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Gene Therapy in Liver Diseases: State-of-the-Art and Future Perspectives

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Abstract: Gene therapy is a fundamentally novel therapeutic approach that involves introducing genetic material into target cells in order to fight or prevent disease. A number of different strategies of gene therapy are tested at experimental and clinical levels, including: a) replacing a mutated gene that causes disease with a healthy copy of the gene, b) inactivating a mutated gene that its improper function causes pathogenesis, c) introducing a new gene coding a therapeutic compound to fight a disease, d) introducing to the target organ an enzyme converting an inactive pro-drug to its cytotoxic metabolite. In gene therapy, the transcriptional machinery of the patient is used to produce the active factor that exerts the intended therapeutic effect, ideally in a permanent, tissue-specific and manageable way. The liver is a major target for gene therapy, presenting inherited metabolic defects of single-gene etiology, but also severe multifactorial pathologies with limited therapeutic options such as hepatocellular carcinoma. The initial promising results from gene therapy strategies in liver diseases were followed by skepticism on the actual clinical value due to specificity, efficacy, toxicity and immune limitations, but are recently re-evaluated due to progress in vector technology and monitoring techniques. The significant amount of experimental data along with the available information from clinical trials are systematically reviewed here and presented per pathological entity. Finally, future perspectives of gene therapy protocols in hepatology are summarized.

Keywords: Hepatocellular, gene therapy, liver, metabolic disorder, vector.

INTRODUCTION

Despite the immense advances in the understanding of the etiology of several liver pathologies at a molecular level, treatment responses remain poor and outcome limited, imposing emerging need for novel therapeutic alternatives. Genetic manipulations for altering gene expression pattern *in vivo* with therapeutic outcome, known as gene therapy, offer a fundamentally different to pharmacotherapy approach for inherited as well as acquired diseases. Gene therapy, one of the greatest achievements of biomedical science, is based on genetic material transfer techniques in the affected or other relevant tissues, in order to stop the pathogenetic process. Using the host's cell protein synthetic machinery, permanent, tissue specific and controllable changes in gene expression are attempted, that aim to overcome the limitations of classical pharmacology at the level of chemical synthesis and stability of therapeutically useful substances, toxicity, even cost.

Many liver diseases such as hereditary metabolic defects, hepatitis, cirrhosis and primary or metastatic cancer are amenable for gene therapy approaches. The pathology of liver offers theoretically ideal candidates for gene therapy strategies: serious, life-threatening disease, often with known or of single-gene etiology, and limited, ineffective treatment options, either surgical or pharmacological. Numerous experimental data have been accumulated, which argue that genetic strategies can be tailored to liver disorders, some of those seen in clinical trials underway. In most studies, the therapeutic nucleic acid is delivered directly or using viral vectors with better transfection efficiency, usually adenoviruses because they exhibit tropism for the liver. Administration can be via intravascular, intra-hepatic or biliary routes. In addition, due to the physiology of the tissue, gene modification of the hepatic parenchyma could turn it into an endogenous laboratory for the production of protein-drugs to treat diseases that do not directly affect its function.

In spite of several approaches, human gene therapy in liver diseases is still in its infancy. The experience to apply protocols in clinical practice that derives from impressive initial results from *in vitro* and *in vivo* preclinical studies shows significant expected and unexpected barriers and poses questions about their future. The challenges associated with liver-directed gene therapy are efficient targeting of hepatocytes, stability of the vector genome, and persistent high level expression [1]. In this review, we present the knowledge acquired: what are the strategies developed, the technical possibilities and obstacles, and finally, what seems to be of therapeutic value of gene therapy in liver diseases?

VECTORS AND TREATMENT STRATEGIES

A gene that is inserted directly into a cell usually does not function. A carrier called vector is often used to intro-

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duce a therapeutic gene into the host's target cells. The most common vector is a virus that has been genetically altered to carry normal human DNA. Target cells such as hepatocytes are infected by the vector, which unloads its genetic material containing the therapeutic human gene into the cell genome. The generation of a functional protein product from the therapeutic gene restores the target cell or the adjunct tissue to a normal state.

Gene therapy vectors can be constructed on the basis of viral or non-viral molecular structures [2]. Integrating RNA-viruses and the non-integrating DNA-viruses have been used as viral vectors. The first are subcategorized in a) retroviruses that deliver copies of their RNA genomes that is reverse transcribed to DNA and b) lentiviruses which can deliver large amounts of DNA into the cell and replicate in non-dividing cells. Non-integrating DNA-viruses are subcategorized in: a) Adenoviruses (Ads), with double-stranded DNA genomes; their drawback is respiratory, intestinal and eye infections in humans, b) Adeno-Associated Viruses (AAV) are small, single-stranded DNA viruses; their genetic material can be inserted at a specific site on chromosome 19, and currently, they are among the most frequently used viral vectors for gene therapy and c) Herpes simplex viruses (HSV) are a class of double-stranded viruses that can infect a particular cell type, i.e. neurons.

Adenovirus (Ad) based gene transfer vectors continue to be the platform of choice for an increasing number of clinical trials worldwide. In fact, within the last years, the number of clinical trials that utilize Ad based vectors has increased dramatically, indicating growing enthusiasm for the numerous positive characteristics of this gene transfer platform. However, for the human adenovirus serotype 5 (Ad5), the most frequently used vector prototype; three major limitations have been identified: widespread pre-existing anti-Ad5 immunity in humans [3, 4], an unacceptably large amount of damage to normal non-target tissues, which causes severe toxicity [5, 6], and low levels of target-specific gene delivery [7-9]. Several efforts have been made towards diminishing the immune response. These strategies include, modification of viral capsids [10], covalent conjugation of polyethleneglycol (PEG) to Adv [11-14], hydrodynamic injections towards local delivery of low vector doses directly into the liver [15], pretreatment with glucocorticoids, use of lipid bilayer envelopes, and preventing binding to the CAR receptor by masking the adenoviral fiber knob [16-18]. Recent data suggest that combination of immunotherapy with chemotherapy can overcome the inability to give multiple doses, resulting in the boosting of the initial vaccination of the adenovirus [19]. Collectively, these findings indicate modified Ad vector protocols better suited for gene transfer in humans.

In the non-viral vectors for gene delivery the transferred gene is in the form of a plasmid [20] that is on the surface or in the interior of the vector. Such vectors include liposomes [21], molecular conjugates [22], nanoparticles [23], naked DNA [24] and complex DNA [25]. In order for more efficient transfection to be achieved, delivery systems, such as cationic lipids and cationic polymers were used [26]. In this case, the carrier is an artificial lipid sphere (a liposome) with an aqueous core carrying the therapeutic DNA, which is capable of transporting the DNA through the target's cell membrane.

Another way of introducing therapeutic DNA into target cells is by chemically linking the DNA to a molecule that will bind to specific cell receptors where it constructs and is engulfed by the cell membrane and then is passed into the interior of the target cell. This delivery system is however less effective than the other options.

In addition, experiments with the introduction of a 47^{th} chromosome (an artificial, human techno-chromosome) into target cells are being carried out [2]. This chromosome would exist autonomously alongside the standard 46, without affecting their functions or causing any mutations. The construction and the autonomy of this large vector, not only would make it invisible to the body's immune system, but also able to carry substantial amounts of the genetic code. A disadvantage with this method is the difficulty in delivering such a large molecule into the nucleus of a target cell.

Another delivery method is the Sleeping Beauty Transposon System (SBTS), a non-viral gene delivery vehicle found ubiquitously in nature. Transposon-based vectors have the capacity of stable genomic integration and long-lasting expression of transgene constructs in cells [27]. The SBTS consists of the SB Transposon, the SB Transposase – specific to the SB Transposon and a therapeutic gene.

An alternative non-viral option is RNA interference (RNAi), which includes a sequence-specific RNA degradation process in the cytoplasm of eukaryotic cells that is induced by double-stranded RNA (dsRNA). This endogenous regulatory RNA silencing mechanism, which was first described in *Caenorhabditis elegans* and *Drosophila melanogaster*, also possesses many similarities with post-transcriptional gene silencing in plants, and the process of quelling in *Neurospora crassa* [28, 29]. RNAi and related RNA silencing mechanisms are supposed to act as a natural defense against incoming viruses and the expression of transposable elements [30]. Moreover, with the antiviral function of RNAi, there is evidence that RNAi plays an important role in regulating cellular gene expression. The use of RNAi in gene therapy is to disrupt the translation of the faulty mRNA, which is induced by the signal to the signal, to cleave specific unique sequences in the mRNA transcript of the faulty gene. An alternative for gene silencing is the use of specific oligonucleotides antisense to the target gene [2]. In order to overcome the low efficiency of editing genes at their natural location, zinc-finger nucleases (ZFNs) have been constructed which augment this efficacy by making a break in the target site. The ability to modify the DNA-binding specificity of the ZFNs makes them applicable to targeting essentially any desired gene [31]. Despite the fact that the ZFN field is still in its early stages, the potential of ZFNs for gene targeting is indeed successfully used, including mostly animal and human genes [32]. There is some danger, however, that the use of additional fingers will lead to cleavage more sequences that are off target, since a subset may be sufficient to direct binding [31]. Pruett-Miller *et al.,* [33] for example, has shown greater cytotoxicity with a particular pair of 4-finger ZFNs.

Finally, another approach is gene-directed enzyme-prodrug therapy (GDEPT) or suicide gene therapy, which is comprised of three components; the pro-drug to be activated, the enzyme (usually nonhuman) used for activation, and the delivery system for the corresponding gene [34]. Pro-drugs can be considered as a combination of two major domains; a "trigger" unit that is the substrate for the activating enzyme, and an "effector" unit that is activated or released by this metabolic process, sometimes joined by a definable linker [35]. The most prominent GDEPT therapy has been the use of the herpes simplex type-1 thymidine kinase enzyme (HSV-Tk) in conjunction with a variety of guanosine based pro-drugs, compounds originally developed as antiviral (anti-herpes) agents [36-38]. Ganciclovir (GCV; 1a) is the most widely used pro-drug for HSV-Tk, and is well known as an antiviral agent [38]. The *cytosine deaminase* gene is the next most widely studied for GDEPT.

Tested approaches for DNA delivery in liver diseases include nanoparticles, DNA plasmids with cationic lipids, liposomes and others. AAV vectors offer a more promising alternative, as they are capable of maintaining high levels of hepatic transgene expression for prolonged periods of time, particularly when regulated by tissue-specific enhancers and promoters [39-41]. In addition, significantly higher hepatic transduction efficiencies by administrating relatively low viral dosages has been succeeded, since the discoveries of novel AAV serotypes, such as AAV8 and 9 [42]. Compared to other viral-based gene therapy vectors, AAV vectors have a favorable biosafety profile, because they are less inflammatory and the wild-type virus is nonpathogenic as well as replication-deficient [43]. AAV8 has been shown to be the most efficient vector for liver-directed gene transfer and is currently being evaluated in a clinical trial for treating hemophilia B [44, 45] (Table **1**).

SEARCH STRATEGY

We performed an electronic article search through PubMed, Google Scholar, Medscape and Scopus databases, using combinations of the following keywords: Gene Therapy, Lysosomal storage disease, Acute intermittent porphyria, Ornithine transcarbamylase deficiency, α(1)-antitrypsin deficiency, acute liver failure, PompeDisease, Tyrosinemia, Crigler-Najjar, Hepatitis, Cirrhosis, and hepatocellular carcinoma. All types of articles (randomized controlled trials, clinical observational cohort studies, review articles, case reports) were included. Selected references from identified articles were searched for further consideration.

LYSOSOMAL STORAGE DISEASES (LSDs)

LSDs are a group of approximately 50 rare inherited metabolic disorders that result from defects in lysosomal function [46]. Being in most cases of single-gene mutation etiology, they present ideal candidates to replacement gene therapy, i.e. introducing a wild type gene, the product of which substitutes the defective or inadequate enzyme. Herein, we examine the prospects of gene therapy in Acute intermittent porphyria, Ornithine transcarbamylase, Wilson's disease, Hurler syndrome, Sly syndrome, Pompe disease, Tyrosinemia type I, α(1)-antitrypsin deficiency, Crigler–Najjar. Naturally occurring animal homologues of LSDs have been described in all common domestic animals, presenting models with critical role in evaluating the efficacy and safety of gene therapy (Table **2**).

ACUTE INTERMITTENT PORPHYRIA

Acute intermittent porphyria (AIP) is a rare autosomal dominant affecting the biosynthesis of, the oxygen-binding prosthetic group of hemoglobin [47] due to a deficiency of the enzymehydrogymethylbilane (HMB) synthase, usually attributed to a mutation that causes decreased amounts of the enzyme, and to a lesser degree by a mutation that causes decreased activity. Acute intermittent porphyria is the second most common form of porphyria [48]. The acute attacks are currently treated with intravenousheme, but a more continuous therapy is needed, particularly for patients experiencing frequent attacks [49]. To date, several efforts have been made by using non-viral methods, which were unsuccessful, as they were incapable of achieving sufficient HMB-synthase levels due to their poor transfection efficiency *in vivo* [50]. In addition, although adenoviral vectors resulted in therapeutic HMB-synthase levels in experimental mice, its expression was transient, thus making them impractical for clinical applications [51]. Administration of recombinant adenoviral, AAV8-based andrecombinant AAV (rAAV) vectors in murine models containing the HMB-synthase complementary DNA, resulted in increased levels of hepatic HMB-synthase activity, inhibiting the phenobarbital-induced ALA and PBG accumulation and thus, indicating a potential for future gene therapy[51-53]. Finally, Johansson *et al*., 2003 and 2002 showed in mammalian cells that non-viral gene delivery encoding HMB-synthase results in high expression of functional enzyme [54, 55], suggesting enzyme-replacement gene therapy as a feasible proposal.

ORNITHINE TRANSCARBAMYLASE DEFICIENCY

Ornithine transcarbamylase deficiency (OTCD), the most common of the genetic results in an ineffective form of the enzyme. Urea cycle defects presenting early in life with hyperammonemia remain difficult to treat and commonly necessitate liver transplantation. Gene therapy has the potential to prevent hyperammonemic episodes while awaiting liver transplantation, and possibly also to avert the need for transplantation altogether. Ornithinetranscarbamylase (OTC) deficiency provides an ideal model for the development of liver-targeted gene therapy.

To date, several studies have been performed in OTC-deficient mouse models (i.e. spfash) using adenoviral and more recently rAAV vectors [56-63]. Although a major limitation of this model is the presence of residual OTC enzymatic activity, which confers a mild phenotype without clinically significant hyperammonemia. Initial poor results with early generation adenoviral vectors [60] have reduced cytotoxicity, but have not overcome limitations imposed by considerable intrinsic immunogenicity [62, 63]. In the only study reported to date using rAAV, treatment of adult OTC-deficient mice was investigated using vectors pseudo- serotyped with type 7, 8, and 9 capsids [61]. Promising metabolic correction was achieved, albeit at high vector doses, and declining therapeutic efficacy was observed beyond 250 days. The type 8 capsid proved most effective although the

Table 1. Studies for Gene Therapy in Liver Cancer using Viral Vectors

Author	Cell line-Animal Model	Viral Vector	Transfer gene	Combination	Overall	Application	Refs
Xu *et al.,* 2011	mice	onco-lytic adenovirus	*HCCS1*	-	improved	theurapeutic	178
Kanai *et al.,* 1997	HuH-7, HLF	rAAV	*CD*	-	improved	theurapeutic	180
Gan *et al.,* 2008	mice	oncolytic adenovi-rus	*HCCS1*	-	improved	theurapeutic	161
Wei *et al.,* 2011	HCC cell lines	onco-lytic adenovirus	*SOCS3, TNF*	-	improved	theurapeutic	162
Xue *et al.,* 2010	HepG2, Hep3B, MHCC97L, L02	oncolytic adenovi-rus	*MDA-7*	-	improved	theurapeutic	163
Takahashi *et al.,* 2002	HuH7, HepG2, PLC/PRF/5 (P5)	rAAVs	*E1A-13S*	-	improved	theurapeutic	179
Sagawa *et al.,* 2008	Hep3B	Adenovirus	*p53*	-	improved	theurapeutic	165
Guo *et al.,* 2001	PLC/PRF/5 PLC/PRF/5	rAAV	*p53*	-	improved	theurapeutic	187
Chen *et al.,* 2011	Hep3B	rAAV2	*p53*	DOX	improved	theurapeutic	182
Ohashi *et al.,* 2001	mice	E1B55k-attenuated adenovirus	*E1B*	-	improved	theurapeutic	188
Wang *et al.,* 2007	SMMC-7721 mice	rAAV	*IL-24*	-	improved	theurapeutic	186
Song *et al.,* 2008	HEK-293 cells	rAAVs	*NT4p53(N15)An*	-	improved	theurapeutic	185
Xiangji *et al.,* 2011	HepG2.2.15 cells	Lentiviral miR-based system	*HBsAg*	-	improved	theurapeutic	255
Jin *et al.,* 2011	L-02, WRL-68	oncolytic adenovi-rus	*let-7*	-	improved	theurapeutic	230
Xu *et al.,* 2011	HCC cells in G2/M phase	adenovirus	MiR-122	ADM or VCR	improved	theurapeutic	231
Ma *et al.,* 2010	HepG2, Hep3B, Huh7, PLC/PRF/5	rAAVs	miR-122	-	improved	theurapeutic	243
Barajas *et al.,* 2001	rats	adenovirus	*IL-12*	-	improved	theurapeutic	206
Pützer *et al.,* 2001	woodchucks	adenovirus	*IL12/B7.1*	-	improved	theurapeutic	209
Wei *et al.,* 2011	HCC cell lines	oncolytic adenovi-rus	*SOCS3, TRAIL*	-	improved	theurapeutic	162
Xu *et al.,* 2011	Cancer and normal cell lines	quadruple-regulated oncolytic adenovi-rus	HCCS1	-	improved	theurapeutic	178
Lam *et al.,* 2007	HCC cells	HSV-1	chimeric *Gal4/NF-YA*	-	improved	theurapeutic	167
Argnani *et al.,* 2011	mice	HSV-1	luciferase reporter gene	-	improved	theurapeutic	171
Foka *et al.,* 2010	HCC cell lines	HSV-1	luciferase reporter gene		-	Promoter ident/tion	168
Sia *et al.,* 2011	HCC and non-HCC cell lines	HSV-1	luciferase reporter gene, (*yCD*) genes	yeast yCD -FC5-FC prodrug	improved	theurapeutic	170

(Table 1) contd….

Author	Cell line-Animal Model	Viral Vector	Transfer gene	Combination	Overall	Application	Refs
Song *et al.,* 2011	HCC cell lines	HSV- G207	diploid ICP6	-	improved	theurapeutic	172
Wills *et al.,* 1995	HCC cell lines and mice	adenovirus	*Tk*	ganciclovir treatment	improved	theurapeutic	173
Iwai *et al.,* 2002	mice	EBV-based plasmid vector	*HSV-1 Tk*	PEI	improved	theurapeutic	174
Harada *et al.,* 2000	HCC cells lines	EBV-based plasmid vectors	*β-gal*	EBV/lipoplex-PAAD	improved	theurapeutic	175
Chen *et al.,* 2011	HCC cell lines	oncolytic adenovirus	*p53*	-	improved	theurapeutic	182
Inoue *et al.,* 2004	HCC cell lines	adenovirus	*p53*	TRAIL-induced apoptosis	improved	theurapeutic	183
Yang *et al.,* 2010	40 Patients	adenovirus	*p53*	fractionated stereotactic radiotherapy	efficient	Clinical trial	190
Tian *et al.,* 2009	1 Patient	adenovirus	*p53*	Oxaliplatin, chemotherapy and TACE	efficient	Clinical trial	191
Tian *et al.,* 2009	46 Patients	rAAV	*p53*	TACE	efficient	pilot phase II trial	192
Habib *et al.,* 2002	10 Patients	E1B-deleted adenovirus (dl1520)	*p53*	-	NSD	Clinical trial	189
Guan *et al.,* 2005	1 Patient	adenovirus	*p53*	TACE	efficient	Clinical trial	193

HCCS1; hepatocellular carcinoma suppressor 1, CD; cytosine deaminase gene, SOCS3; suppressor of cytokine signaling 3, TNF; tumor necrosis factor, HSV-Tk; Doxorubicine; DOX, herpes simplex virus thymidine kinase gene, IL-24; interleukin-24, HBsAg; surface antigen of hepatitis B virus, IL-12; interleukin-12, MiR; microRNA, TRAIL; tumor necrosis factor-related apoptosis-inducing ligand, HSV-1; herpes simplex virus type 1, Adriamycin; ADM, vincristine ;VCR, TK; thymidine kinase, EBV; epstein barr virus, HSV-1 Tk; herpes simplex virus-1 thymidine kinase, polyethylenimine, PEI; EBV/lipoplex; cationic liposome, PAAD; polyamidoamine dendrimer, FC-5; fluorocitosine, TACE; transcatheter arterial chemoembolization.

Table 2. Gene Therapy Published Studies for Metabolic Liver Diseases and Hepatitis

Author	Disease Target	Therapeutic molecule	Animal Model	Vector	Delivery system	Overall	Refs
Johansson *et al.,* 2003	Acute intermittent porphyria	*PBGD*	murine	Non viral (naked DNA)	intravenous or portal vein injection	Not improved	54
Johansson *et al.,* 2004	Acute intermittent porphyria	*PBGD*	mice	adenovirus	intravenous	NSD	50
Unzu *et al.,* 2011	Acute intermittent porphyria	*PBGD*	mice	rAAV	Phenobarbital injections	improved	53
Johansson *et al.,* 2003	Acute intermittent porphyria	*PBGD*	mammalian cells	non-viral	intravenous	improved	55
Johansson *et al.,* 2004	Acute intermittent porphyria	*PBGD*	mammalian cells	non-viral	intravenous	improved	51
Cunningham *et al.,* 2011	Ornithine transcarbamylase	*OTC*	mice	rAAV	intraperitoneal	improved	56
Kiwaki *et al.,* 1996	Ornithine transcarbamylase	*OTC*	mice	rAAV	intravenous	improved	60

(Table 2) contd….

Author	Disease Target	Therapeutic molecule	Animal Model	Vector	Delivery system	Overall	Refs
Mian *et al.,* 2004	Ornithine transcarbamylase	*OTC*	mice	adenovirus	tail-vein injection	improved	62
Brunetti-Pierri *et al.,* 2008	Ornithine transcarbamylase	OTC	mice	adenovirus	tail-vein injection	improved	63
Moscioni *et al.,* 2006	Ornithine transcarbamylase	OTC	mice	AAV	portal vein injection	improved	61
Wang *et al.,* 2012	Ornithine transcarbamylase	*OTC*	mice	AAV8	intravenous	improved	58
Wang *et al.,* 2011	Ornithine transcarbamylase	*OTC*	mice	AAV	intravenous	improved	59
Roybal *et al.,* 2011	Wilson's disease	*ATP7B*	murine	lentivirus	prenatal	improved	68
Zhang *et al.,* 2011	Wilson's disease	*ATP7B*	Human iPSCs	lentivirus	*Ex vivo*	improved	69
Merle *et al.,* 2006	Wilson's disease	*ATP7B*	rat	lentivirus	systemic application	improved	70
Ha-Hao *et al.,* 2002	Wilson's disease	ATP7B	rat	adenovirus	tail-vein injection	improved	71
Liu *et al.,* 2005	Hurler syndrome	*IDUA*	mice	retroviral	intravenous	improved	78
Ma *et al.,* 2007	Hurler syndrome	*IDU*A	mice	retroviral	intravenous	improved	80
Metcalf *et al.,* 2009	Hurler syndrome	*IDUA*	mice	retroviral	intravenous	improved	81
Kobayashi *et al.,* 2005	Hurler syndrome	*IDUA*	mice	lentiviral	intravenous	improved	86
Hartung *et al.,* 2004	Hurler syndrome	*IDUA*	mice	rAAV	intravenous	improved	85
Ponder *et al.,* 2006	Hurler syndrome	*IDUA*	Cats, dogs	retroviral	intravenous	improved	82
Traas *et al.,* 2007	Hurler syndrome	*IDUA*	Canine dogs	retroviral	intravenous	improved	83
Herati *et al.,* 2008	Hurler syndrome	*IDUA*	dogs	retroviral	intravenous	improved	84
Di Domenico *et al.,* 2006	Hurler syndrome	*IDUA*	mice	retroviral	intravenous	improved	87
Ma *et al.,* 2007	Hurler syndrome	*IDUA*	mice	retroviral	intravenous	improved	80
Daly *et al.,* 2001	Sly syndrome	*GUS*	mice	AAV	intravenous	improved	93
Elliger *et al.,* 2002	Sly syndrome	*GUS*	mice	AAV	intrathecal, intravenous	improved	94
Cotugno *et al.,* 2011	Sly syndrome	*GUS*	feline	AAV	intravascular	improved	91
Ponder *et al.,* 2002	Sly syndrome	*GUS*	dogs	retroviral	intravenous	improved	96
Xu *et al.,* 2002	Sly syndrome	*GUS*	mice	retroviral	intravenous	improved	95
Xu *et al.,* 2002	Sly syndrome	*GUS*	dogs	retroviral	intravenous	improved	97

(Table 2) contd….

Author	Disease Target	Therapeutic molecule	Animal Model	Vector	Delivery system	Overall	Refs
Sun *et al.,* 2010	Pompe disease	*GAA*	mice	AAV	retro-orbital injection	improved	101
Sun *et al.,* 2003	Pompe disease	*GAA*	mice	AAV	intravenous	improved	99
Sun *et al.,* 2008	Pompe disease	*GAA*	mice	AAV	intravenous	improved	100
Sun *et al.,* 2010	Pompe disease	*GAA*	mice	AAV	intravenous	improved	101
Paulk *et al.,* 2010	Tyrosinemia	*Fah*	mice	AAV	intravenous facial vein injection	improved	103
Chen *et al.,* 2000	Tyrosinemia	*Fah*	rabbits	AAV	intravenous	improved	104
Overturf *et al.,* 1996	Tyrosinemia	*Fah*	mice	retroviral	portal vein injection	improved	105
Overturf *et al.,* 1998	Tyrosinemia	*Fah*	mice	retroviral	Ex vivo	improved	106
Wilber *et al.,* 2007	Tyrosinemia	*Fah*	murine	Non viral (transposase)	tail-vein injection	improved	107
Montini *et al.,* 2002	Tyrosinemia	*Fah*	mice	Non viral (transposase)	tail vein injection	improved	110
Held *et al.,* 2005	Tyrosinemia	*Fah*	murine	Phage phiC31 integrase	tail vein injection	improved	111
Overturf *et al.,* 1997	Tyrosinemia	*Fah*	mice	adenovirus	Tail or portal vein injection	improved	107
Grompe 1997	Tyrosinemia	*Fah*	mice	retrovirus	portal vein injection	improved	108
Yusa *et al.,* 2011	α(1)-antitrypsin deficiency	*SERPINA1*	human iPSCs	Non viral (ZFNs)	*Ex vivo*	improved	113
Bortolussi *et al.,* 2011	Crigler-Najjar (CNI) syndrome	*hUGT1A1*	mice	AAV	intraperitoneal	improved	114
Birraux *et al.,* 2009	Crigler-Najjar (CNI) syndrome	*hUGT1A1*	human cells	lentiviral	*Ex vivo*	improved	115
Van der Wegen *et al.,* 2006	Crigler-Najjar (CNI) syndrome	*hUGT1A1*	rat	lentiviral	intravenous	improved	116
Kren *et al.,* 1999	Crigler-Najjar (CNI) syndrome	*hUGT1A1*	Gunn rat	Non viral (chimeric oligonucleotide)	tail vein injection	improved	117
Morrissey *et al.,* 2005	Hepatitis B virus	*HBV*	mice	Non viral (siRNA)	intravenous	improved	127
Hsu *et al.,* 2003	Hepatitis C virus	serine protease (*NS3/NS4A*)	Mice (chimeric human livers)	adenovirus	infected with HCV using the serum	improved	123
Lang *et al.,* 2011	Hepatitis C Virus	*HCV* genotype *1a/1b*	rhesus macaques	NS3/NS3A DNA Vaccine	intramuscular intramuscular	improved	129
Li *et al.,* 2008	Hepatitis B virus	*HBV*	mice	AAV	tail vein injection	improved	125
Ebert *et al.,* 2011	Hepatitis B virus	HBV transcripts	human liver cells and mice	Non viral (siRNAs)	Intravenous	improved	126
Zhou *et al.,* 2010	Hepatitis B virus	CTLA4-fused DNA	mice	Non viral (plasmid)	injection at multiple sites	improved	130

NSD; not significant difference, PBGD; porphobilinogen deaminase, AAV; adeno-associated virus, rAAV; recombinant adeno- associated virus, iPSCs; human induced pluripotent stem cells, IDUA; gene responsible for the production of iduronidase enzyme protein production (mapped to the 4p16.3 site on chromosome 4, GUS; beta-glucuronidase reporter gene, Fah; furmarylacetoacetate gene, ZFNs; zinc finger nucleases, A1AT or SERPINA1; a(1)-antitrypsin gene, 1A1(hUGT1A1); uridine disphoshate glucuronyltransferase

maximal OTC activities achieved were sub-physiological. Finally, Cunningham *et al*., [64] showed that AAV2/8-mediated transgene delivery is an extremely powerful tool for life-long correction of OTC deficiency in the adult mouse, but is less robust in neonatal mice, where substantial loss of transgene expression occurs during liver growth.

WILSON'S DISEASE

Wilson's disease is a genetic disease caused by mutations in the *ATP7B* gene, which is responsible for the expression of a liver transporter protein that coordinates copper export into bile and blood [65]. Current medical treatments including chelating agents and zinc salts are not effective in all Wilson disease patients. Liver transplantation is the alternative option [65-67]. To date, few gene therapy efforts have been tested in animal models of Wilson disease. In the murine model, adenoviral and lentiviral gene transfer studies have shown that viral gene transfer is therapeutically effective and can reverse clinical symptoms [68-71]. However, these approaches were limited by a more or less transient transgene expression. Other researchers demonstrated a self-inactivating lentiviral vector that expresses codon optimized-*ATP7B* or treatment with the chaperone drug curcumin showing reduction of the functional defect *in vitro* [69]. In conclusion, there is still a long way to go until gene therapy can be used for safe treatment of Wilson disease in humans.

MUCOPOLYSACCHARIDOSES

Mucopolysaccharidoses (MPSs) form a group of more than 40 caused by the absence or malfunction of enzymes. Here we provide information for three of them; MPS I, MPS VII and MPS IV concerning results from gene therapy approaches.

MPS I

Mucopolysaccharidosis type I (MPS IH; Hurler syndrome or α-L-iduronidase deficiency) is a rare genetic disorder caused by mutations in the *IDUA* gene, resulting the deficiency of α-L-iduronidase enzyme activity and intracellular accumulation of glycosaminoglycans (GAGs) [72]. The low or absent lysosomal enzyme activity leads to this accumulation which is essential to the GAG catabolism, and results in systemic impairment of organs and tissues. A characteristic skeletal phenotype is one of the many clinical manifestations in Hurler disease. Available treatments for patients with MPS I currently consist of hematopoietic stem cell transplantation [73] or enzyme replacement therapy [74]. Gene therapy as a promising alternative to existing treatments for MPS I, has improved clinical manifestations of the disease in a variety of animal models [75]. There are several recent reports which designed viral and non-viral vectors for Hurler syndrome. For example, Aronovich *et al*., [76, 77] demonstrated the reversion of MPS I phenotypein adult mice, using therapeutic SB transposon plasmids encoding human *IDUA*.

However, most of the approaches include viral vectors. In particular, neonatal administration of γ-retroviral vectors (γ-RV) [78-81], lentiviral vectors, or AAV vectors expressing the *IDUA* gene have prevented many aspects of MPS I disease in mice, while γ-RV have been effective in dogs and cats [82-85]. Administration of a γ-RV7 or a lentiviral vector [86, 87] to adult mice without immunosuppression, had no therapeutic effect. Ma *et al*., [80] showed improvement in MPSI symtopatology in adult mice, after retroviral vector-mediated gene therapy with immunomodulation.

MPS VI

Mucopolysaccharidosis type VI (MPS-VI) is an autosomal recessive lysosomal storage disorder caused by the deficiency of N-acetylgalactosamine-4-sulfatase (*4S; or ARSB*). Mutations in the *4S* gene are responsible for 4S deficiency, which leads to the intralysosomal storage of partially degraded glycosaminoglycans, dermatan sulfate, and chondroitin 4-sulfate [88]. The disorder shows a wide spectrum of symptoms from slowly to rapidly progressing forms. The characteristic skeletal dysplasia includes short stature, dysostosis multiplex and degenerative joint disease. Moreover, before enzyme replacement therapy (ERT) with galsulfase (Naglazyme), clinical management was limited to supportive care and hematopoietic stem cell transplantation. Galsulfase is now widely available and is a specific therapy providing improved endurance with an acceptable safety profile.

Few reports have demonstrated the utility of viral vectors in MPS VI. Byers *et al*., [89], indicated that the lentiviral vector can be used to deliver *4S* to a range of joint tissues *in vitro* and efficiently transduce synovial cells to express beta-galactosidase *in vivo*. A study by Ho *et al*., [90], supported the utility of AAV as a vector for the treatment of retinal pigment epithelium (RPE) cells of feline MPS VI. Finally, Cotugno *et al*., [91] showed that intravascular administration of AAV in MPS VI cats, represents a promising therapeutic strategy for MPS VI patients.

SLY SYNDROME

Another example of treating LSDs with gene therapy is MPS type VII or Sly syndrome. Here, the gene of interest encodes β-glucuronidase (GUS), whose absence or functional defectiveness causes a buildup of glycosaminoglycan (GAG) storage granules within the lysosomes of most cell types [92]. In humans, this disease is characterized by mental retardation, abnormal bone development, distorted features, and organ malfunctions leading to organ failure and early death. Fortunately, Sly syndrome is rare; however, because a great deal is known about GUS and because there are animal models of this disease, Sly syndrome has become a paradigm for the study of lysosomal storage diseases in general and for gene therapy in particular. Furthermore, complete regulation of expression is not necessary, as has been found that overexpression does not cause problems, and if 2% of normal levels are restored the abnormal phenotype is reversed. These features make this disease an ideal target for the application of gene technology.

Attempts for gene therapy in MPS VII in animal models involve intravenous injection of vectors derived from AAV, adenovirus, retrovirus or a plasmid. Daly *et al*., [93] reported that AAV-mediated gene transfer in the neonatal period can lead to the prevention of many of the clinical symptoms as-

sociated with MPS VII in the murine model. Indeed, therapeutic levels of enzyme persist for at least 1 year following a single intravenous injection of virus in neonatal MPS VII mice. The level and distribution of GUSB expression were sufficient to prevent many clinical symptoms over the life of the animal. By adding coding sequences for secretion (Igκ) and uptake (HIV-1 TAT) signals to the *GUS* gene delivered by AAV, and treated mice both intrathecally and intravenously as newborns, Elliger *et al*., [94] have increased the GUS enzyme levels in more tissues and have improved the health of the mice so much that they were able to breed [94]. Several more recent reports attempted gene therapy for MPS VII in animal models; one approach involves neonatal i.v. injection of a γ-retroviral vector expressing β-glucuronidase, which results in transduction of liver cells and secretion of enzyme into blood in significant levels [95-97]. In conclusion, gene therapy holds great promise for providing a long-lasting therapeutic effect for MPS VII if safety and higher expression levels issues can be resolved [75].

POMPE DISEASE

The Glycogen storage disease type II (GSD II) is another LSD, also known as acid maltase deficiency or Pompe disease. It is caused by mutations in the gene of acid alpha-glucosidase (GAA), the enzyme responsible for breaking down glycogen into glucose, causing absence or significantly reduction in enzyme levels. Classic infantile-onset Pompe disease may be apparent *in utero* but it more often presents in the first month of life with hypotonia, generalized muscle weakness, cardiomegaly and hypertrophic cardiomyopathy, feeding difficulties, failure to thrive, respiratory distress, and hearing loss. The existing available treatment reverses cardiac muscle damage and enhances life expectancy in those with the infantile form of the disease, but is less effective in skeletal muscle.

Some recent approaches include gene therapy by using viral vectors. Koeberl *et al*., [98] reported that immune tolerance to the transgene could be achieved by liver-specific expression with AAV vectors by enhancing the efficacy. Sun *et al*., [99-101] made several efforts in murine Pompe disease by administrating AVV vectors with promising results. Despite the presence of immune responses, the successful development of gene therapy in mice is a fact, which indicates that curative therapy for Pompe disease may become available in the foreseeable future.

TYROSINEMIA

Tyrosinemia is caused by a shortage of enzyme fumarylacetoacetate hydrolase (FAH) [102]. The usual manifestations appear in the first few months of life and include failure to gain weight and grow at the expected rate (failure to thrive), diarrhea, vomiting, yellowing of the skin and whites of the eyes (jaundice), cabbage-like odor, and increased tendency to bleed (particularly nosebleeds). Tyrosinemia can lead to liver and kidney failure, problems affecting the nervous system, and an increased risk of liver cancer. Toxic metabolites such as fumarylacetoacetate accumulate in hepatocytes and renal proximal tubules, causing death in a cell-autonomous manner [102].

Paulk *et al.,* [103] demonstrated stable hepatic gene repair in both adult and neonatal mice with AAV-FAH serotypes 2 and 8, showing that AAV-mediated gene repair is feasible *in vivo* and can functionally correct enzyme-deficiency consequences. In a study by Chen *et al*., [104] a murine model for hereditary tyrosinemia was used to evaluate *in vivo* gene therapy with AAV vectors expressing FAH. Results reported that after 9 months, vector-treated animals showed benign hepatomas, whereas in untreated animals areas of marked dysplasia were present within hepatomas. Furthermore, Overturf *et al*., [105, 106] demonstrated *in vivo* and *ex vivo* successful effects in murine models by administrating adenoviral vector [107] but in therapeutic trials, Grompe *et al*., showed that 2/3 treated animals developed hepatocarcinoma after 1 year retroviral gene transfer [108]. Wilber *et al.,* [109] tested the use of transposase-encoding RNA plus transposon DNA for correction of murine FAH deficiency. The authors concluded that transposase-encoding messenger RNA (mRNA) can be used to mediate stable non-viral gene therapy, resulting in complete phenotypic correction. Using the same strategy, Montini *et al.,* [110] demonstrated long-lasting correction of the FAH-deficiency phenotype.

Finally, Held *et al.,* [111] used Phage phiC31 integrase, which is a site-specific recombinase that mediates efficient integration of circular extra-chromosomal DNA into the host genome. They used this integrase system to transfer the *FAH* gene into the liver of mice affected with hereditary tyrosinemia, suggesting that it may have utility in many gene therapy settings. Overall, several gene therapy strategies have shown therapeutic potential for tyrosinemia, which still needs to be proven in humans.

α(1)-ANTITRYPSIN DEFICIENCY

Alpha 1-antitrypsin deficiency (α1-antitrypsin deficiency, A1AD or simply Alpha-1) is a recessive caused by defective production of (A1AT also known as SERPINA1). This miss expression leads to decreased A1AT activity in lungs, and deposition of excessive abnormal A1AT protein in liver cells [112]. The enzyme deficiency causes pulmonary emphysema, while 10% of patients develop cirrhosis due to accumulation of defective folded protein. The administration of the enzyme gene with adenovirus could reverse the lung damage in mice and baboons, while for liver cirrhosis gene repair is essential.

Yusa *et al*., [113] showed that a combination of zinc finger nucleases (ZFNs) and piggyBac technology in human induced pluripotent stem cells and achieved biallelic correction of a point mutation (Glu342Lys) in the *A1AT*. Genetic correction of human induced pluripotent stem cells restored the structure and function of A1AT in subsequently derived liver cells *in vitro* and *in vivo*. This approach is significantly more efficient than any other gene-targeting technology that is currently available and prevents contamination of the host genome with residual non-human sequences.

CRIGLER–NAJJAR

Crigler–Najjar syndrome is characterized by unconjugated hyperbilirubinemia resulting from an autosomal recessive inherited deficiency of hepatic UDP-glucuronosyl-

transferase (UGT) 1A1 activity. The enzyme is essential for glucuronidation and biliary excretion of bilirubin, and its absence can be fatal. Bortolussi *et al.,* [114] by using AAV9-mediated gene transfer, showed the rescue of bilirubin-induced neonatal lethality in a mouse model of Crigler-Najjar syndrome. In addition, Birraux *et al.,* [115] and Wegen *et al.,* [116] showed successful treatment of Crigler-Najjar syndrome, by administrating the human *UGT1A1* with lentiviral vectors in mice and rat animal models respectively. Kren *et al.,* [117] showed permanent correction of the *UGT1A1* genetic defect in Gunn rat liver with site-specific replacement of the absent G residue at nucleotide 1206 by using an RNA/DNA oligonucleotide designed to promote endogenous repair of genomic DNA. However, none of these protocols have so far been translated into a clinical trial [118].

HEPATITIS C AND B

Hepatitis C virus (HCV) infection is a serious global health problem that affects 180 million people worldwide [119]. Hepatitis C virus is a major cause of chronic liver diseases which can lead to permanent liver damage, hepatocellular carcinoma and death [120]. The present available treatment with interferon plus ribavirin, has limited benefits due to adverse side effects such as anemia, depression and "flu-like" symptoms. Hence, the development of a new treatment is crucial, and gene therapy is on target. In this case, the goal of treatment is to resolve the viral potential for infection, cirrhosis and liver failure and not develop liver cancer.

An option for HCV eradication is RNA which is an attractive target for RNAi, as the single positive-stranded viral transcript functions both as genomic RNA and a replication template, and also because of its localization in the infected liver, an organ that can be readily targeted by nucleic acid molecules and viral vectors. Several reports demonstrated potent RNAi activity against HCV in sub-genomic replicon and fully infectious HCV particles [121]. A review of Ashfaq *et al*., [122] concluded that combination of siRNA against virus and host genes will be a better option to treat HCV.

Alternatively, suicide gene therapy is based on administration of genes encoding cytotoxic agents that have been modified to be expressed only in cells infected by the virus of hepatitis. For example, a chimeric molecule that when cut by a protease of HCV releases BID, an agent that induces apoptosis in infected cells. Another report demonstrated that modified apoptotic molecule (BID) reduces hepatitis C virus infection in mice with chimeric human livers [123].

Hepatitis B is also an infectious illness caused by (HBV) which infects the liver of hominoidea, including humans, and causes hepatitis [124]. Interferon-alpha2 (IFNalpha2) is routinely used for anti-hepatitis B virus (HBV) treatment. However, as in HCV, the therapeutic efficiency is unsatisfactory too. Liz *et al.,* [125] managed to decrease the level of viral DNA over 30-fold for several weeks by testing a single dose of vector-delivered IFNalpha1 for its anti-HBV effects. Another recent report combined gene silencing by using 5' Triphosphorylated siRNAs control replication of hepatitis B virus with induction of IFN in the liver [126]. As in HCV, there is also a report demostrating that siRNA may be a clinically viable therapeutic approach for HBV [127].

An alternative therapeutic approach that combines gene silencing with induction of IFN in the liver was demonstrated by Ebert *et al*., [126]. He reported that this combination in HBV-infected primary human hepatocytes and in HBV transgenic mice controlled replication of HBV efficiently. In general, it is well-recognized that a combination of gene therapy antiviral treatment and immunomodulation is essential to achieve a sustained control of HBV and HCV infection.

Another way for activation anti-viral immunity, which may have therapeutic and preventive clinical value, is DNA-vaccines that induce the synthesis of viral antigens of hepatitis B or C - surface and central. There are plenty of advantages for such a vaccine; the easy production and storage - since the DNA is much more stable than the respective protein antigens, the potential for epidermal (particle-mediated epidermal delivery, PMED), the reduction of contamination, the possibility of parallel immunization with different genes, and the use of DNA induces cytotoxic immune response. This response is induced because the antigen is expressed intracellularly just as in cells infected with actual virus and thus immunity is more effective and more permanent. Clinical trials of DNA vaccines given intramuscularly, or transdermal method of gene-gun-show absence of toxicity and development of effective immunity [128]. The parallel administration of interferon genes has also been suggested as a treatment regimen. Numerous studies have suggested that an effective hepatitis C virus (HCV) vaccine must induce a strong T helper 1 (Th1) T cell response. Lang *et al.,* [129] used the rhesus macaque vaccination model in assessing HCV DNA vaccine-induced T cell responses and presented a useful tool in the evaluation of immune responses induced by HCV immunogens. Finally, the increased immunogenicity of DNA vaccines can be achieved by the fusion of specific antigens to extracellular domain of cytotoxic-T-lymphocyte-associated antigen 4 (CTLA4) which represents a promising approach. Zhou *et al.,* [130] evaluated this interesting approach for its enhancement on HBV-specific immune responses and its antiviral effects in HBV transgenic mice. Thus, the CTLA4-fused DNA vaccine led to breakdown of immune tolerance to viral infection in HBV transgenic mice, which might be used as a therapeutic vaccine in HBV infection.

LIVER CIRRHOSIS

Liver cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration, characterized by diffuse disorganization of the normal hepatic structure of regenerative nodules and fibrotic tissue. It is associated with prominent morbidity and mortality, and is induced by many factors, including chronic hepatitis virus infections, alcohol drinking and drug abuse. The treatment aims at limiting hepatocyte destruction and decreasing fibrosis. Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes, shows mitogenic, motogenic and morphogenic activities for a wide variety of cells. Moreover, HGF plays an essential part in the development and regeneration of the liver, and shows anti-apoptotic activ-

ity in hepatocytes. In a rat model of lethal liver cirrhosis produced by dimethylnitrosamine administration, repeated transfections of the human HGF gene into skeletal muscles induced a high plasma level of human as well as enodogenous rat HGF, and tyrosine phosphorylation of the c-Met/HGF receptor. Transduction with the HGF gene also suppressed the increase of transforming growth factor-beta1 (TGF-β1), which plays an essential part in the progression of liver cirrhosis, inhibited fibrogenesis and hepatocyte apoptosis, and produced the complete resolution of fibrosis in the cirrhotic liver, thereby improving the survival rate of rats with this severe illness. Thus, HGF gene therapy may be potentially useful for the treatment of patients with liver cirrhosis, which is otherwise fatal and untreatable by conventional therapy [131].

TGF-β1 and its signaling pathway also present targets for gene therapy. One approach is to deliver dominant negative gene for defective receptor in order to dimerize with the wild type receptor and inactivate it, or alternatively hyper-expression of Smad7, a signal transduction element that antagonizes secondary TGF-β1 messengers. The inhibition of TGF-β1 has been demonstrated by several authors either by using adenoviral vectors [132-135], recombinant plasmid [136] or siRNA [137] *in vivo*, resulting in the prevention of liver fibrosis. The second approach which includes the hyper-expression of Smad7, totally blocks TGF-β1 signal transduction. Indeed, several studies demonstrated that adenovirus-mediated overexpression of Smad7 in rats [138-141] resulted in significant inhibition of fibrosis. Besides, TGF-β1 is also a key mediator of liver fibrosis [142-144]. Arias *et al.,* [145, 146] demonstrated that adenoviral expression of a TGF-β1 antisense mRNA is effective in preventing liver fibrosis in bile-duct ligated rats. Furthermore, Yeom *et al.,* [147] constructed a siRNA targeting Smad3 which prevented the activity of TGF-β1.

In conclusion, gene therapy may be potentially useful for the treatment of patients with liver cirrhosis, which however remains to be proven in human.

LIVER CANCER

Liver cancer is one of the most common malignant tumors worldwide and the third leading cause of cancer-related death. Because of its high malignancy and fast progression, most high-grade patients have tumors that are unresectable. Plenty of pathways are involved in the development of hepatocellular carcinoma (HCC) and are depicted as follows: (a) activation of the Wnt/Frizzled/catenin pathway through mutations in catenin as well as up-regulation of upstream elements, such as Frizzled receptor, (b) alteration of the MAPK signaling pathway through HBV or HCV infection, (c) activation of the JAK/STAT pathway through inactivation of JAK-binding proteins, (d) inactivation of the tumor suppressor gene *p53* through gene mutation and posttranscriptional interaction with viral proteins as well as oxidative stress, (e) alteration of the tumor suppressor retinoblastoma (pRb pathway) and p16INK4 genes through mutations or promoter methylation, and (f) alteration of the TGF-β pathway [148]. Furthermore, many HCC-related oncogenes, including *AFP,RAS, c-FOS, c-JUN, RHO, TGF-a, HGF, CerbB2, HER-2,HER-2/neu, NEU, NGL, MDM2, MMP,* and *IGF*, have been found. The abnormal expression of these genes with regard to uncontrolled cell proliferation results ultimately in carcinogenesis [149].

The best curative options for the treatment of liver cancer are the surgical resection and liver transplantation. Chemotherapy and radiotherapy are also applied, however with major drawbacks such as the high frequency of tumor recurrence, metastasis and generally low responses. Moreover, recent experimental treatment approaches, including hormonal therapy, biologic, biochemical therapy [150-154], and molecular targeted therapy [155-158], are still needed to be verified in clinical application. Thus, there is an urgent need to develop novel treatments for recurrent and advanced HCC. Gene therapy suggests a novel and promising approach for liver cancer via a variety of gene transfer strategies aiming to the treatment of patients with primary and secondary liver tumors, including gene directed enzyme/prodrug therapy, inhibition of oncogenes and restoration of tumor-suppressor genes, immunotherapy, anti-angiogenesis and virotherapy (Fig. **1**). Some of these strategies have reached early clinical development with diverting success.

INHIBITION OF ONCOGENES AND RESTORATION OF TUMOR-SUPPRESSOR GENES BY ONCOLYTIC AND OTHER VIRUSES

The strategy of "Cancer Targeting Gene-Viro-Therapy" (CTGVT) was developed in 2001 [159] and has increased expectations in the treatment of cancer. It combines the advantages of gene therapy and oncolytic viral therapy. Oncolytic adenoviruses can replicate themselves in cancer cells and lyse the carcinoma, whereas they are very inefficient in normal cells. Tumor suppressor genes can replicate together with oncolytic viral vectors, thereby greatly enhancing the ability to induce cancer cell death [160]. In published studies, oncolytic adenovirus-mediated gene therapy has produced good results in targeting cancer cells. However, safety and efficacy, the two most important aspects in cancer therapy, remain serious challenges.

Gan *et al.,* [161] inserted the novel tumor suppressor gene *hepatocellular carcinoma suppressor 1* (*HCCS1*) into an oncolytic adenovirus and exhibited a dramatic inhibitory effect on cancer cells *in vitro* and *in vivo*, leading to a complete regression of 50% of established tumor xenografts in nude mice. However, the antitumor efficacy of oncolytic adenoviruses on human hepatocellular carcinoma (HCC) cells was compromised due to low expression of the adenovirus serotype 5 (Ad5) receptor on the target cells.

Wei *et al.,* [162] incorporated two therapeutic genes, *suppressor of cytokine signaling 3 (SOCS3)* and *tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)* into a double-regulated oncolytic adenovirus vector. Their results exhibited potent antitumor activity in *carcinoplacental antigen α-fetoprotein (*AFP)-positive HCC cell lines both *in vitro* and *in vivo*. Another report also tested the oncolytic adenovirus vector, enhancing the antitumor activity in HCC cell lines with similar results [163].

Moreover, to specifically target liver malignant cells, cancer gene therapy needs to combine highly selective gene delivery with highly specific gene expression. Towards this

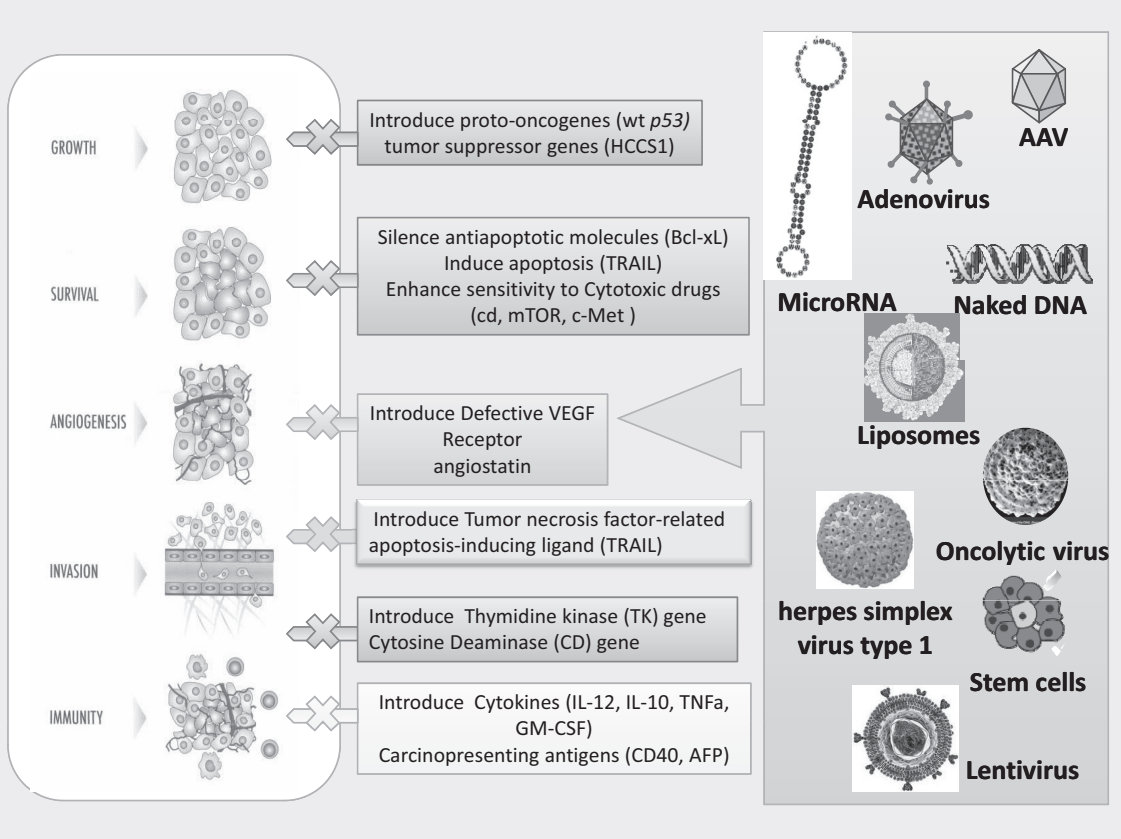

Fig. (1). Targets for gene therapy in liver cancer.

target, several approaches have been made [164-169]. Herpes virus simplex type-1 (HSV-1) is one of the most promising viral platforms for transferring therapeutic genes and the development of oncolytic vectors that can target, multiply in, and eradicate hepatoma cells via their lytic cycle. More specifically, Foka *et al.,* [168] identified and experimentally characterized novel hepatoma-specific promoters, which were valuable for cancer-specific gene therapy, by using HSV-1 vectors. Sia *et al.,* [170] used as a viral vector the HSV-1 amplicon in HCC and non-HCC cell lines *in vitro* and *in vivo* with promising results. Several other reports characterized different kinds of HSV-1 vectors as platforms for virotherapy against liver cancer [167, 168, 171-173].

Researchers have also used Epstein-Barr virus (EBV)-based plasmid vectors and demonstrated suicide gene therapy *in vitro* and *in vivo* suggesting efficient expression of the gene [174-176]. More specifically, these reports demonstrated in mice and in HCC cells that (EBV)-based plasmid vectors coupled with polyamidoamine dendrimer or polyethylenimine-mediated is a very useful tool in suicide gene therapy of cancer.

The gene of the *carcinoplacental antigen α-fetoprotein (AFP)* is overexpressed in HCC which makes it also a target of gene therapy. Kanai *et al.,* [177] demonstrated that replication defective recombinant adenoviral vectors, containing the human AFP promoter/enhancer, can be used to express the *Escherichia coli* cytosine deaminase (CD) gene (AdAFPCD) and the β-galactosidase gene (AdAF-PlacZ) in AFP-producing HCC cell lines. Hai-Neng Xu *et al.,* [178] showed reduced toxicity and excellent anti-liver cancer efficacy by using quadruple-regulated adenoviruses carrying an AFP promoter-controlled *HCCS1* gene both *in vitro* and *in vivo.* Other reports also used adenoviral vectors for AFP delivery in hepatocellular carcinoma cells *in vitro* or *in vivo* in animal models showing promising strategies for cancer specific therapy which may have clinical use in the future [179, 180].

Abnormalities of *p53* are also considered a predisposition factor for hepatocarcinogenesis as *p53* is frequently mutated in HCC [181]. *p53* gene transfer has been proposed as a potential therapeutic option for treatment of HCC. Some researchers used adenoviral-mediated transfer or oncolytic adenovirus by expressing *p53* fiber chimeric oncolytic adenovirus in combination with *p53* expression, promising a safe anticancer agent for the treatment of HCC [182, 183]. Furthermore, Gu *et al.,* [184] studied the effect of adenovirus (Ad)-*p53* gene therapy on HCC in a rabbit model and concluded that trans-arterial Ad-p53 gene therapy can reduce tumor growth of HCC. In a series of published studies data were presented of a constructed recombinant adenovirus vectors by replacing E1 region by exogenous DNA, demonstrating induction of HCC cell cytotoxicity and apoptosis *in vitro* and *in vivo* [185-188]. Habib *et al.,* [189] attempted a clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for HCC with no effective antitumor results. This study

showed that the adenovirus was well tolerated, but did not seem to offer significant tumor control. Several clinical studies of (Ad)-*p53* combined with fractionated stereotactic radiotherapy or with chemotherapy and trans-catheter arterial chemo-embolization for HCC were conducted and presented promising results [190-193]. Recombinant adenoviral vector expressing wt-*p53* (Ad-*p53*) [209] yield a relatively low degree of acute toxicity, compared to other commonly used gene transfer vectors such as adenovirus and retrovirus, recombinant AAV serotype 2 (rAAV2) [214] has shown promising results in human clinical trials. Significant enhancement in the gene transfer efficiency is needed, however, for HCC applications.

IMMUNOTHERAPY

Cancer immunotherapy can be defined as the techniques used to eliminate malignant tumors through mechanisms through immune system responses [194, 195]. The goal of cancer immunotherapy is to direct against tumors extremely potent immune responses such as those naturally occurring against microbial antigens, and subsequently apply these results to human cancer diseases [196]. Cancer represents almost 70% of the clinical trials conducted in patients and 25% of these studies consisted in the application of cytokine genes [196]. Two main approaches have been used in gene transfer of immunostimulatory cytokines (e.g. IL-2, IL-4, IL-6, IL-7; IL-12, INF-γ, TNF-α, GM-CSF) [144]: i) vectors expressing cytokines/chemokines/costimulatory molecules injected directly into tumor lesions, or ii) transduction of tumor /dendritic cells *ex vivo* with vectors expressing cytokines/costimulatory molecules [196]. Several reports demonstrated the potential usefulness of IL-12 gene transfer for liver tumors treatment in animal models, mostly by using adenovirus vectors [197-211]. Recent studies showed that the combination of cytokines with anti-angiogenic gene therapies achieved better antitumor effects on large tumors [162].

The other immunotherapy strategy includes the use of oncolytic viruses for cytokine gene delivery which also offers a promising treatment of liver tumors [189, 212-215]. As discussed earlier, these vectors are considered quite effective, associated with higher infectious rate and prolonged expression. However, gene therapy with oncolytic viruses in hepatoceccular carcinoma induced also immune responses, expression of heterologous genes in unwanted tissues or cells that could shut down the function gene expression [216].

microRNA

One of the most promising anticancer therapies involves the use of microRNA (miRNA), the efficacy of which mainly depends on the efficiency of the delivery system [217, 218]. miRNAs are a class of short endogenous RNAs. There are about 1000 known miRNA today. A lot of findings suggest the involvement of miRNAs in the pathogenesis of HCC, i.e. the irregular expression of miRNAs [219-227] and the association with the clinical outcome of cancer patients [228, 229]. miRNAs may function as oncogenes while others act as tumor suppressors; studies in animal models treated with miRNAs or in combination with chemotherapy showed promising results [230-251] (Table **3**). The identification of miRNA function involved in liver cancer has provided an important knowledge regarding miRNA tumor associated gene interactions and revealed many potential therapeutic targets.

CONTROL OF TUMOR MICROENVIRONMENT

The microenvironment of the HCC is composed of non-tumor cells and their stroma, with the stroma having been implicated in the regulation of tumor growth, metastatic potential and outcome following therapy. Niess *et al.,* [252] used engineered mesenchymal stem cells (MSCs) as therapeutic vehicles for the treatment of HCC. They concluded that stem cell-mediated introduction of suicide genes into the tumor followed by pro-drug administration was effective towards liver cancer [252].

ACUTE LIVER FAILURE

Acute liver failure (ALF) is a life-threatening medical emergency and occurs when the liver rapidly looses its function within a short period. ALF is mainly caused by viral infections, primarily HBV (hepatitis B virus), HAV (hepatitis A virus) and HEV (hepatitis E virus) in the East and developing countries, such as China and India [253, 254]. In contrast, the majority of ALF is resulted from a drug overdose in the West and developed countries. ALF can also be developed secondarily due to a variety of causes such as infection with non-hepatotropic viruses, including cytomegalovirus, Epstein-Barr virus, adenovirus and hemorrhagic fever virus, vascular diseases, such as Budd-Chiari syndrome and venoocclusive disease, metabolic diseases, such as Wilson's syndrome, Reye's syndrome, acute fatty liver of pregnancy and galactosemia, and idiosyncratic drug/toxin reactions [255]. Currently, the orthotopic liver transplantation (OLT) is the "Gold Standard" therapy for the disease. However, due to the limited availability of donor organs and rapid progression of the disease, the mortality of ALF remains high. Therefore, it is imperative to develop novel therapeutic reagents for ALF. Gene therapy by delivering a target gene to the patients appears to be a promising approach for the treatment of ALF. Besides, the asialoglycoprotein receptor (ASGP-R) is uniquely and highly expressed on hepatocytes, offering a mechanism for glycoprotein-mediated hepatocyte-specific delivery [256]. In contrast, the major drawback for ALF gene therapy is that multiple factors contribute for its development, such as apoptosis, necrosis, inflammation and liver regeneration.

Therefore, gene therapy for treating ALF is still in its infancy. Several studies report successful development of gene therapy for ALF in animals, mostly mice [257-267]. The main non viral vectors used in theses references are siRNA antisense oligonucleotides and Zinc-finger nucleases (ZFNs). Currently, there is no large-scale clinical trial to test a specific gene. However, gene therapy holds great promise in inhibition of the disease progression, benefiting the ALF patients. Potentially, gene therapy may become an alternative or at least complement to OLT for intervention of ALF patients. Zhu *et al*., [268] proposed targeting *fgl2* for inhibiting necrosis, together with *Fas* to reduce apoptosis and HGF to promote liver regeneration; this multitarget approach could synergistically block the progression of ALF.

Table 3. MicroRNA (miR) for Hepatocellular Carcinoma (HCC)

Author	Cell line-Animal model-clinical trial	Non-viral vector- miR	Overall	Refs
Fang *et al.,* 2011	mice	miR-29b	improved	232
Xu *et al.,* 2011	HCC cells in G2/M phase	miR-122	improved	231
Yang *et al.,* 2011	HCC cells	miR-122	improved	233
Li *et al.,* 2011	mice	miR-99a	improved	221
Zhou *et al.,* 2011	HCC cells	miR-1274a	improved	235
Young *et al.,* 2010	HCC cells	miR-122	improved	239
Fornari *et al.,* 2010	HCC cells	miR-199a-3p	improved	240
Shimizu *et al.,* 2010	Huh7 and HepG2 cell lines, primary human hepatocytes	miR let-7	improved	241
Liu *et al.,* 2010	non-tumor tissues of HCC patients	miR-375	improved	242
Ma *et al.,* 2010	Cancer cells HepG2, Hep3B, Huh7 and PLC/PRF/5	miR-122	improved	243
Xu *et al.,* 2009	HCC patients	miR-195	improved	246
Suzuki *et al.,* 2008	HCC	miR-122a	improved	248

CONCLUSIONS

In this review, we have focused on the ongoing development of gene therapy in liver diseases. We summarized the different strategies that have been explored to improve the efficacy of gene therapy in these diseases. During the past decade, gene therapy has offered plenty of therapeutic options. Unfortunately, a clear therapeutic effect for most of the liver diseases has not yet been achieved. Several reasons could explain these results; the neutralization of the virus by the immune system, severe toxicity, and low levels of target-specific gene delivery. Despite several efforts in clinical research trials the effectiveness and clinical utility were undermined also by several limitations, such as insufficient delivery, intravascular administration and short-lived transgene expression. Still, gene therapy for liver cancer is moving towards clinical application with promising results and especially when the virus is administered in combination with existing chemotherapy and radiation therapy. Hopefully, the ever growing knowledge on molecular pathways and the construction of improved and more effective vectors will shed new light on the right path in order to ensure the best clinical efficacy.

Nonviral vectors are simpler, safer, and less expensive than viruses and offer very large carrying capacities. Transfection efficiency can be increased by associating the DNA with a carrier, such as a liposome or a polymer, or through the use of a physical stimulus, such as an electric pulse (electroporation). Nonviral gene delivery (transfection) remains much less efficient than viral gene delivery (transduction) and this remains a barrier to its wider use. Recent progress in vector technology and imaging techniques, allowing *in vivo* assessment of gene expression, will facilitate the development of clinical applications of gene therapy. Although only a small number of patients were treated, it becomes apparent that more effective vectors are needed to achieve a useful clinical impact. Overall, the numerous experimental strategies addressing gene therapy efficacy in liver diseases need to be evaluated in clinical trials and may offer successful therapeutic alternatives.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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