive evaluation of its safety and efficacy over time. It is essential to prioritize patient safety and well-being in the development and application of gene therapy for cancer treatment [63].

4.2. Strategies for Preventing the Off-Target Effects of Therapies

Minimizing off-target effects is crucial for enhancing the efficacy and safety of gene therapy for cancer treatment. Various strategies are being explored to address this challenge. One approach involves improving the delivery system used to transport therapeutic agents to their target cells. By enhancing the specificity of the delivery system, off-target effects can be minimized, ensuring that the therapeutic agents reach only the intended cancer cells.

Furthermore, leveraging computational tools and software can aid in predicting the potential outcomes of anticancer gene therapy, allowing researchers to anticipate and mitigate off-target effects before administering the treatment to patients. These predictive models enable a more precise and targeted approach to therapy, minimizing the risk of unintended consequences.

Another strategy involves integrating multiple anticancer genomic treatments, such as combining chemotherapy and immunotherapy. This approach can enhance the effectiveness of treatment while reducing the likelihood of off-target effects by targeting multiple pathways involved in cancer progression.

Additionally, implementing thorough monitoring and observation protocols during therapy is essential for promptly identifying and addressing any off-target effects that may arise. Regular monitoring allows healthcare professionals to intervene quickly if adverse reactions occur, ensuring patient safety throughout the treatment process.

Overall, by employing a combination of improved delivery systems, predictive modeling, integrated therapies, and diligent monitoring, researchers can work towards minimizing off-target effects and maximizing the therapeutic benefits of gene therapy for cancer treatment [64].

5. CONCLUSION

Your report provides a comprehensive overview of various gene therapy techniques used to treat cancer, highlighting their benefits and potential risks. It's important to consider the specific characteristics of each therapy and tailor them to the individual needs of patients based on the type of carcinoma they are diagnosed with. Indeed, mitigating the potential risks associated with gene therapy is crucial for ensuring patient safety and optimizing treatment outcomes. Developing more advanced delivery systems for therapeutic agents can help improve the specificity of treatment, reducing the likelihood of off-target effects and minimizing damage to healthy tissues. Furthermore, leveraging computer software and predictive modeling tools can aid in predicting and monitoring the outcomes of gene therapy, allowing healthcare professionals to intervene promptly if adverse reactions occur. This proactive approach to monitoring can help mitigate risks and ensure patient safety throughout the treatment process. Additionally, integrating multiple therapeutic agents simultaneously, such as combining gene therapy with other treatment modalities like chemotherapy or immunotherapy, may enhance the effectiveness of treatment while reducing the potential for adverse effects. By implementing these strategies and continuously refining gene therapy techniques, we can strive to maximize the benefits of these innovative treatments while

minimizing the associated risks, ultimately offering better outcomes for patients battling cancer.

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Enhancing Therapeutic Approaches for SMA: Progress in Gene and RNA Therapies

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Abstract: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterized by high incidence and severity. While the disease affects approximately 1 in 6,000-10,000 live births, the carrier frequency is observed at a rate of 1 in 40 to 1 in 60 individuals. SMA is the leading genetic cause of infant mortality. This review explores advancements in gene and RNA therapies for both advanced and developing stages of SMA, considering the evolving landscape of biotechnology.

Key words: Spinal Muscular Atrophy (SMA), Gene therapy, RNA therapy

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterized by the degeneration of alpha motor neurons in the spinal cord, leading to muscle weakness and paralysis (1). It is among the most common genetic causes of infant mortality. SMA results from loss or mutation of the SMN1 gene, resulting in a decrease in SMN protein levels, which in turn leads to motor neuron death and progressive muscle atrophy. The disease was first described by Guido Werding and Johann Hoffmann in the 1890s. SMA has an incidence of 1 in 6,000-10,000 live births, with carrier frequency observed at a rate of 1 in 40 to 1 in 60 individuals. It is the leading genetic cause of infant death.

CLINICAL CLASSIFICATION

In SMA disease, prominent atrophy and widespread muscular weakness primarily affect the proximal extremity muscles. The severity of the disease varies depending on phenotypic factors, age at onset, and acquired motor function. SMA is classified into five groups based on severity, with three main groups distinguished by the degree of severity: SMA type 1, SMA type 2, and SMA type 3 (2-3). Additionally, there are other subgroups known as SMA type 0 and SMA type 4 (Table 1).

SMA Type	Other Names	Age of Onset	Life Span	Highest Motor Milestone Achieved	Other Features
Type 0	Prenatal, congenital SMA	Prenatal	<6 months	Mostly unable to achieve motor milestones	Severe weakness at birth Profound hypotonia Facial diplegia Areflexia Early respiratory failure Joint contractures
Type I	Werdnig-Hoffman disease, severe SMA ("nonsitters")	0-6 months	<2 years without respiratory support	Never sits supported	Weakness "Frogleg" posture, hypotonia Tongue fasciculations Hyporeflexia, areflexia Suck and swallow difficulties Respiratory failure
Type II	Intermediate SMA ("sitters")	6-18 months	Approximately 70% alive at age 25 years	Sits independently, never stands or walks	Proximal weakness, hypotonia Postural hand tremor Hyporeflexia Average or above average intellectual skills by adolescence Scoliosis
Type III	Kugelberg-Welander disease, mild SMA ("walkers")	>18 months	Almost normal	Stands and walks	May manifest hand tremor Resembles muscular dystrophy
Type IV	Adult SMA	>21 years	Normal	Normal	

Abbreviation:
SMA = Spinal muscular atrophy

Table 1. Types of SMA disease (4).

SMA Type 0

SMA type 0 is characterized by severe weakness and hypotonia present at birth, often accompanied by reduced fetal movements. The weakness is presumed to have onset during prenatal development. Symptoms of SMA type 0 in infants include facial diplegia, areflexia, joint contractures, and atrial septal defects. Unfortunately, life expectancy is significantly shortened due to respiratory failure issues that arise early in life. Most infants with SMA type 0 do not survive beyond 6 months of age.

SMA Type 1

SMA type 1, also known as Werding-Hoffmann disease, manifests with poor head control, hypotonia, and decreased or absent tendon reflexes in infants before the age of 6 months. These infants, often categorized as non-sitters, are unable to achieve independent sitting without assistance due to profound weakness and hypotonia. The characteristic "frog legs" posture results from weakness or absence of head control. Weakness in the intercostal muscles leads to a bell-shaped chest structure and sometimes paradoxical breathing, known as "belly breathing," due to the relatively spared diaphragm.

In SMA type 1 infants, fasciculation in the tongue is common due to weakness in the tongue and swallowing muscles. Facial weakness may develop in later stages of the disease. The risk of aspiration and failure to thrive increases as the tongue and pharyngeal muscles weaken. Respiratory failure typically occurs before the age of 2, necessitating ventilatory support. Despite profound physical weakness, infants with SMA type 1 typically have normal cognition, remaining alert and intellectually intact at the time of diagnosis.

SMA Type 2

SMA type 2, also referred to as intermediate SMA, encompasses infants with onset between the 7th and 18th months of life. In this group, some infants are able to sit without support, while others may gain the ability to stand but cannot walk independently. Fine tremors are common in the upper extremities, and deep tendon reflexes are typically absent.

Kyphoscoliosis and joint contractures are frequently observed, particularly in patients with more severe SMA type 2, and may manifest within the first years of life. Although poor swallowing is less common in SMA type 2 patients, weakness of the chewing muscles can affect their ability to chew effectively. The severity of SMA type 2 varies widely among patients. Thinner children with SMA type 2 tend to have greater difficulty sitting without support, experience respiratory symptoms earlier, and may develop scoliosis sooner. Conversely, relatively stronger children with SMA type 2 exhibit greater trunk, limb, and respiratory muscle strength. However, even in these cases, respiratory failure requiring mechanical ventilation may still occur, particularly in patients on the weaker end of the spectrum.

SMA Type 3

SMA type 3, also known as Kugelberg-Welander disease; It manifests itself with slowly progressive weakness, muscle density, and atrophy in the lower extremities after gaining the ability to move independently. Patients with SMA type 3 disease are ambulatory, that is, they have the ability to stand or walk without support, but the majority of patients lose their ability to walk over time. It is difficult to diagnose children with SMA type 3 disease because the symptoms of the disease mimic muscular dystrophy. Although the course of SMA type 3 is slow, the disease progresses over time. The incidence of SMA type 3 is less than other SMA types (<15%). SMA type 3 disease occurs in babies after the 18th month. Children first experience difficulties such as getting up from the ground, having difficulty jumping, running or climbing stairs. Distal limbs are also affected, showing hand tremors, weakness of forearm muscles, pes cavus deformity in the feet, and short and rapid movements of the fingers due to fasciculation. The reason why walking distances become shorter over time in children with SMA type 3 disease is that waddle gait occurs due to pelvic muscle stiffness, causing fatigue in children. Loss of walking movement in SMA type 3 patients occurs as children gain weight due to rapid growth during adolescence. In SMA type 3, unlike SMA types 1 and 2, tongue fasciculation is not seen until the later stages of the disease. Respiratory muscle weakness symptoms are less common or absent in patients with SMA type 3. Visceral involvement, which is common in SMA type 3, is rare. Absence of osteotendosis, hypotonia and hyperlaxity reflexes are typical features. Unlike what is seen in SMA types 1 and 2, visceral involvement, which is common in SMA type 3, is rare. Absence of osteotendosis, hypotonia and hyperlaxity reflexes are typical features. Their lifespan is normal compared to SMA type 0, 1 and 2 disease.

SMA Type 4

SMA type 4, also known as adult SMA, occurs when SMA disease, typically seen in children, manifests in adults. SMA type 4 is diagnosed when the symptoms of SMA appear after the age of 18, with disease onset typically occurring after the age of 30. This type of SMA is the least common (2-5%) and tends to progress slowly with relatively mild characteristics.

The slow progression of type 4 SMA is attributed to the higher copy number of SMN2 (4-8). Patients with SMA type 4 commonly experience atrophy with proximal weakness, with the initial symptom often being hyperlordosis and difficulty squatting or standing due to symmetrical weakness in the thigh and leg muscles, resulting in a duck-like gait. Notably, involvement of the quadriceps femoris muscle is particularly pronounced.

Individuals with SMA type 4 may walk slowly and experience fatigue more readily compared to their healthy counterparts. Fasciculation occurs in approximately 75% of cases, while muscle cramps are less common but may be present. Rare symptoms include bulbar findings, respiratory muscle weakness, and scoliosis. Although wheelchair use may become necessary in severe cases, it is uncommon due to the relatively mild nature of the disease progression.

GENETIC BASIS

The survival motor gene (SMN), responsible for SMA disease, was discovered in 1995 and subsequently located on chromosome 5q11.2-q13.3 (Figure 1) (5-9). Interestingly, chromosome 5q13 harbors an almost identical copy of the SMN gene. The distinction between the telomeric (SMN1) and centromeric (SMN2) genes within this region is crucial in determining spinal muscular atrophy (5-9).

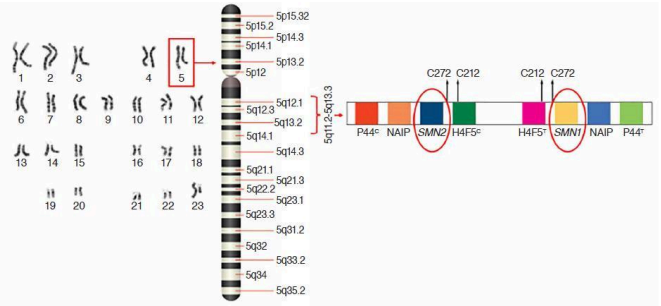

Figure 1. The location of the SMN protein-encoding genes on chromosome 5q (Rao et al, 2018).

SMN2, distinguished from SMN1 by a single nucleotide (840C>T) in its coding sequence, undergoes alternative splicing of exon 7, resulting in mRNA transcripts with exon 7 missing (referred to as SMN-del7). This alteration does not affect the amino acid sequence but leads to the production of a shorter and less stable protein, as well as reduced levels of full-length transcripts (SMN-fl) and protein (5-9).

In approximately 95% of cases, homozygous disruption of SMN1 occurs due to deletion or gene conversion, while compound heterozygotes, comprising around 3% of affected individuals, possess one SMN1 allele deletion along with minor intragenic alterations. However, each patient retains one or more copies of SMN2, typically ranging from 2 to 4.

Although the pathophysiology of SMA involves the loss of SMN1, the severity of the disease is largely determined by the number of SMN2 copies present. SMA type I patients commonly have two copies of SMN2, while SMA type II patients often have three copies. In contrast, SMA type III and IV patients typically possess three or four copies of SMN2 (5-9).

The SMN protein, encoded by SMN genes, exhibits widespread expression and is present in both the cytoplasm and nucleus, with particularly high levels in spinal cord motor neurons (10-15). Within the nucleus, the SMN protein is localized within structures known as "gems" (gemini of coiled bodies), which are dot-like formations associated with coiled (Cajal) bodies.

While the exact biological function of the SMN protein in the pathophysiology of spinal muscular atrophy (SMA) is not fully elucidated, cells from SMA patients typically exhibit fewer gems compared to those from unaffected individuals and carriers (16-19). This observation suggests a potential role for the SMN protein and gems in the disease process, although further research is needed to fully understand their significance.

NUSINERSEN (SPINRAZA)

Preclinical Discovery of Nusinersen

The first medication approved for treating spinal muscular atrophy (SMA) in both adults and children is nusinersen, also known by the trade name Spinraza (20-25). Nusinersen is an antisense oligonucleotide designed to increase the expression of the SMN protein, which is deficient in SMA. It received approval from the US Food and Drug Administration in late December 2016 and from the European Medicines Agency in June 2017.

The development of nusinersen involved years of preclinical and clinical research. In a pivotal study in 2006, researchers identified an intron-splicing silencer N1 sequence within intron 7 of the SMN2 gene (20-25). This sequence was found to enhance exon 7 skipping and the production of SMNΔ7, a shorter form of the SMN protein. Subsequent investigations revealed that antisense oligonucleotides targeting the intron-splicing silencer N1 region in the SMN2 pre-messenger RNA could promote the generation of full-length SMN protein and increase exon 7 inclusion.

Further studies demonstrated that antisense oligonucleotides directed at the intron splicing silencer N1 region could extend lifespan in a mouse model of severe SMA. These findings paved the way for the development of nusinersen as a targeted therapy for SMA, ultimately leading to its approval for commercial use.

Clinical Development of Nusinersen

The transition from preclinical to clinical studies for nusinersen involved several phases of research. Initially, an open-label phase 1 to 2 study was conducted for patients with type 1 SMA, focusing on assessing the safety, tolerability, and pharmacokinetics of intrathecal dosages of nusinersen (20-25). This trial involved testing four different dosages of nusinersen (1 mg, 3 mg, 6 mg, and 9 mg) on 28 individuals to determine their safety profiles.

Following the phase 1 study, an open-label extension was conducted for patients with types 2 and 3 SMA. This extension study allowed for further evaluation of the safety and efficacy of nusinersen in a broader spectrum of SMA patients. Subsequently, a double-blind placebo-controlled study was conducted for patients with types 1 and 2 SMA to rigorously assess the efficacy of nusinersen. This study aimed to determine whether nusinersen treatment could improve motor skills and increase survival rates in patients with SMA. During these clinical trials, notable improvements were observed in juvenile SMA type 2 patients receiving nusinersen over a 15-month therapy period. Additionally, an open-label phase 2 trial involving 20 pediatric patients with SMA type 1 demonstrated that nusinersen therapy could enhance motor skills and increase survival rates in this patient population. These findings collectively supported the progression of nusinersen through clinical development towards regulatory approval for the treatment of SMA.

The treatment landscape for spinal muscular atrophy (SMA) varies depending on the disease subtype. Here's an overview of the current understanding of treatment options for each SMA subtype:

SMA Type 0: Nusinersen has not been utilized for individuals with SMA symptoms appearing at birth or within the first week of life, nor for children with a single copy of SMN2.

SMA Type 1: In a Phase 3 clinical trial, infants treated with nusinersen demonstrated significant improvement in motor function within six months of treatment initiation, meeting the primary endpoint of the trial early.

SMA Type 2: Patients with SMA type 2 treated with nusinersen have shown an increase in scores on the Hammersmith Functional Motor Scale (HFMSE), indicating improved motor function. This increase in motor function has also been associated with improved independence in daily mobility and increased survival rates.

SMA Type 3: While Spinraza (nusinersen) has demonstrated superiority over placebo in terms of achieving new milestones such as walking with assistance and standing alone, it has also shown statistically significant improvements in changes from baseline motor function in patients with SMA type 3.

SMA Type 4: There is currently no published information available on the use of nusinersen in individuals with SMA type 4.

Overall, nusinersen has shown promising results in improving motor function and achieving developmental milestones in patients with SMA types 1, 2, and 3. However, further research is needed to explore its efficacy and safety in other SMA subtypes, such as type 0 and type 4.

ONASEMNOGENE ABEPARVOVEC (ZOLGENSMA)

Preclinical Discovery of Onasemnogene Abeparvovec

Gene therapy has marked a significant milestone with the development of onasemnogene abeparvovec-xioi (AVXS-101), commonly known as Zolgensma, as the first treatment for SMA that addresses the underlying genetic cause of the disease (26-28). Zolgensma works by delivering a functional copy of the SMN1 gene, which is essential for producing the SMN protein, directly into the patient's cells.

The FDA has approved onasemnogene as a one-time intravenous infusion for SMA patients under two years old who possess mutations in both copies of the SMN1 gene. Additionally, patients with SMA type 1 who have two to three copies of the SMN2 gene but do not exhibit symptoms are also recommended to initiate treatment promptly.

Onasemnogene utilizes a nonreplicating adeno-associated virus 9 (AAV9) vector to deliver the SMN1 gene into the patient's cells. Once administered, the therapy provides a continuous expression of the SMN protein, compensating for the deficiency seen in SMA patients. Due to its design for sustained expression and rapid onset, the effects of onasemnogene are expected to persist long after administration. However, the duration of its efficacy beyond the initial treatment remains uncertain and requires further study.

Clinical Development of Onasemnogene Abeparvovec

Clinical studies have demonstrated the potential of Zolgensma (onasemnogene abeparvovec) to enhance motor function and improve survival rates in patients with SMA type 1 (26-28).

In the START study conducted on 15 patients with SMA type 1 between 2014 and 2017, 100% of the SMA type 1 patients survived at 20 months of age. While no significant improvement in motor function was observed in SMA type 1 patients receiving low-dose Zolgensma, 11 out of 11 SMA type 1 patients receiving high-dose Zolgensma were able to sit unaided for at least 5 seconds (26-28).

The STR1VE study, conducted between 2017 and 2022, involved 180 patients with SMA type 1 who were younger than 6 months old. By the conclusion of the research, 91% of treated patients had survived 14 months without experiencing any events. At 18 months, 59% of patients could sit for at least 30 seconds without assistance, and at 6 months, 91% of patients had increased their CHOP INTEND score by 14.6 points.

However, the effectiveness of intrathecal application of Zolgensma in patients with SMA type 1 is not yet fully understood (26-28). The STRONG study, conducted in 2019 on patients with SMA type 1 aged 6 to 60 months, revealed that Hammersmith Functional Motor Scale Expanded (HFMSE) scores increased by 6 points in patients with SMA type 1 between 24 and 60 months of age following intrathecal administration of onasemnogene abeparvovec. This suggests potential improvements in motor functions for SMA type 1 patients after intrathecal administration of Zolgensma.

Treatment of SMA type 1

Onasemnogene abeparvovec, also known as Zolgensma, is a gene therapy that has demonstrated efficacy in improving motor function and survival rates in patients with SMA type 1 (20-28). Patients treated with Zolgensma have shown a slowing down in the progression of SMA disease, leading to an improvement in their quality of life and prolonging survival. In infant patients with SMA type 1, early administration of Zolgensma has been associated with a rapid improvement in motor functions. This highlights the potential benefits of early intervention with gene therapy for SMA type 1 patients.

RISDIPLAM (EVRYSDI)

Preclinical Discovery of Risdiplam

Risdiplam, also known as Evrysdi, is the first oral medication approved for the treatment of spinal muscular atrophy (SMA) (20-28). It addresses the deficiency of survival motor neuron (SMN) protein caused by defects in the SMN1 gene. By targeting the SMN2 gene, risdiplam enhances the production of functional SMN protein. Clinical trials such as FIREFISH and SUNFISH have demonstrated that risdiplam improves motor function in patients of all ages, with these improvements being sustained even after 24 months of treatment. Risdiplam has shown good tolerability and a favorable benefit-to-risk ratio in these trials. As an oral medication, risdiplam offers a practical and beneficial treatment option for various subtypes of SMA and patients across different age groups. Additionally, as an SMN2 pre-mRNA splicing regulator, risdiplam can penetrate the blood-brain barrier, making it effective in treating SMA associated with SMN1 biallelic gene mutations.

Clinical Development of Risdiplam

In clinical trials, risdiplam has demonstrated the ability to increase levels of survival motor neuron (SMN) protein in the blood of patients with spinal muscular atrophy (SMA). For instance, in the phase 2/3 FIREFISH study, newborns with SMA type 1 who received risdiplam showed a significant increase in median blood SMN protein levels after 12 months of treatment. In the low-dose cohort, median SMN protein levels rose from 1.31 ng/mL at baseline to 3.05 ng/mL, while in the high-dose cohort, levels increased from 2.54 ng/mL to 5.66 ng/mL. Similarly, in the phase 2/3 SUNFISH study involving individuals with SMA type 2 and 3, those treated with risdiplam at therapeutic doses experienced a doubling or more in median SMN protein levels compared to baseline. This increase was sustained for up to 24 months of therapy. Ongoing studies like the phase 2 JEWELFISH trial are further investigating risdiplam's effects. Importantly, in patients previously treated with nusinersen or onasemnogene abeparvovec, risdiplam therapy resulted in at least a twofold increase in SMN protein levels compared to baseline, with sustained elevation observed after 12 months of treatment.

Treatment of SMA type 1

The open-label FIREFISH study aimed to assess the efficacy of risdiplam in treating infants with spinal muscular atrophy (SMA) type 1 (20-28). Eligible participants had two copies of the SMN2 gene, a bodyweight at or above the 3rd percentile for their age, and confirmed genetic diagnosis of 5q-autosomal recessive SMA (5q SMA). Enrollment criteria included infants aged 1 to 7 months. Patients who were able to swallow received risdiplam orally once daily, while those unable to swallow were administered the medication via a feeding tube.

The primary outcome measure after one year of treatment was the percentage of infants who could sit without assistance for at least five seconds, as determined by Item 22 of the Bayley Scales of Infant and Toddler Development – Third Edition (BSID-III) scale. Important secondary outcomes included motor milestone response based on Hammersmith Infant Neurological Examination – Module 2 (HINE-2) criteria, an increase of ≥ 4 points from baseline in CHOP-INTEND score, achievement of a score of ≥ 40 on the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) scale, and event-free survival, defined as remaining alive without continuous ventilation. The significance of the results was determined based on a predetermined performance requirement of 5%, as derived from natural history data.

Treatment of SMA type 2 and 3

The study included eligible patients aged 2 to 25 years who were non-ambulatory and presented clinical symptoms consistent with either type 2 or type 3 spinal muscular atrophy (SMA), along with a genetically confirmed diagnosis of 5q-autosomal recessive SMA.

Results from the study indicated that risdiplam treatment resulted in improved motor abilities among these patients. Significant enhancements were observed in MFM32 (Motor Function Measure 32) and RULM (Revised Upper Limb Module) scores, with the most substantial improvements noted in patients under the age of eighteen. Furthermore, patients treated with risdiplam demonstrated sustained or improved motor skills over the 12-month therapy period, and these results were consistent with the outcomes observed at the 24-month mark. However, individuals aged 18 to 25 did not exhibit the same level of improvement.

Compared to the placebo group, patients receiving risdiplam showed more pronounced and significant improvements in both RULM and MFM32 scores. These findings suggest that risdiplam therapy may offer benefits for individuals diagnosed with SMA type 2 and type 3.

BRANAPLAM

Preclinical Discovery of Branaplam

Branaplam, an mRNA splicing corrector, is designed to promote the production of functional survival motor neuron (SMN) protein and full-length SMN2 mRNA by modulating SMN2 splicing (20-28). This modulation occurs through the sequence-selective enhanced binding affinity of U1 small nuclear ribonucleic protein (snRNP) to the 5′ splice site of SMN2 in the presence of branaplam. By interacting with SMN2 pre-mRNA, branaplam, a pyridazine derivative administered orally, facilitates exon 7 inclusion, thereby increasing the quantity of functional SMN protein.

Preclinical studies in mice have indicated that branaplam is likely to be distributed similarly in humans. However, it is important to note that branaplam has exhibited adverse effects both in vivo and in vitro. These effects include aneugenic effects and cell-cycle inhibition. Further research and clinical trials are necessary to fully understand the safety and efficacy profile of branaplam in humans.

Clinical Development of Branaplam

The clinical research on branaplam, initiated in 2014, aimed to evaluate its safety, tolerability, early efficacy, pharmacokinetics, and pharmacodynamics in infants with SMA type 1, a severe form of the condition, who were less than six months old (20-28). The trial involved administering increasing dosages of branaplam once a week to determine the maximum tolerated dosage in the first phase. Subsequently, the second phase would involve a new cohort of patients to evaluate up to three dosages based on the information gathered.

However, recruitment for the trial was halted by Novartis in 2016 due to safety concerns arising from animal studies conducted during the trial. These concerns included reports of adverse effects on the testes, renal blood vessels, spinal cord, and nerves in animals receiving a daily regimen of branaplam. After modifying the study's design, which included incorporating additional safety measures such as nerve testing and allowing oral administration of the therapy, enrollment was permitted to resume in late 2017.

The recruitment process concluded in May 2019, with 13 infants enrolled in part 1 and 25 in part 2 of the trial. Subsequently, 29 babies received the treatment, some for more than four years, and the company reported positive progress in the research later that year. However, the results of the ongoing trial have not been disclosed to the public.

ADVANCE OF GENE AND RNA THERAPY OF SMA

Gene therapy of SMA

Onasemnogene Abeparvovec (Zolgensma)

The FDA approved the use of Zolgensma in 2019 for infants under the age of two whose genetic testing verified their diagnosis of SMA. Gene replacement therapy (GRT) was developed for Zolgensma as a result of the disease's genetic basis becoming understood. Notably, Zolgensma penetrates the blood-brain barrier, unlike nusinersen, and

one intravenous infusion every hour is sufficient for the systemic production of SMN protein (20-28).

Using a ubiquitous promoter, Zolgensma delivers the transgene into motor neuron cells via a non-replicating adeno-associated virus capsid (scAAV9). To far, more than a dozen distinct AAV serotypes have been identified. They vary in immunological response capability, transduction effectiveness, and cell tropism based on the kind of capsid surface proteins. In therapeutic applications, all AAVs combine low immunogenicity and decreased pathogenicity with long-term transgene expression. They can also transduce specific dividing and nondividing cells. Furthermore, a large number of AAVs are capable of transducing neurons and glial cells, which makes it possible to employ vectors made from them to treat neurodegenerative illnesses. In this case, we can differentiate between serotype AAV2, which is exclusive to cerebral endothelial cells, and serotype AAV9, which, upon systemic treatment, causes high expression in the motor cortex, cerebellum, substantia nigra, and cervical spinal cord neurons.

However, these advancements come with limitations. One significant restriction is the production of neutralizing antibodies, which often reduces the efficiency of AAVs. Moreover, the target specificity of AAVs is constrained by the presence of receptors for multiple AAV serotypes in various organs. Nevertheless, the use of an organ-specific promoter can enhance the gene expression specificity of AAV transmission. It's noteworthy that AAVs demonstrate superior transgene expression levels and safety profiles compared to LV vectors. Additionally, AAVs mitigate the risk of insertional mutagenesis due to their enhanced genome stability and increased vector propagation, primarily occurring as extrachromosomal episomes.

The effectiveness of Zolgensma has been established through studies such as START and STR1VE. Participants in the START trial did not require continuous mechanical ventilation and survived for at least 20 months. This marked a significant improvement compared to a previous cohort, where only 8% of patients survived beyond the 20-month mark without continuous ventilation, indicating substantial progress. Those in the historical cohort also showed better motor function, longer event-free periods, and quicker attainment of milestones. Over a three-month period, children receiving higher dosages experienced an average increase of 15.4 points on the Children's Hospital of Philadelphia Neuromuscular Disorders Infant Test (CHOP-INTEND). Among the high-dose group, 92% could sit without assistance, 92% could speak and swallow food, 75% could turn over, and 17% could walk independently. Subsequent studies revealed that children receiving early GRT with Zolgensma exhibited a greater increase in CHOP-INTEND scores compared to older patients.

The STR1VE trial included patients with the SMN1 mutation who possessed one or two copies of SMN2 from diverse geographic locations, aiming to evaluate the safety and efficacy of Zolgensma. By comparing outcomes from 22 patients in the USA (STR1VE-US) with data from cohorts reflecting the natural history of the disease, the effectiveness of gene replacement therapy (GRT) was confirmed. Eighteen months post-treatment, 59% of patients could sit without assistance for at least 30 seconds, and 91% did not require ongoing respiratory support at 14 months. Furthermore, research has shown that the therapeutic benefits of Zolgensma outweigh potential risks of adverse effects.

Currently, clinical trials are underway to explore the long-term safety and efficacy of intrathecal administration of Zolgensma, as well as the therapeutic potential of various delivery routes.

Treatment	Modality	Indicated Population	Pros	Cons
Nusinersen	*SMN2* mRNA splicing modifier	Infants to adults	Option available for adults, maintenance intrathecal injection every 4 mo	Expensive, non-systemic delivery, complicated administration
Risdiplam	*SMN2* mRNA splicing modifier	≥2 mo	Systemic delivery, simple oral administration	Numerous adverse events, possibly due to off-target splicing effect
Onasemnogene abeparvovec	*SMN1* gene replacement via AAV9 vector	<2 y	Systemic delivery, simple one-time injection	May not be effective in presence of anti-AAV antibodies; hepatotoxicity risk

Abbreviations: AAV, adeno-associated virus; FDA, US Food and Drug Administration; mRNA, modified RNA; SMA, small muscular atrophy; SMN, survival motor neuron.

Table 2. Therapies Approved by the FDA for Treatment of SMA

RNA therapy of SMA

Nusinersen

Nusinersen became one of the earliest medications approved by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for treating SMA, with approvals granted in December 2016 and June 2017, respectively (Table 2) (20-28). Studies validating the effectiveness of Nusinersen include ENDEAR, NURTURE, CHERISH, and DEVOTE.

In the ENDEAR trial, newborns treated with nusinersen exhibited significant improvements in motor milestone achievement compared to controls, as assessed by the Hammersmith Infant Neurological Examination (HINE). These children also had a higher likelihood of event-free survival, indicating a longer period before requiring assisted ventilation or experiencing mortality.

Results from the 25-month analysis of the NURTURE study were promising when compared to expected outcomes from the natural disease progression. Each patient survived this duration without requiring continuous ventilation. Furthermore, research has shown that 88% of patients, with an average age of 34.8 months, are capable of walking independently, while 92% can walk with the assistance of a caregiver. All patients survived the study period.

After 15 months of treatment, children receiving nusinersen showed an average increase of 4 points on the Hammersmith Functional Motor Scale-Extended (HFMSE) scale, as indicated by analysis of data from the CHERISH clinical trial.

The efficacy and safety of nusinersen have been validated through the aforementioned clinical trials (20-28). Ongoing research, such as the DEVOTE clinical study, is investigating the efficacy and safety of higher dosages of nusinersen. Following DEVOTE, an open-label ONWARD trial is assessing the long-term effects of increased nusinersen dosages.

Recent research has also focused on identifying novel biomarkers to assess patient improvement. Studies suggest that miRNAs may play a significant role as key modulators of SMN-mediated biological pathways. Additionally, inflammatory molecules are being explored as potential therapeutic targets and reliable biomarkers for patient classification, disease progression prediction, therapy response monitoring, and ultimately, improved care for SMA patients.

In a study involving 21 individuals with SMA types 2 and 3, nusinersen was found to decrease skeletal muscle-specific miRNA levels, which are implicated in the pathogenic process of neuromuscular diseases. The improvement in patients' motor functions, as measured by the HFMSE, correlated with the downregulation of these miRNAs. Furthermore, nusinersen therapy demonstrated potential benefits for the peripheral immune system, as evidenced by decreased blood levels of pro-inflammatory cytokines from the Th1/Th17 pathway in 12 SMA patients after six months of treatment. Notably, IL-10 has been identified as a potential biomarker for treatment monitoring, while miR-133a and IL-23 molecules hold promise as predictive biomarkers of nusinersen therapy.

Risdiplam (Evrysdi)

Oral administration of risdiplam (Evrysdi) represents a significant advancement in SMA treatment, especially for patients with SMN1 mutations (20-28). Approved by the FDA on August 7, 2020, risdiplam is now authorized for the management of SMA across all age groups, including infants as young as two months old. Risdiplam functions as an SMN2-directed splicing modifier, targeting exon 7 and the 5′ splice site of intron 7 in the SMN2 transcript to enhance exonic splicing.

By facilitating the inclusion of exon 7, risdiplam promotes the production of full-length SMN protein (FL-SMN). Additionally, risdiplam impacts genes like FOXM1 and MADD, which play roles in apoptosis and cell cycle regulation. Risdiplam binds to splicing modulators of the pre-mRNA SMN2 complex, such as KHSRP and FUBP1, further facilitating SMN2 splicing activation.

One of risdiplam's key advantages is its ability to cross the blood-brain barrier, allowing it to increase SMN protein levels not

only in peripheral organs but also in the central nervous system. Administered orally, risdiplam is typically taken either before or after a meal.

Clinical studies such as FIREFISH, SUNFISH, JEWELFISH, and RAINBOWFISH have demonstrated the efficacy and safety of risdiplam in SMA patients, further supporting its use as a therapeutic option for SMA management (20-28).

The FIREFISH trial, a multicenter clinical study, was initiated to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of risdiplam in its first phase. The cohort receiving the higher dosage demonstrated increased levels of SMN protein in their blood. Notably, seven infants in the high-dose cohort were able to sit without assistance for at least five seconds, whereas none in the low-dose group achieved this milestone. In the natural history of infantile-onset SMA, the median age of survival is typically around 8 months. However, in the FIREFISH trial, two infants in the high-dose group achieved significant milestones on the HINE-2 gait examination, and 14 children in the group attained a CHOP-INTEND score of 40 or higher at 16 months.

The efficacy and safety of the increased dosage were further confirmed in the trial's second phase (20-28). Among the 41 patients enrolled, 38 were still alive after 12 months, with only three requiring ventilator support. Additionally, twelve children were able to sit without assistance.

In the SUNFISH trial, which included patients with delayed onset of symptoms, the investigation began with dosage escalation. Motor function significantly improved over 24 months, as evidenced by better Motor Function Measurement (MFM) scores compared to the control group. The chosen risdiplam dosage from the first phase was then compared to a placebo in the trial's second phase. Patients receiving risdiplam exhibited notably greater improvements in Motor Function Measure 32 (MFM32), Hammersmith Functional Motor Scale Expanded (HFMSE), SMA Independence Scale (SMAIS), and Revised Upper Limb Module (RULM) scores after 12 months of treatment compared to the placebo group.

In the multicenter open-label trial JEWELFISH, individuals diagnosed with 5q-autosomal recessive SMA undergo assessment for risdiplam's pharmacokinetics, pharmacodynamics, efficacy, and safety (20-28). Following a 12-month treatment regimen, patients showed increased levels of SMN protein in their blood.

The ongoing RAINBOWFISH study is recruiting pre-symptomatic infants with genetically confirmed SMA from birth to 42 months of age. The study aims to evaluate pharmacodynamics, pharmacokinetics, safety, and efficacy. Findings from this study will provide valuable insights into the effects of treatment in young infants with SMA and aid decision-makers in determining the significance and value of pre-symptomatic treatment for children. Additionally, the study will help determine the most appropriate method for administering risdiplam therapy during the pre-symptomatic phase.

CONCLUSION

Recent advancements in treating SMA have significantly increased the synthesis of SMN protein. These treatments include SMN gene replacement therapy, such as onasemnogene abeparvovec, and the modulation of SMN2 gene splicing using medications like nusinersen and risdiplam. Despite these breakthroughs, there are still unmet needs, such as addressing the impact of SMA on peripheral tissues and achieving developmental milestones appropriate for the patient's age. Additionally, greater improvements in motor function may be achieved through complementary approaches that target the entire motor unit. Furthermore, ongoing developments in treatments like Branaplam offer promising prospects for the future of SMA therapy.

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