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Review article. Gene therapy, recent developments and future prospects in gastrointestinal oncology.

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Short Title

Gene therapy, liver neoplasms, colorectal neoplasms, pancreatic neoplasms

For Peer Review

Abstract

Background Gene therapy consists of the introduction of genetic material into cells for a therapeutic purpose. A wide range of gene therapy vectors has been developed and used for applications in gastrointestinal oncology.

Aim To review recent developments and published clinical trials concerning the application of gene therapy in the treatment of liver, colon and pancreatic cancers.

Methods Search of the literature published in English using the PubMed database.

Results A large variety of therapeutic genes are under investigation, such as tumour suppressor, suicide, antiangiogenesis, inflammatory cytokine and micro-RNA genes. Recent progress concerns new vectors, such as oncolytic viruses, and the synergy between viral gene therapy, chemotherapy and radiation therapy. As evidence of these basic developments, recently-published phase I and II clinical trials, using both single agents and combination strategies, in adjuvant or advanced disease settings, have shown encouraging results and good safety records.

Conclusion Cancer gene therapy is not yet indicated in clinical practice. However, basic and clinical advances have been reported and gene therapy is a promising, new therapeutic approach for the treatment of gastrointestinal tumours.

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INTRODUCTION

Despite recent progress, there is an important need for new therapeutic agents in gastrointestinal oncology. Gene therapy consists of the introduction of genetic material into cells for a therapeutic purpose. The initial aim of the field of gene therapy was the correction of inherited genetic diseases by providing to the targeted cells a functional copy of the deficient gene responsible for the disease. However, gene therapy can also be applied to the treatment of acquired diseases, in particular cancers. Indeed, more than 1500 gene therapy clinical trials have been recorded worldwide and about two thirds of these concern cancers (**http://www.wiley.co.uk/genetherapy/clinical/**). In gastrointestinal oncology, most approaches have targeted liver, colon, and pancreatic tumours, and this review will focus on recent knowledge concerning the use of gene therapy in the treatment of these cancers.

LITERATURE SEARCH

A literature search was performed using the PubMed database, limited to documents published in the English language. Searches were not date-restricted. Search terms included free text words and combinations of the following Mesh terms: [Digestive system neoplasms], [Colorectal Neoplasms], [Liver Neoplasms], [Pancreatic Neoplasms], [Gene Therapy], [Genetic Vectors], [Clinical trial]. The 'Gene Therapy Clinical Trial Worldwide' website was also used to search for clinical trials.

PRINCIPLES OF GENE THERAPY: SELECTION OF A GENE, A VECTOR AND A MANAGEMENT STRATEGY

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Selection of the gene

Mutant Gene correction

The principles of gene selection strategies are illustrated in Figure 1. In the case of inherited monogenic diseases, the aim of gene therapy is to transfer and express the defective gene. The situation is more complex in cancer gene therapy because cancer most often results from sequential genetic and epigenetic alterations, affecting oncogenes, tumour-suppressor genes and microRNAs. One gene therapy approach is, thus, to restore tumour-suppressor gene expression or to inhibit oncogene expression. About 11 % of transferred genes in gene therapy clinical trials are tumour-suppressor genes, and many trials have been performed in cancer gene therapy using the p53 gene, mostly including patients with lung or head and neck cancers **(http : //www.wiley.co.uk/genetherapy/clinical/).**

Suicide genes

The aim of suicide gene therapy is to enable, selectively, the transfected cell to transform a prodrug into a toxic metabolite, resulting in cell death. The most widely described suicide gene is the herpes simplex virus thymidine kinase (HSV-tk) gene. HSV-tk can phosphorylate ganciclovir which is a poor substrate for mammalian thymidine kinases. Ganciclovir can, therefore, be transformed into ganciclovir triphosphate which is cytotoxic to the transfected cell, resulting in cell death [1]. This cell death can also affect neighbouring cells which do not express HSV-tk. This phenomenon is called a local bystander effect, as opposed to a bystander effect that can be observed in distant, non-transduced tumour sites [2]. This distant bystander effect involves the immune system.

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Immunotherapy

Cancer immunotherapy has been developed to stimulate the immune response against tumour cells. Gene therapy can be used to transfer genes into tumour cells to render them more highly immunogenic. Gene transfer of tumour-specific antigens, co-stimulatory molecules and/or inflammatory cytokines has been assessed. Tumour-associated antigens can be recognised by T lymphocytes. These antigens can be derived from oncogenic viruses (Epstein-Barr virus, human papillomavirus) or can be self-antigens. These self-antigens can be overexpressed antigens or antigens which are altered by virtue of a gene mutation or a post-translational modification. They can also be onco-fetal antigens such as 5T4 antigen and carcinoembryonic antigen (CEA) [3]. Immunisation against a specific antigen can induce cellular and/or humoral immune responses. T-cell activation requires not only the interaction between major histocompatibility molecules bearing a specific peptide and the T-cell receptor, but also non-antigen-specific co-stimulatory activation by interaction of molecules expressed on the T-cell and the antigen presenting cells (such as interactions between CD28 and CTLA-4 expressed on the T-cell, and B7 expressed on the antigen presenting cells). Finally, vectors encoding inflammatory molecule genes (such as interleukin-2, interleukin-12, TNF-α, interferon-γ, GM-CSF) have been engineered. In terms of the site of gene transfer, immunisation can be carried out *in situ* in the tumour or at a distant site [4].

RNA interference

RNA interference is a promising new therapeutic approach for many diseases, including cancer. MicroRNAs (miRNA) are short, endogenous ~22 nucleotide RNAs. They can regulate gene expression at the post-transcriptional level by binding to the 3' untranslated region of the target mRNA, resulting in either mRNA degradation or inhibition of translation [5, 6]. They act as fine-regulators of the proteome [7]. MiRNAs can have oncogenic activities

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when they are up-regulated and target tumour suppressor genes [8]. MiRNAs can also have a tumour suppressor potential [9]. Furthermore, miRNAs can modify the response to therapeutic agents. For example, the Let-7 family of miRNAs can modify the response of cancer cells to radiation therapy [10]. The endogenous RNA interference pathway has been exploited to develop other RNA interference molecules: synthetic, exogenous, double-stranded, short, interfering RNA (siRNA) and vectors expressing short hairpin RNAs (shRNA). As opposed to siRNAs, shRNAs are synthesised in the nucleus and use maturation pathways similar to the miRNA maturation pathways [11]. As cancer cells present many well-known RNA interference targets, there are multiple opportunities for therapeutic gene silencing in oncology [12].

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The choice of the vector

The effectiveness of gene therapy is highly dependent on the efficacy of gene transfer. The vector has to be safe, has to protect the genetic material from degradation in the extracellular environment, and must release the genetic material to the target cell.

Viral Vectors

Recombinant viruses have been shown to be efficient for gene transfer both *in vitro* and in *vivo*. Many viruses such as adenoviruses, adeno-associated viruses (AAV), retroviruses, lentiviruses, herpes viruses, poxviruses, measles viruses, simian virus 40 recombinant (SV40r) and vesicular stomatitis viruses have been proposed for preclinical studies (Table 1). Other viruses are used as virotherapy, rather than gene therapy, agents, because they cannot be genetically modified (reoviruses) or because they have not been modified with the insertion of a therapeutic transgene. Data and clinical trials related to these

viruses are not reported in this review. Viruses can be either replication-defective or replication-competent. To engineer replication-defective viruses, one or several genes required for viral replication can be deleted. Vectors able to replicate selectively in cancer cells would be of great interest in oncology. A new strategy involving so-called "oncolytic" viruses has been proposed. In this context, the aim is to generate a pathogen for the tumour, which is unable to propagate in, and to harm, normal tissues. Two main characteristics of oncolytic viruses are: (1) they replicate selectively in cancer cells and have self-amplification properties; (2) they have cancer-cell specific toxicity [13]. Some viruses, such as reoviruses, have natural, inherent tumour selectivity [14, 15]. From a general point of view, oncolytic viruses can be engineered in three ways. (1) Viruses can be deleted for genes that are necessary for their replication in normal cells but not in cancer cells. (2) Another possibility is to engineer a virus with genes under the control of a tumour-specific promoter. (3) Viruses can also be engineered to bind to specific tumour antigens on the cell surface [13, 16]. In the first two approaches, the aim is to restrict the replication of the viruses, while the third strategy aims at selective infection of cancer cells. However, viral vectors have many limitations. There is a limiting immunogenicity and there are safety considerations, including a possible insertional mutagenesis for retroviruses and lentiviruses.

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Non-viral Vectors

Naked DNA

Naked or plasmid DNA has a rapid clearance, and a low cellular uptake. As a consequence, the main application of naked DNA injection is in vaccination and immunotherapy strategies. Electroporation involves the generation of a transient increase of the permeability of cell membranes by the use of electric pulses and is more efficient than a simple injection of naked DNA. Electroporation increases the transfer efficiency of drugs,

DNA or RNA into the cells, *in vitro* and *in vivo* in the case of intratumoural injections [17-19]. However, the safety and efficiency of this procedure need to be assessed in humans.

Synthetic vectors

Cationic liposomes comprise a lipid bilayer membrane. They are amphiphilic molecules and can interact with negatively-charged DNA, compacting it and protecting it from circulating endonucleases. The structure generated upon incubation of DNA with liposome is called a lipoplex. Cationic lipoplexes can bind to negatively-charged cell membranes and induce cellular uptake. However, *in-vivo* transfer efficiency remains low [20].

Blood vessels in tumours can have gaps between endothelial cells, up to 800 nm in size. Nanoparticles can extravasate through these gaps and accumulate selectively in tumours. This enhanced permeability and retention (EPR) effect could explain the tumour selectivity of several gene therapy vectors [21]. Nanoparticles are small-sized vectors which can be lipoplexes, other polymers, or metal- and ceramic-based. They can be used as gene therapy vectors and can accumulate in tumours as a result of the EPR effect [22-23].

Biological non-viral vectors

Bacteria can be used as gene therapy vectors. They can be used as gene transfer vectors or protein delivery systems when the therapeutic transgene is already expressed in the bacterium [24]. In addition, many mammalian cell types can be used as carriers of gene therapy vectors [25]. Haematological and progenitor mesenchymal cells are promising agents for use in cancer gene therapy. For example, cytokine-induced killer (CIK) cells can be obtained from mouse splenocytes or human peripheral blood, after *in-vitro* stimulation and culture with interferon-γ, interleukin-2 and anti-CD3 antibodies. These cells naturally target tumours and have cytotoxic effects. They have been combined with the oncolytic vaccinia virus and a synergistic antitumour effect has been observed in mouse models [26].

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Choice of strategy: *in-vivo* versus *ex-vivo*

The *ex-vivo* strategy consists of collecting a cell type or its precursor from the subject, culturing and transducing the cells with the vector and then reintroducing the genetically-modified cells into the subject. The *in-vivo* strategy consists of direct injection of the vector into the subject (either intratumourally or systemically). The *ex-vivo* strategy allows control of the transduction, and limitation of dissemination of the vector. The *in-vivo* strategy does not require a cell culture step but cell targeting and transduction efficiency are more difficult to control.

GENE THERAPY IN GASTROINTESTINAL ONCOLOGY: CLINICAL TRIALS AND FUTURE PROSPECTS

Liver Tumours

Clinical trials

Based on preclinical data, several clinical phase I and phase II trials concerning gene therapy of liver tumours have been published (Table 2) [27-34]. Re-expression of tumour-suppressor genes has been evaluated *in vitro*, *in vivo* and in clinical trials, mainly with vectors encoding wild-type p53. In a recently-published, phase II trial, 46 patients with unresectable hepatocellular carcinoma were randomised to either group 1 (multiple hepatic arterial injections of 5-FU and a recombinant adenovirus encoding the p53 gene, after transcatheter arterial chemoembolisation (TACE)) or group 2 (TACE alone). Both treatments were safe. Partial response or stable disease were reported for 69.5 % of subjects in group 1

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and 65.2 % of subjects in group 2. Times to progression were 9.6 months in group 1 and 8.3 months in group 2. Overall survival times were 12.8 months in group 1 and 10.4 months in group 2 [34].

Other gene therapy trials have concerned suicide therapy strategies. A recent, phase II clinical trial compared patients with hepatocellular carcinoma and tumours bigger than 5 cm, treated by liver transplantation, with or without adenoviral-mediated HSV-tk therapy. During transplantation surgery, adenoviruses encoding HSV-tk were injected into the peritoneal cavity around the liver and ganciclovir therapy was started 24 hours after surgery and continued for 10 days afterwards. Forty-five patients were included in the trial, 22 received liver transplantation only, and 23 received liver transplantation and gene therapy. Recurrence-free survival and overall survival were significantly higher in the gene therapy group, particularly in patients without vascular invasion before treatment [32].

A phase I trial included 21 patients (9 patients with primary liver cancers, 5 with metastatic colorectal cancers and 7 with pancreatic cancer) in an immunotherapy approach. Intratumoural injections of an adenovirus encoding the interleukin 12 gene were well tolerated, and a partial, objective remission was observed in 1 patient with hepatocellular carcinoma [30]. Another phase I trial included 17 patients (9 patients with primary liver cancers, 5 with colorectal cancers and 3 with pancreatic cancer). Patients were treated with intratumoural injections of autologous dendritic cells transfected with an adenovirus-encoding IL-12 gene. The treatment was well tolerated. It was associated with a marked increase of infiltrating CD8+ T lymphocytes in 3 of 11 tumour biopsies analysed. Eleven patients could be assessed for response. One partial response was observed in a patient with pancreatic carcinoma. Stable disease was observed in two patients and progression in eight patients [31].

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Oncolytic viruses have also been evaluated in clinical trials. In a phase II study, 19 patients with advanced or metastatic hepatobiliary cancers (tumours of the liver, gall bladder, or bile duct) received intratumoural injections of ONYX-015, a first-generation, replication-selective adenovirus. Sixteen patients could be evaluated for response. One patient had a partial response, one patient had prolonged disease stabilisation (49 weeks), and 8 patients had a biological response with a decrease in tumour markers of more than 50 % [28]. Recent clinical studies with other therapeutic viruses have been reported, highlighting the interest in oncolytic viruses for cancer gene therapy. A phase I trial, including 14 patients with refractory primary or metastatic liver tumours, evaluated the maximum tolerated dose of intratumoural injection of JX-594. JX-594 is a targeted, oncolytic poxvirus (vaccinia virus) modified by disruption of the thymidine kinase gene and insertion of the human granulocyte macrophage colony-stimulating factor (GM-CSF). Patients received a mean of 3.4 cycles of intratumoural injections. Four patients received only one cycle (2 because of toxicity and 2 because of self-withdrawal from the study) and were not analysed for tumour response. Among the 10 patients analysed, 3 showed a partial response, 6 had stable disease and 1 showed progression of disease after CT scan examination. Hyperbilirubinaemia was a limiting factor in patients treated with the highest dose. Flu-like symptoms were the most frequent adverse event. Of particular interest, JX-594 was found in peripheral blood after the initial injection and delayed viraemia was detected several days afterwards. JX-594 was found in non-injected tumours, suggesting that the virus could target disseminated tumours after systemic injection [33]. Another study included 3 patients with concomitant hepatocellular carcinoma and hepatitis B virus infection (HBV) with active viral replication. JX-594 intratumoural injections were associated with tumour response and a decrease in HBV DNA levels [35].

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Perspectives from preclinical models

New strategies aiming at improving the rate of transduction of cells have been developed for gene re-expression strategies, with interesting preclinical results. For example, an adenoviral vector encoding a fusion protein, p53-VP22, has been engineered. VP22 is a herpes simplex virus type 1 tegument protein, exhibiting the capacity to be exported from the cell to neighbouring cells. Some VP22 fusion proteins, such as p53, can retain this capacity. In a model of heterotopic, subcutaneous hepatocellular carcinoma xenograft in mice, intratumoural injections of VP22-p53 adenoviruses were more efficient than those of p53-alone adenoviruses, leading to partial tumour regression [36]. The most widely-described suicide gene is the herpes simplex virus thymidine kinase (HSV-tk) gene. However, other suicide gene strategies have been developed. For example, an adenoviral vector encoding carboxypeptidase G2 (CPG2) has been engineered. CPG2 is an enzyme which catalyses the conversion of alkylating prodrugs such as ZD2767P into cytotoxic agents. Tumour regression was observed in a model of subcutaneous hepatocellular carcinoma xenograft, after intratumoural injection of the vector and treatment with the prodrug, ZD2767P [37]. Other immune therapy strategies have also been evaluated *in vitro*. Adenoviruses encoding CD40 ligand were injected into orthotopic hepatocellular carcinomas in rats. The treatment caused a tumour response, with 10 of 23 treated animals showing a complete response and 9 animals a partial response, and an improved survival of treated rats. The treatment induced lymphocytic infiltration of the tumour, and the tumour response was dependent on CD8+ T cells [38]. In a model of alpha-fetoprotein-expressing hepatocellular carcinoma spontaneously developing in mice (BW7756 tumours), the combination of alpha-fetoprotein vaccination (by injection of expression plasmids into muscles) and an adenoviral-mediated chemokine IP-10 and interleukin-12 expression (intratumoural injection) resulted in a synergistic effect with improved survival [39].

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The sodium-iodide symporter (NIS) gene encodes a membrane glycoprotein that mediates active iodide transport across the basolateral membrane of thyroid follicular cells. NIS can drive the uptake of radioisotopes (^{123}Iodide, ^{124}Iodide, ^{125}Iodide, ^{131}Iodide, ^{188}Rhenium, ^{211}Astatine) into the cells [40]. A viral-guided NIS gene therapy is an innovative approach of radio-iodine therapy. Such an approach was assessed in a rat model of hepatocarcinoma (diethylnitrosamine-treated rats). Animals were treated by intraportal injections of an adenovirus encoding rat NIS. Long-term retention of radioiodine was demonstrated after viral transduction, both in treated, tumour-bearing rats and in healthy, treated rats, but not in control, non-treated rats. After intratumoural injection of the virus, intraperitoneal injections of ^{131}I were associated with inhibition of tumour growth and prevention of the formation of new tumour nodules around the injected nodule. Furthermore, intraperitoneal injection of the virus followed by ^{131}I therapy was associated with improved survival of diethylnitrosamine-treated rats [41].

New strategies include anti-angiogenic gene therapy and therapeutic RNAi. Anti-angiogenic therapies are currently well developed and widely-used in oncology. Endostatin is an inhibitor of angiogenesis and tumour growth [42]. An adenovirus encoding human endostatin was tested in a model of subcutaneous hepatocellular carcinoma xenograft in mice and demonstrated an antitumour effect [43]. Pigment epithelium-derived factor (PEDF) is another recently-discovered, anti-angiogenic factor. In a model of heterotopic, subcutaneous hepatocellular carcinoma xenograft in mice, intratumoural injections of a plasmid expressing PEDF resulted in a significant reduction of tumour volume compared with controls [44]. Kallistatin is another example of new antiangiogenic inhibitors. Intraportal injections of a recombinant AAV encoding kallistatin were performed in hepatic and subcutaneous

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hepatocarcinoma murine models. Treatment with the recombinant virus resulted in significant tumour growth inhibition, as compared with treatment with empty AAV [45].

There has been recent interest in the RNAi strategy. The pituitary tumour-transforming gene 1 (PTTG1) is an oncogene which is frequently expressed in human liver cancers. An adenovirus encoding an RNAi targeting PTTG1 (Ad.PTTG1-siRNA) has been engineered. *In vitro*, the virus could deplete PTTG1 in hepatoma cells, resulting in apoptosis. Furthermore, intratumoural injections of the virus led to inhibition of tumour growth in a xenograft tumour in mice [46]. Another recent study concerns RNAi cancer gene therapy in a mouse model of hepatocellular carcinoma (tet-O-MYC; LAP-tTa). Hepatocellular carcinoma cells exhibit a down-regulation of the expression of miR-26a in human and mouse liver tumours. This miRNA plays a role in cell cycle arrest. Systemic administration by tail vein injection of an adeno-associated virus encoding miR-26a resulted in the inhibition of cancer proliferation, with 6 out of 8 treated mice exhibiting only small tumours or no tumour and 6 out of 8 mice treated with control virus developing a fulminant disease [47].

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Colorectal Cancer

Clinical trials

Clinical trials concerning the gene therapy of metastatic colorectal cancers have also been published (Table 3) [48-55]. Re-expression of tumour-suppressor genes has been evaluated in a phase I trial including 17 patients with advanced cancers, among them 5 patients with colorectal cancer and 1 patient with pancreatic cancer. Patients were treated with escalating doses of an adenovirus encoding a wild-type p53 gene (Ad5CMV-p53). The

treatment was administered intravenously, daily for 3 consecutive days, every 28 days. There were no objective responses; one patient with metastatic and progressive colorectal carcinoma had a 10-month period of stable disease. The virus was detectable in plasma 14 and 28 days after treatment in the majority of patients treated at the highest dose. Seven patients had tumour biopsies before and after treatment. Six of these patients had undetectable Ad5CMV-p53 expression before treatment that became detectable after treatment. This study showed that this treatment was feasible, with no major toxicity [53].

A phase I clinical trial including 16 patients with hepatic metastases from colorectal carcinomas assessed the safety of intratumoural injections of an adenovirus encoding the HSV-tk gene. Injections were followed by systemic ganciclovir administration. The treatment was safe. However, there was no complete or partial tumour response [49].

A p53-specific immunity can be observed in advanced cancers. In a phase I/II, dose-escalation trial, 15 patients with advanced colorectal cancers were treated with systemic injections of a poxvirus encoding a wild-type p53 gene, aiming to induce p53-specific immunity. The treatment could induce p53-specific antibodies and, in 2 out of 5 patients treated with the highest dose of virus, a specific T-cell response [51]. TROVAX is a modified vaccinia virus encoding the tumour antigen, 5T4, aiming to produce an anti-5T4 immune response. Trovax is used in several cancer gene therapy protocols. A phase I/II trial was carried out in 22 patients with metastatic colorectal cancer. Repeated intramuscular injections of TROVAX were safe and induced an anti-5T4 cellular response. Five out of 22 patients had stable disease for a period ranging from 3 to 18 months [54]. A 5T4-specific cellular or antibody response was still detectable when given alongside a chemotherapy regimen [56].

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A phase II trial included 27 patients with colorectal cancer liver metastases. Infusions of ONYX-015 into the hepatic artery, in combination with 5-FU, were well tolerated with fever, rigors and fatigue as the most common side effects. The antitumoural effect of the virus could not be assessed in patients who had not previously received this chemotherapy regimen. Interestingly, 3 patients with 5-FU-refractory disease had a 30-50 % regression of the tumour mass [52]. Another phase I, dose-escalation trial included 12 patients with liver colorectal cancer metastases. All patients were refractory to a first line chemotherapy. NV1020 is an oncolytic herpes simplex virus type 1. Patients had a single NV1020 injection into the hepatic artery at 4 different doses (3 patients in each group). NV1020 administration was well tolerated with fever, rigors and headache as the most common side effects. Antitumour activity was assessed by CT-scan at day 28, even if this was a toxicity study. Two patients had a 39 % and a 20 % reduction, respectively, in tumour size, 7 patients showed stable disease and 3 a progressive disease [55]. Floxuridin chemotherapy was administered after day 28 by an intrahepatic arterial pump, in association with intravenous irinotecan. All 12 patients had a partial response to subsequent chemotherapy [57].

Perspectives from preclinical models

In a recent study, a p53 gene re-expression strategy was improved in a combination of viral-directed therapies. An AAV vector expressing the kringle 1 domain of the human hepatocyte growth factor (AAV-HGFK1) and an adenovirus expressing p53 were combined in a murine model of colorectal cancer xenograft. The combination treatment increased the survival time, inducing apoptosis, necrosis and suppressing angiogenesis, as compared with single agent therapies. *In vitro*, this antiangiogenic effect was related to an inhibitory effect on endothelial cell migration [58].

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The HSV-tk/ganciclovir suicide strategy could also be improved. An adenovirus encoding the monocyte chemoattractant protein-1 (MCP-1) gene has been engineered. In a model of subcutaneous colorectal cell line tumours in immunocompetent mice, intratumoural injections of both MCP-1-encoding adenovirus and of HSV-tk-encoding adenovirus reduced tumour growth significantly more than HSV-tk adenovirus alone [59]. Another suicide system has been developed in a murine model of colorectal cancer. A retrovirus encoding the cytosine deaminase gene was engineered. Cytosine deaminase converts the non-toxic 5-fluorocytosine into toxic 5-fluorouracil. In a model of orthotopic liver metastasis (CT26 cells) in mice, the viral vector was administered into the portal vein *via* intrasplenic injection. 5-fluorocytosine was administered into the peritoneal cavity. All mice in the control groups had tumour progression, but tumour growth was significantly inhibited in the treated mice [60].

Chemo-gene and radiation-gene therapies are the combinations of gene therapy with chemotherapy or radiation therapy, respectively. Gene therapy can sensitise colon cancer cells to chemotherapy *in vitro* [61, 62]. Cytosine deaminase (CD) converts 5-fluorocytosine into the toxic metabolite, 5-fluorouracil. Intratumoural injection of free plasmids expressing CD in liver metastasis rat models, in association with an oral treatment with 5-fluorocytosine, resulted in a systemic antitumour effect [63]. Flt3 Ligand (Flt3L) is an activator of dendritic cells and NK cells. Intratumoural injections of an adenovirus encoding Flt3L produced a synergistic effect in association with 5-FU therapy in subcutaneous models of hepatocarcinoma and colon cancer and induced long-term immunity against further parental cancer cell injections [64]. In a recent study, cultured human colorectal cancer cells and tumour xenografts were infected with an engineered adenovirus expressing the NIS gene. Tumour xenografts concentrated ^{99m}Technetium, allowing quantitative imaging by SPECT/CT. NIS expression reached a peak 48 hours after intratumoural injection.

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Administration of a single dose of virus combined with a single ^{131}Iodide dose 48 hours after virus injection induced tumour regression with an additional effect. [65].

Recently, the effect of miRNA targeting c-myc was assessed *in vitro* and *in vivo*. *In vitro*, miRNA inhibited c-myc expression, reduced HT 29 cell proliferation and induced apoptosis. *In vivo*, intratumoural injections of miRNA were performed in a model of subcutaneous HT 29 tumour in athymic nude mice. Treatment significantly reduced tumour volume [66]. In another study, a replication-deficient adenovirus expressing p53 and an miRNA targeting the cyclin-dependent kinase inhibitor p 21 (Adp53/mIR-p21) have been engineered. In tumour xenografts in nude mice, intratumoural injections resulted in a significant decrease in tumour volume, as compared with a control virus expressing p53 but not mIR-p21. *In vitro*, Adp53/mIR-p21 increased apoptosis as compared with the control virus, but also increased the sensitivity of cancer cells to doxorubicin [67].

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Pancreatic Cancer

Clinical trials

Clinical studies have also been carried out in pancreatic cancer (Table 4) [68-71]. A phase I study assessed the safety and side effects of a subcutaneous vaccination regimen using 2 viruses (vaccinia virus and fowlpox virus) expressing CEA and mucin-1 with 3 co-stimulatory molecules (B7.1, ICAM-1 and LFA-3), in association with GM-CSF therapy, in 10 patients with advanced pancreatic cancer. The most common side effects were injection site reactions, fatigue, headache and nausea. Survival was significantly better in patients who developed anti-CEA- or anti-MUC-1-specific immune responses; however these are

preliminary data [70]. A phase III study in metastatic pancreatic cancer has been carried out (US 635 phase III trial) but results have not yet been published.

A phase I/II study showed the feasibility and tolerability of endoscopic ultrasound-guided, repeated administrations of the ONYX-015 virus into carcinomas of the pancreas [69]. An interim analysis of the PACT study, a multi-centre, randomised, controlled, phase III clinical trial, was presented at the American Society of Clinical Oncology meeting 2009. The study included 330 patients with advanced pancreatic cancers, with 2 arms of treatment. The control arm received standard, 5-week chemo-radiation therapy, the experimental arm received chemo-radiation plus weekly, intratumoural injections of TNF-erade™, an adenovirus encoding the TNF-α gene. Both arms received adjuvant gemcitabine with the option of erlotinib. An interim analysis of overall survival was conducted after the 92nd death (one-third of total expected events) had occurred. The analysis concluded that TNF-erade™ appeared to be safe and well-tolerated. The overall survival interim analysis indicated a trend in favour of TNF-erade™ therapy, with a late effect on Kaplan Meyer curves [72].

Perspectives from preclinical models

Transduction efficiency is a major concern in gene therapy. A recently-published study showed high transduction efficiency for gene transfer in cells derived from pancreatic cancers, using a lentiviral vector, both *in vitro* (from 68 to 98 %, depending on the cell line and the multiplicity of infection) and *in vivo*. In tumour xenografts in mice, intratumoural injections of a lentivirus encoding human interferon-β resulted in a significant inhibition of apoptosis [73].

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Peritoneal dissemination is a poor prognostic event in pancreatic cancer. Intraperitoneal vector injections may therefore be of great interest. Intraperitoneal injection of retrovirus encoding the HSV-tk gene has been effective in treating nude mice with a model of metastatic pancreatic cancer disseminated to the peritoneal cavity [74]. The K-ras oncogene is mutated in more than 80 % of ductal pancreatic adenocarcinomas [75]. An adenovirus expressing K-ras anti-sense RNA can induce apoptosis *in vitro* in human pancreatic cancer cells. Intraperitoneal injections of this adenovirus inhibited peritoneal dissemination in hamster pancreatic cancer models [76].

We will focus on the promising approach of chemo- and radiation gene therapy. Adenoviruses encoding p53 genes showed a synergistic antitumour effect with 5-FU therapy, *in vitro* and *in vivo*, in heterotopic models in rats [77]. A recombinant adenovirus encoding deoxycytidine kinase and uridine monophosphate kinase (enzymes converting gemcitabine into toxic metabolites) sensitised pancreatic cancer cells to gemcitabine, *in vitro* and in an orthotopic tumour hamster model [78]. In a subcutaneous model of pancreatic carcinoma in mice, a single, intravenous injection of the vaccinia GLV-1h68 virus, an oncolytic, engineered poxvirus, was able to reverse the course of tumour growth. The efficacy of viral therapy was enhanced by repeated injections of gemcitabine or cisplatin. Seven out of 8 mice treated with the virus and cisplatin had complete tumour regression at the end of the observation period, whereas 1 out of 8 mice treated with the virus alone had complete tumour regression. The viral therapy was safe, with a high tumour selectivity of the virus after systemic administration [79]. Another study demonstrated that an oncolytic virus was able to synergise with gemcitabine to kill pancreatic cancer cells *in vitro* and *in vivo* by a synergistic effect on apoptotic pathways [80]. In another study, cultured human pancreatic cancer cells and tumour xenografts were infected with an engineered measles virus expressing the NIS gene.

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Intratumoural injections resulted in a significant reduction in tumour volume and increased survival time of the treated mice compared with the control mice. Tumour xenografts also concentrated radioiodine, allowing quantitative imaging with ^{123}I micro-SPECT/CT [81]. Pancreatic cancer is, therefore, a candidate for specific, viral-driven delivery of therapeutic radioisotopes.

CONCLUSION

The safety profile of gene therapy is a major concern. Recently-published trials have shown good safety records. The most commonly-reported side effects related to treatments involving a viral vector were fever, headache, fatigue, nausea and vomiting. Intratumoural injections were used in most studies. In trials using replication-competent agents, viruses were found in non-injected tumours and regression of both injected and distant disease occurred. This effect could be related to targeting of disseminated tumours by the vector and to local and systemic immune responses. Thus, intratumoural injections could be efficient, even for the treatment of disseminated tumours. However, lesions can be difficult, or impossible, to inject, and repeated intratumoural injections using invasive procedures could be a limiting factor for this approach. Systemic administration has also shown a good safety record, and the development of oncolytic viruses which selectively target tumour cells makes this mode of administration of great interest. These data regarding oncolytic viruses may pave the way for more widespread clinical applications of this approach.

Gene therapy will need to be positioned in the context of existing cancer therapy strategies. Good safety records could make gene therapy a good candidate in all therapeutic settings (neo-adjuvant, adjuvant, and advanced disease), probably in combination therapies.

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Combining gene therapy with chemotherapy or radiation therapy may be very interesting, as gene therapy can act as a chemo- or radio-sensitiser, and because chemotherapy and radiation therapy can improve gene transfer efficiency and gene expression [82-86].

Gene therapy is not yet suitable for use in routine clinical practice, mainly because of insufficient levels of gene delivery in patients. However, promising preclinical data include the recent development of new therapeutic targets, new treatments such as miRNA and new vectors and, in particular, vectors that are more highly selective with greater therapeutic potential. Clinical trials are ongoing in gastrointestinal cancers, as well as in other tumours such as melanoma, brain, head and neck, prostate or ovarian cancers. Even if safety profiles, drug-resistance and efficacy have to be elucidated further, cancer gene therapy may become a new weapon in anti-cancer strategies.

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FIGURE LEGEND

Figure 1. Principles of gene therapy. **A**. Gene re-expression. The vector carries a wild-type version of a mutant gene into the tumour. **B**. Suicide therapy. A combination of the systemic administration of a non-toxic prodrug and the tumour-specific delivery of the prodrug-activating enzyme gene. **C**. Immune therapy. The vector carries a gene encoding a specific immunogenic tumour antigen (1), a co-stimulatory signalling molecule (2) or an inflammatory cytokine (3), leading to immune stimulation. **D.** Oncolytic viruses. Oncolytic viruses specifically replicate in, and kill, tumour cells. **E**. Therapeutic RNA interference. The RNAi (siRNA) or the RNAi gene (shRNA) is delivered into the cell. After maturation and incorporation in the RNA-induced silencing complex (RISC), RNAi can bind an oncogene RNA, leading to oncogene repression and cell death.

Review

Vector	Advantages	Disadvantage
Viral Vectors		
Adenovirus	Large transgene insert capacity Biologically safe	High immunogenicity
Herpes simplex virus	Large transgene insert capacity Availability of anti-herpetic drugs	Immunogenicity
Adeno-associated viruses	Stable transgene expression Reduced immunogenicity	Small transgene insert size capacity Need for helper viruses during manufacture
Retrovirus	Stable transgene expression Only infects dividing cells	Small transgene insert size capacity Possible insertional mutagenesis (integration)
Lentivirus	Infects both dividing and non-dividing cells	Possible insertional mutagenesis (integration)
Vaccinia virus	Long history of safe human use Large transgene insert capacity	High immunogenicity Productive infection in immune suppressed patients Replication in skin lesions (eczema, psoriasis)
Vesicular stomatitis virus	Selective replication-competence in cells with defective interferon response (tumour cells)	High immunogenicity Animal pathogen (safety/environmental concerns)
Measles Virus	Long history of safe human use (Edmonston vaccine strain)	Most adults are immune Wild-type virus is immunosuppressive Rare measles-like illness with vaccine strains
Non-Viral Vectors		
Naked DNA	"Easy" engineering Low immunogenicity	Rapid clearance, low transfection efficiency *in vivo*
Synthetic vectors	Large gene carrying capacity Nanoparticles can accumulate into tumours as a result of the enhanced permeability and retention effect.	Cationic liposomes have an inflammatory toxicity and a low transfection efficiency *in vivo*

Table 1. Some advantages and disadvantages of different gene therapy vectors

For Peer Review

Author	Clinical phase	Number of patients	Clinical setting	Vector and gene	Treatment Protocol	Comments
Habib et al, 2002 [27]	II Randomised	10	Hepatocellular carcinoma	E1B-deleted adenovirus	Intratumoural ethanol (Group 1) or adenovirus (Group 2) injections	Tumour response assessment: 2 SD and 3 PD in group 1, 1 PR and 4 PD in group 2
Makower et al, 2003 [28]	II	19	Advanced or metastatic hepatobiliary cancer	ONYX-015 adenovirus	Intratumoral injections	Good safety records with two grade 4 toxicities likely related to disease progression Sixteen patients assessed for response: 1 PR, 12 SD, 3 PD
Palmer et al, 2004 [29]	I	18	Resectable primary or metastatic liver cancers	Adenovirus encoding nitroreductase (prodrug activating enzyme)	Intratumoural injection, 3 to 8 days before hepatic resection	Good safety records Transgene expression in the tumour
Sangro et al, 2004 [30]	I	21	Advanced liver, colorectal and pancreatic cancers	IL-12-encoding adenovirus	Intratumoural injections	Good safety records Ninety-nine patients assessed for response at day 30: 1 PR, 10 SD, 8 PD
Mazzolini et al, 2005 [31]	I	17	Nine patients with primary liver tumour, 5 with colorectal cancer, 3 with pancreatic cancers	Autologous dendritic cells transfected with an adenovirus encoding the IL-12 gene	Intratumoural injections	Good safety records 11 patients assessed for response: 1 PR, 2 SD, 8 PD
Li et al, 2007 [32]	II Randomised	45	Hepatocellular carcinoma, adjuvant therapy after liver transplantation	HSV-tk-encoding adenovirus	Group 1: Liver transplantation + postoperative systemic epirubicin injections Group 2: Same procedure + postoperative peritoneal injections of HSV viruses and systemic ganciclovir injections	RFS and OS at 2 years, respectively: 9.1% and 19.9% (Group 1), 43.5% and 69.6% (Group 2)
Park et al, 2008 [33]	I	14	Advanced primary or metastatic liver cancers	Oncolytic vaccinia virus, JX-594	Intratumoural injections	Good safety records 10 patients assessed for response: 3 PR, 6 SD, 1 PD
Tian et al, 2009 [34]	II Randomised	46	Advanced hepatocellular carcinoma, in association with transcatheter arterial chemoembolisation (TACE)	Adenovirus encoding the p53 gene	Group 1: TACE alone Group 2: adenovirus + 5-FU hepatic infusions	OS: 10.4 months (group 1) versus 12.8 months (Group 2)

Table 2. Clinical trials concerning gene therapy in primary liver tumours

For Peer Review

PR: Partial Response, SD: Stable Disease, PD: Progressive Disease, RFS: Recurrence Free Survival, OS: Overall Survival

For Peer Review

Author	Clinical phase	Number of patients	Clinical setting	Vector and gene	Treatment protocol	Comments
Rubin et al, 1997 [48]	I	15	Advanced colorectal cancers	Liposomal vector, HLA-B7 gene transfer	Intratumoural injections	Good safety record Feasible DNA transfection with gene expression
Sung et al, 2001 [49]	I	16	Advanced colorectal cancers	HSV-tk-encoding adenovirus	Intratumoural injections into liver metastases, followed by systemic ganciclovir administration	Good safety record Tumour response of injected tumour: 11 SD, 5 PD
Reid et al, 2001 [50]	I	11	Advanced colorectal cancers	ONYX-015 adenovirus	Infusions of ONYX-015 into the hepatic artery, in combination with intravenous 5-FU and leucovorin	Good safety record, no dose-limiting toxicity
Van der Burg et al, 2002 [51]	I/II	15	Advanced colorectal cancers	p53-encoding canarypoxvirus	Intravenous injections	Good safety record Induction of p53-specific antibodies and a specific T-cell response with the highest dose of virus
Reid et al, 2002 [52]	II	27	Gastrointestinal carcinoma metastatic to the liver	ONYX-015 adenovirus	Infusions of ONYX-015 into the hepatic artery, in combination with intravenous 5-FU and leucovorin	Three patients with 5-FU refractory disease had a 30-50% tumour regression
Tolcher et al, 2006 [53]	I	17	Advanced cancers, including 5 colorectal and 1 pancreatic cancers	Adenovirus encoding wild -type p53 gene (Ad5CMV-p53)	Intravenous injections	Fever, nausea, vomiting and fatigue were common but rarely severe Tumour response assessment: no objective response, 1 SD.
Harrop et al, 2006 [54]	I/II	22	Advanced colorectal cancers	5T4-encoding vaccinia virus	Intramuscular or intradermal injections	Good safety record Five patients had stable disease for a period ranging from 3 to 18 months
Kemeny et al, 2006 [55]	I	12	Advanced colorectal cancers	NV1020 oncolytic herpes simplex virus	NV1020 injection into the hepatic artery	Good safety record with fever, rigors and headache as most-common adverse effects. Tumour response assessment at Day 28: 2 PR, 7 SD, 3PD

Table 3. Clinical trials concerning gene therapy in metastatic colorectal tumours

PR: Partial Response, SD: Stable Disease, PD: Progressive Disease

For Peer Review

Author	Clinical phase	Number of patients	Clinical setting	Vector and gene	Treatment protocol	Comments
Mulvihill et al, 2001 [68]	I	23	Locally advanced cancer	ONYX-015 intratumoural injections	CT-guided injections (n = 22 patients) or intraoperative injections (n = 1) into pancreatic primary tumours every 4 weeks until tumour progression	Good safety record, no clinically significant pancreatitis No detectable viral replication No objective response documented
Hecht et al, 2003 [69]	I/II	21	Locally advanced or metastatic cancer	ONYX-015 intratumoural injections	Endoscopic ultrasound-guided ONYX-015 injections on days 1, 5, 8, 15, 36, 43, 50, and 57, in association with intravenous gemcitabine therapy on days 36, 43, 50, and 57	Two duodenal perforations when transduodenal approach was used. Two instances of sepsis before institution of systematic antibiotic prophylaxis No clinically significant pancreatitis No objective response on day 35 After combined treatment: 2 PR and 8 SD of the targeted lesion
Kaufman et al, 2007 [70]	I	10	Locally advanced or metastatic cancer	Subcutaneous vaccination with vaccinia virus and fowlpox virus, both expressing tumour antigens and co-stimulatory molecules	Subcutaneous vaccination, in association with GM-CSF therapy	Site reaction, fatigue, headache and nausea as most-common side effects Significantly better survival in patients developing anti-CEA or anti-1-specific immune responses
Galanis et al, 2008 [71]	I	12	Gemcitabine-refractory, metastatic pancreatic cancer	Intravenous injections of Rexin-G, a non replicative, retroviral vector expressing cyclin G1 gene	Repeated intravenous injections	Good safety record No evidence of clinical antitumour activity

Table 4. Clinical trials concerning gene therapy in pancreatic cancer

PR: Partial Response, SD: Stable Disease, PD

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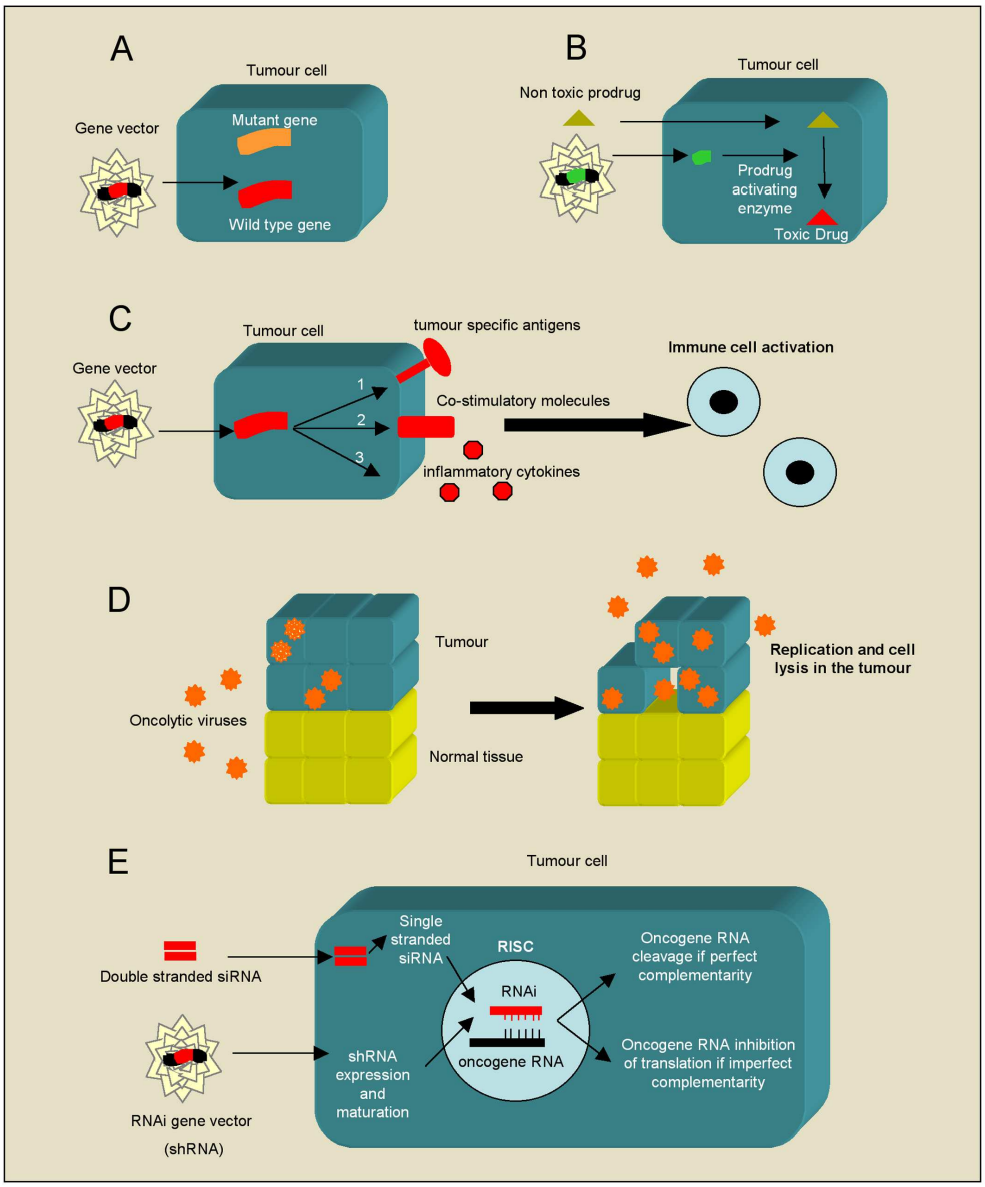

Principles of gene therapy. A. Gene re-expression. The vector carries a wild-type version of a mutant gene into the tumour. B. Suicide therapy. A combination of the systemic administration of a non-toxic prodrug and the tumour-specific delivery of the prodrug-activating enzyme gene. C. Immune therapy. The vector carries a gene encoding a specific immunogenic tumour antigen (1), a co-stimulatory signalling molecule (2) or an inflammatory cytokine (3), leading to immune stimulation. D. Oncolytic viruses. Oncolytic viruses specifically replicate in, and kill, tumour cells. E. Therapeutic RNA interference. The RNAi (siRNA) or the RNAi gene (shRNA) is delivered into the cell. After maturation and incorporation in the RNA-induced silencing complex (RISC), RNAi can bind an oncogene RNA, leading to oncogene repression and cell death.
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