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Molecular Updates in Gene Therapy of Liver Diseases

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***Abstract*: Gene therapy is an advanced treatment approaches which are involved in insertion of genetic materials to the target cells for diseases management. It involves correcting defective genes that are appropriate for treating diseases caused by single gene failure in particularly suitable rare diseases e.g., cystic fibrosis, adrenoleukodystrophy beside liver diseases and cancers. It is looking forward to transfigure modern medicine for cure of numerous inherited metabolic liver disorders. Urgently, effective and continuous advanced biotechnology is required in developing nanoparticles vehicles, deliver mRNA biomolecules and target host cell genome. This is to reinsert the missed gene expressions, functional proteins within the target cells and disabling many obstacles. Gene editing aims to change the microorganism genetic material of DNA. It permits insertion of genetic materials, deletion, or alterations within a particular location within the gene. Many procedures have been investigated for getting gene editing. Currently, CRISPR-Cas9 is well designed DNA editing system and identified as regularly interspaced short palindromic repeats and associated with protein-9. The CRISPR-Cas9 system is cheap and fast, with high accuracy and efficiency comparing to other genetic editing tools. Molecular progress will avoid stimulating innate immunological responses. Many research studies started clinical trials based on the allowed advance in nanotechnology and molecular biology tools. They highlighted therapies to treat such liver disorders with safe and effective approaches. This study summarizes and discusses the progress in liver diseases gene therapy with a hope for success of clinical trials applications through advanced therapeutic potentials and gene editing.**

***Keywords*: gene therapy, monogenic and polygenic disorders, CRISPR-Cas9, genome editing delivery system, recombinant adeno-associated virus vectors, exosome, genetic disorders, liver diseases, hemophilia, FDA.**

Abbreviations

Gene therapy (GT), Monogenic disorders (MD), hematopoietic stem cells (HSC), heat shock proteins (HSP47), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR-associated) nucleases systems, TT-lipid containing LNPs (TTLLNs), mobile genetic elements (MGEs), Develop recombinant adeno-associated virus (rAAV).

1. Introduction

Gene therapy (GT) based on shifting the affected genes inside the human organ to control and manage the disease by the way of improving the organ's ability to attack a wide series of diseases, such as liver diseases, cancer, diabetic problems, hemophilia, cystic fibrosis, AIDS, heart disease, and management related to most inherited metabolic liver disorders [1].

GT started with understanding and recognition of DNA used in transformation process to change the genome code and the phenotype related body systems disorders. It is known that viral oncogenes can be inserted into the human genome leading to new engineered genome that code the viral proteins, including human hematopoietic stem cells (HSC) [2]. Proper engineered vectors are able to effectively transducing HSC safely. Successful GT requires developing vectors, design effective materials, methods, equipment and effective biotechnology tools to be able to manipulate and exchange genomic DNA, improving, acquiring, purifying and maintaining of cultures HSC [3].

Clinical approaches-based gene editing technologies of recombinant adeno-associated virus (rAAV) vectors related management of liver inherited disorders will have promising consequences in GT. But the DNA translational processes related GT are still unclear. This is because of raising patient's immune system interactions with the vector conditions, efficiency of management duration and safety. In addition, it is suggested that GT based rAAV with a highly used doses is suggesting more innate immune responses effects. Similarly, the genomic integration of rAAV and the edited gene sequences will raise the probability for getting carcinogenicity risks [4].

Currently, GT is accessible only for clinical trials, more excessive understanding of the preparation of patients and tools were required to enhance engraftment manipulations of the modified HSC [5]. Challenging ideas in essential clinical and experimental trials are assessing the efficacies and causes related adversative events has been advanced. Generally, abnormal proteins production in the infected cells would demonstrate cells to be fatal causing diseases. Therefore, the dented bio- products of proteins are substituted by insertion of genetic engineered DNA into these cells based on GT tools and

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techniques. It is urgent to consequence the behavior in clinical trials, observation of achievements and difficulties happening in performing these trials [6]. Many experimental research studies provide a new approach in gene therapy with mRNA using multi-gene editing strategies as novel trend for management of rare genetic metabolic disorders leading to high morbidity with limited chances of therapy [7].

Mainly, the liver takes part in biosynthesis of many biological materials e.g. bile salts, plasma proteins and detoxifications process. In addition, it is responsible for storage of glycogens, fat soluble vitamins and hormonal regulations. In addition, it takes part in metabolic pathways of iron carbohydrates, fat and proteins [8]. Thus, liver organ is considered the original site for various inherited metabolic disease related to protein secretions as evaluated and characterized in hemophilia [9]-[14]. Research studies explored in vivo and in vitro results of codon and non-coding transgenic sequences are revealing a clear deal about vector constructions, mapping design and constructions of long-lasting diseases corrections within many of animal models [15], [16].

Consequently, the liver organ is the major target for GT. GT tools supply genes for getting treatment of lost functions related to diseases. Developing new approaches for getting precised modified cellular genomes [17] and silencing the defective genes causing pathology [18]. New system for DNA sequences editing biotechnology [CRISPR–Cas9] was developed which is the best exciting scientific discoveries of the recent decade. It is looking forward giving promising improvements of versatility and precision via base editing.

The main target for GT development for achieving the allowed targets are; i) Improvement to understand genetic causes of different inherited diseases -based on advances in sequencing data base of different bioinformatics molecular tools. ii) More clearance for genetic vectors delivery among cytotoxicity, pharmacokinetics and stability iii) Developing more efficient genetic-based in vitro and in vivo therapeutic strategics models. iv) Developing advanced tools for gene delivery into the target tissues. v) Saving wide scale for viral vectors manufacturing skills [19].

Liver gene therapies updates avoid all complications like immune response and cloning mutagenesis-based nanotechnology tools during application. It clears the importance of non-coding segments of a genome, thereby enchanting gene therapy efficiency [20]. GT is intermediated by rAAV vectors as a new trend of therapeutic choices among genetic liver diseases. The most recorded wide experience has been expanded in management of hemophilia. In such disorder there is no liver pathologic lesion, but it is noted that the liver is not able to synthesize the coagulating factors related to blood clot formation.

Current issue discusses gene therapy in liver disease based on nanotechnology for different RNA and DNA sequences editing tools in research and focusing on both the efficacy and safety of therapeutic tools.

2. Strategies of Gene Therapy

Recently, conventional GT based on supplementation of gene-based adeno-associated viral vectors [21]. Various strategies for GT that have been experienced in vitro experiments and clinical tails levels based on encapsulated therapeutic mRNA and gene editing [22]. These include i) replacement of a transmuted genes that originate the present disorders based on a strong normal gene copy, ii) disabling transmuted genes that causing the pathogenesis, iii) presenting an original gene coding a therapeutic material to contest diseases, iv) supply of a converting enzyme to the target organ cells to inactivate its cytotoxic metabolite [23].

Experimental in vivo cells treatment procedures necessitate surgical tools to get cells, then extend and infest them within viral vectors. The DNA holders comprising missed functional gene is implanted within the vectors for transmission, then target cells are returned to the operating system. The direct transports to vivo require accessing the target tissue to avoid gene overexpressing, DNA cassettes deliver proper inhibitors based on small inhibitory RNA or antisense oligonucleotides [24].

Gene or DNA cassette or DNA holder system contains a gene and a recombination site. It is a form of transferrable genetic component that contains a genetic sequence with a site for recombination of the new DNA sequences edit. It is usually holding a single gene with 500–1000 base pairs of DNA sequences. They are incorporated into the intron or freely as circular DNA.

3. Monogenic and Polygenic Disorders

Till now, GT technologies are experimental approaches in which therapeutic genes are used for treatment or prevention of various disorders, including monogenic and polygenic disorders and cancers. They comprise detection of mutant genes causing disorders with giving therapeutic genes to patients target cell to reinvest the normal functions. Last decades, research studies investigated many genetic tools and gene transforming systems to manage monogenic diseases among gene therapies. Many problems have been discovered that are related to gene expression, immunogenicity, inefficient delivery, capacity limitation, genomic integration, limited tissue specificity and toxicity [25].

Monogenic disorders (MD) result from mutations in a single recessive or dominant gene with interruption of normal arrangement. They are characterized as “Sex-linked or Autosomal” They include Hemophilia, Osteogenesis Imperfecta, ichthyosis, thalassemia and others [26], [27]. Monogenic recessive disorders need coding genes for expression. Although they often show effective therapy levels, they are lower than those in normal cases. Monogenic dominant diseases involve abnormal gene to be silenced. Thus, replacement of dysfunctional gene with the corresponding healthy one will direct to achievement of the cure [28].

Polygenic disorders arise more frequently in human than monogenic disorders. They result from effective collective actions or interactions of multiple genes. It is noted that genetic interactions complication doesn’t follow the equivalent method related monogenic disorders. Non genetic factors are a part of multiple gene diseases that participate in the disorders

manifestation with increased risk of evolving the disease e.g. coronary heart diseases, autoimmune diseases, obesity, liver hypertension, cancers, atherosclerosis and others [29].

4. Genomic DNA and Recombinant cDNA in Gene Therapy

Active GT protocols depend on significant selected transgenic DNA sequence. Many research studies on gene delivery based on the synthetic recombinant cDNA with limited length sequences and bases. This to fit different virus-related vectors e.g. lentiviruses, retroviruses and adeno associated virus [30], [31].

The most common advantages for recombinant cDNA are i) short sequences, ii) lack of the original gene's controlling base sequences, promoters, enhancers, introns, and/or poly(A) sequences for promotion of experimental transgenic animals [32]. In addition, it is related to some disadvantages like i) causing transgenic nonsense and overexpression of proteins [33], ii) failure for getting applicable and functional gene expression within the target cells [34], [35]. By the same way, the advantages of using genomic DNA applications-based exons and introns or any other non-coding regions are i) saving capacity to improve nuclear mRNA constancy with transportation for getting pure spliceosome complex, ii) getting transgenic sequences like chromatin conformation that prevents protein access to precise DNA regions, to control DNA replications, to support DNA repairs and transcriptions [36], iii) some introns may raise recombinant proteins synthesis [37], iv) improving transcriptional effectiveness among experimental transgenic animals [38], [39], v) removal of several cDNA deficiencies related to nonsense gene, loss of regulatory elements and down regulation pathways by chromatin [40], vi) transgenic locus of the genome protects cells from destructive effects of overexpression's with long term of regulations of transgenic expression [41]. Therefore, practice with transgenic genomes results in more further tissue specificity, endogenous and physiological gene expression regulation based on native elements of regulations. However, genomic DNA with non-coding sequence elements act as predicted talented strategies for disabling many obstacles among GT procedures.

5. Liver Disorders Gene Therapy

In last decades, liver transplantation was the only therapeutic handling. There were significantly drawbacks among orthotropic liver transplantation, [42] with investigating alternate therapeutic methods. Chronic liver disorders directed and encouraged developing future rapid treatment manipulations with stem cell. There are two main mechanisms getting treatment i) with progenitor cells or donor stem cells, ii) replacement of damaged cells function with getting differentiation into practical cells, iii) constructing bioactive factors that induce proliferation, developing progenitor cells and immunoregulatory factors that control inflammation [43].

Liver fibrosis is a consequence of recurrent and persistent hepatocellular damage with getting inflammations. It stimulates extra liver immune cells causing stimulation of hepatic stellate cells. The consequence of determined liver tissues damage over several years causes permanent scarring and liver failure terminating in liver cirrhosis [44]. Liver fibrosis and cirrhosis treatment are based on different types of stem cells, i) bone marrow mesenchymal stem cells, ii) mesenchymal stem cells, iii) umbilical cords mesenchymal stem cells and iv) liver-derived mesenchymal stem cell. Stem cell-based therapy is less invasive among patients more than surgical tools with little risks of immune system rejection compared to traditional treatment tools [45].

The liver organ is the key target for GT, representing different metabolic disorders inheritance of single-gene or multifactorial etiologies and hepatocellular carcinoma. In the past, there was doubt around the clinical values due to efficiency, toxicity, specificity and limits around the immune system responses [46]. Currently, there is a great improvement in vector technologies and molecular biology techniques. Future perceptions of GT protocols in hepatology based on significant information related in-vitro experiments and different clinical trials are expected [47].

CRISPR/Cas9 is a new trend for gene sequences editing biotechnology with getting an important achievement in treatment of various genetic disorders in experimental animal models [48]-[50]. Recently, it has been applied in the transthyretin amyloidosis research [51]. Cas9 ribonucleoprotein RNPs gene (Exosome^{RNP}) was loaded to purified isolated exosomes from hepato-stellate cells-based electroporation. Exosomes are considered extracellular re-formed vesicles within a wide of cells with conjugated with phospholipids bilayer structure known as a "cellular junk [52]. Exosome^{RNP} presented strong therapeutic actions among acute liver injuries, hepatocellular carcinoma and liver fibrosis based experimental mice models based p53 up-regulatory modulator of apoptosis (*PUMA*), K (lysine) acetyltransferase5 (*KAT5*) and cyclin E1 (*CcnE1*) respectively. It takes part in genome-editing delivery system as allowed in figure 1 [53], [54].

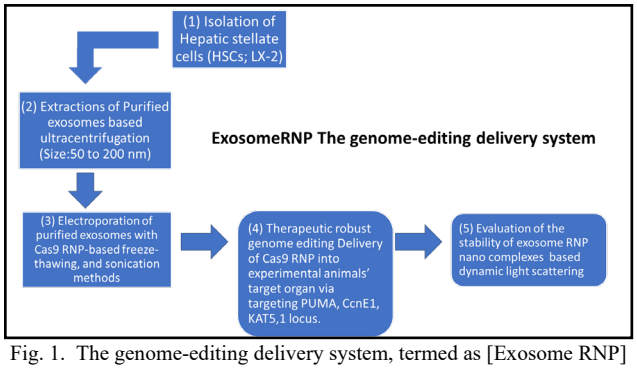

Fig. 1. The genome-editing delivery system, termed as [Exosome RNP] for loading Cas9 RNP into exosomes from hepatic stellate cells (HSCs; LX-2) for the treatment of different liver disorders

Heat shock proteins (HSP47) were applied in GT liver fibrosis. They are collagen-binding molecules sited in endoplasmic reticula. It takes part in collagen biosynthesis and depositions. HSP47 molecules have significant role in fibro genesis. However, HSP47 deficiencies in experimental mice take parts in development of liver fibrosis [55]. Methylmalonic

acidemia is one of rare metabolic liver disorders. Cases treatment based on dosed lipid nanoparticles (LNPs) carrying methyl malonyl-CoA mutase mRNA. There are three nanoparticles' vehicles delivering mRNA to hepatocytes, i) Mtx-LNP, LNP established by Moderna Therapeutics, ii) hybrid mRNA nanotechnology; including a micelle polymer for hepatocyte-specific delivering with endosomal escaping and an inert LNP that protects the mRNA, iii) TT-lipid containing LNPs (TTLLNs) [56].

In hepatocytes cells, LNPs encapsulate mRNA particles then transport into cytoplasm. This is because of LNPs contain four lipids, i) diffusible polyethylene glycol lipid (PEG), ii) an ionizable lipid, iii) cholesterol, iv) neutral helper lipid. Thus, lipid molecules are vital to protect from early phagocytosis capturing. In addition, Ionizable lipids enable cellular uptake of negative charged RNA particles forming mRNA/LNP complex then escaped into cytosol [57]-[58]. Continuous improvement of LNPs as delivery vehicles are required to increase endosomal escaping, to overcome liver injury and to provoke immunological responses [59].

Taking in consideration that liver hepatocytes play a critical role in synthesis of missed proteins responsible for hemophilia A and B. Treatment tools-based on nanoparticles for the transportation of mRNAs particles that are coding for clotting factors VIII and IX, in which hepatocytes secretions can be engineered to synthesize recombinant proteins into circulations [60]. Nanoparticles of LNP and TTLLNs were designed to treat hemophilia in mice using either single doses of them with encapsulating human FIX (hFIX) with hFVIII mRNA or different doses e.g. [0.25-0.50] mg / kg resulted in 12-h plasma levels of hFIX protein [61], [62].

Hemophilia B was recognized as an X-linked recessive genetic bleeding disease due to defects in the factor IX gene. Hemophilia B severity is classified by factor IX activity. It is subsequently causing a partial or complete deficiency of coagulation factor IX. Recently, The US Food and Drug Administration (FDA) has approved GT for Hemophilia B. [63]-[65]. Hemophilia A is still near to acceptance. several trials of rAAV-based liver-targeting GT are in advance with promising consequences [66]. We hope the treatment with gene therapy for hemophilia A will be soon achieved. It seems that we will see an effective GT for hemophilia A with rAAV vector soon in the future according to the current clinical research [67].

6. CRISPR-Cas Systems & Gene Supplementation and Gene Editing in Liver

There is a wide range progress for genome sequences technologies editing. Recently, three genome editing systems were discovered in the world, i) zinc finger nucleases (ZFNs), ii) transcription activator like effector nucleases (TALENs), and iii) clustered regularly interspaced short palindromic repeats nucleases systems (CRISPR-case) [68]-[70]. They are consider to completed correction of different pathogenic mutations, to get aid in immunotherapy by discovering critical genes for cancers then allow solving based problems in xeno-transplantation of organs [71].

CRISPR is an acronym for Clustered Regularly Interspaced Short Palindromic Repeat. The repetitive DNA sequences of CRISPR are detected in the bacterial genomes and a wide range of microorganisms. They act as a vital key component of the adaptive immunity for the bacteria and is responsible for protection against viral invasion-based destructions of viral genome as detected in figure 2 [72].

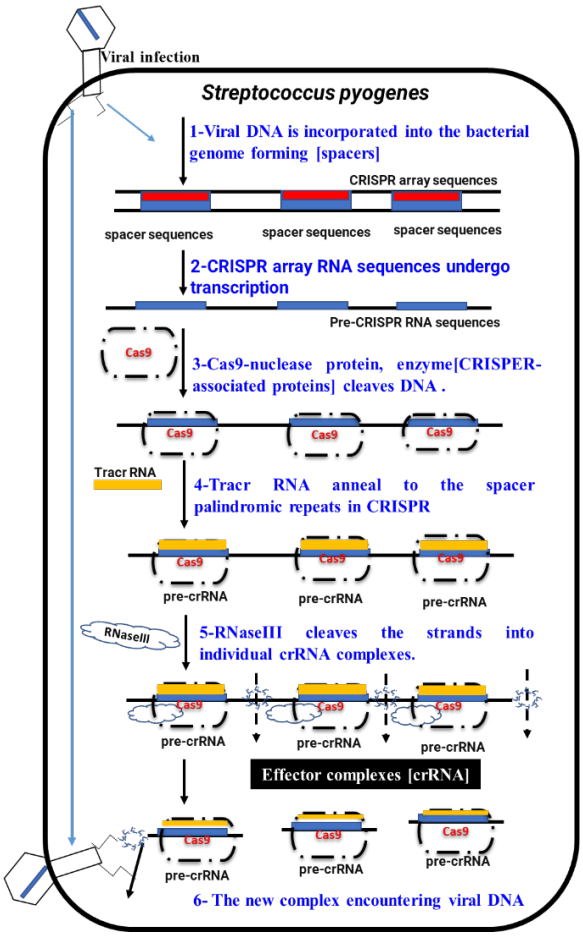

Fig. 2.

Figure 2 is representing CRISPR-Cas9 Genome Editing Technology that investigates role up of CRISPR array sequences for getting genomic crRNA complex. (1) Once the viral DNA is injected into the cell, a section of it can be incorporated into the bacterial genome and will be inserted the repeated palindromic sequences. this will be call spacers [we can see 3 spacers]. Potentially, from 3 different viruses sandwiched in between the repeated palindromic sequences will form CRISPR, (2) CRISPR array RNA sequences undergo transcription to form CRISPR RNA [pre-crRNA], (3) Cas9-nuclease protein, enzyme [CRISPER-associated proteins from S. pyogenes] that are involved DNA cleavage at specific nucleotide sequences like scissors, (4) Tracr RNA: These are complementary sequences and can anneal to the spacer palindromic repeats in CRISPR , A complex of pre-crRNA, a tracr RNA, and a Cas9 protein, (5)Ribonuclease III enzyme [RNaseIII] will cleave the strands in between these complexes leaving us with individual crRNA complexes producing effector complexes, The cell now is ready to defense against

Table 1
Demonstrations for the most common CRISPR editing systems classes, types, subtypes Cas endonuclease and target genetic materials

Classes of CRISPR-Cas Editing System							
Classes	**Type**	**No. of sub types**	**Cas endonuclease**	**Target**	**Requires Trans RNA**	**Target clinical trails**	**References**
Class I	Type I	6	Cas3	DNA or RNA	Target degradation	Influenza	[84]-[85]
		7	Cas9	DNA	No	Hematological malignancies & Hepatocellular carcinoma	[86]-[87]
	Type II	4	Cas10	DNA/RNA	No	Uncharacterized	[88]-[89]
	Type IV	1	--	--	--	Uncharacterized	[90]-[91]
	Type II	3	Cas9	DNA	Yes	Cancer immunotherapy	[92]-[93]
Class II	Type V	3	Cas12	DNA	Yes (subtype I)	Infectious and Zoonotic Diseases	[94]
	Type VI	3	Cas13	RNA	NO	COVID-19	[95]

The Invader whose genome produced that crRNA complex, (6)The new complex encounter a section of the viral DNA which is complementary to the crRNA, the nuclease enzyme will coordinate and recognize a short sequences unique to viral genome [Protospacer adjacent motif: PAM], then snabs both.

Genomic editing system has two groups. First is nuclease-free that include long chain DNA sequences that are homologous to the target cells region. It represents the selected editing by homologous recombination [73]. Although, it is extremely safe and specific, the editing process efficiency is low. Second is nuclease-guided in which integration of nucleases generates breaks double or single stranded DNA (DSB-SSD) in the target cell [74]. CRISPR-Cas9 type2 editing technology is the most generally used in genome editing in different trails in liver research diseases. However, the highest effective genome editing is referred to getting DSB in the among chromosomal sequences target cells [75], [76]. Current research in vivo studies developed direct liver genome editing gene therapies to evaluate the sensitivity and specificity for delivering the system in the liver cells [77], [78].

7. The CRISPR-Cas Gene Editing Technique

The CRISPR-Cas is consisted of, 1) A cas-protein enzyme that can cut DNA and ii) RNA-guided that recognize DNA to be edited in bacterial adaptive immune systems. It stores the memory of foreign DNA in single spacer sequences derivative from movable genetic elements then inserted in CRISPR arrays [79]. It possessed nuclease subunits, termed RuvC and HNH where each one cut one strand of the target double-stranded DNA in the cell [80]. CRISPR-Cas9 system Type II is commonly used in many CRISPR-Cas editing systems and well evaluated [81]. CRISPR spacers Transcripts recognize the related sequences and direct Cas-nucleases to their unique target sites upon new encounters with familiar movable genetic elements [MGEs] [82].

There are two classes of CRISPR/Cas systems I & II with many types and subtypes. Class I effectors contain many subunits while class II effectors are single large proteins. Class II is more developed than class I. CRISPER/Cas types are targeting DNA except type VI is applied to target RNA. Subtypes based on definite Cas endonuclease which is responsible for the cleavage and mode of action as shown in (table 1) [83]. Current liver disease related gene therapies nanotechnologies have sounded advance throughout optimization pf vectors types and advanced new tools e.g. as induction of pluripotent stem cells in mixture with current models of genetic editing (CRISPR-Cas9).

8. Limitations of Liver-Targeted Gene Therapy

Although clinical trials of recombinant adeno-associated virus vectors (rAAV) based GT are welling for getting promising outcomes, there are different significant restrictions. Particularly, the molecular structures vectors e.g. capsid, genome foreign DNA elements, transgenic proteins bio materials products will cause host immune responses, thus it will prevent effective delivering and long-term gene expression of the transgenes. In additions, activation of immune system causes adverse measures and reduces efficacies of GT bioproducts. Also, we must give attention to hepatotoxicity, complement activations, neurotoxicity and genotoxicities [96].

9. Improvement Strategies of the Safety of (rAAV)in Liver Disorders

Although, research studies-based adeno-associated virus gene therapies have rapidly developed among the last decade, more improvement in safety and quality of treatment plan strategies are required. These can be achieved by the allowed suggested study planes; i) more development of (rAAV) vectors to reduce the dose required for the target liver, ii) estimations for quality protocols for detection and eliminations of defects related preparations of rAAV vectors with reducing toxicity. iii) investigations for bioassays foreseeing and detecting hazards of adverse effects based additional prevention measurements. iv) developing estimation tools for of viral genomes doses per kilogram and total administered viral genomes for patients and pediatric patients with the pre-existing liver conditions v) investigations of new management tools for removal or inhibition rAAV-related antibodies and blocking any complement activation. vi) decreasing immunostimulant contents and the elements of potential genotoxicity in different therapeutic vector genomes vii) improvement of preclinical diseases models that mimic human pathological as closely as possible by evaluating their efficacy, safety, identifying their possible adverse effects, and developing mitigation plans [97].

10. Conclusion

Gene therapy in liver disorders is complicated and associated with many genetic risks. Increasing number of successful clinical trials approaches of gene therapy promise more advance. Gene therapy strategies include substitution of defective gene bioproducts, overexpressing of extrinsic or

intrinsic genes and interruption of exact genes expression. Therefore, more intensive experimental therapies are required for getting more advances in clinical trials overcoming different obstacles in liver diseases.

Although currently clinical research studies are using diverse of viral vectors, but challenges like cytotoxicity, carcinogenicity, immunogenicity and still need to be managed.

More advanced research studies are required for getting gene-editing technologies, vectors improvements, suiciding genes, tumor suppressing genes, anti-tumor angiogenesis, gene silencing, and oncolytic virotherapy for monogenic and polygenic disorders. Currently, CRISPR-Cas nanotechnology editing DNA is providing a novel trend for visualizing the genomes. It has been applied in gene function data analysis, gene therapy for some disease and developing drugs that are promising. Although strategies of gene editing export powerful tools for particular correction of genetic disorders among direct developing genome sequences, they pose extra safety risks, like oncogenicity that are owing to mutagenesis insertion.

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